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TITLE

**ORGANOIDS AS A MODEL OF INFLAMMATION IN INTESTINAL
DISEASES: POSTBIOTICS AND PRECISION MEDICINE**

TUTOR

Prof. Maria Vittoria Barone

Maria Vittoria Barone

PH.D. STUDENT

Dr. Claudia Bellomo

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General introduction

Organoids are self-organised 3D tissues, obtained from stem cells. They can mimic the key functional, structural, and biological complexity of an organ^{1,2}. Stem cells could be derived from induced pluripotent stem cells (iPSCs) or tissue-derived cells (TDCs), the second group include normal stem/progenitor cells, differentiated cells and cancer cells³.

The use of organoids gives the possibility to better characterise the physio-pathological mechanisms of diseases. Animal models or secondary tumour-derived cultures cannot reproduce patient specificity, tissue-like structures and functions compared with organoids. They are useful in several diseases, to investigate the possible disease-related mechanisms, and they are more accessible for genetic manipulation, drug-personalised therapies and diagnosis².

The isolation of TDCs made it possible to investigate deeper diseases associated with the small intestine⁴, colon⁵, stomach⁶, tongue⁷, liver⁸, lung⁹, pancreas¹⁰, heart¹¹, ear¹², skin¹³ and brain¹⁴.

Intestinal organoids are a useful model of diseases to be used in research. They can be obtained from TDCs, more specifically from ASCs (adult stem cells), generated from LGR5+ stem cell populations present in crypts isolated from intestinal biopsies¹⁵. This kind of multipotent cells could differentiate toward a limited number of cell types in the presence of specific growth factors¹⁶. They are useful both to investigate intestinal epithelial physiology and most of the diseases related to it and phenotypic and/or genetic characteristics, becoming an optimal tool for the development of precision medicine^{1,17,18}. The stem cells are grown in 3D and enclosed in a matrix, which serves as a basal membrane and by using appropriate stimuli they differentiate into epithelial cells. They can be grown and amplified for several weeks as spheroids. However, to know the response to stimuli of a pro-inflammatory nature or to investigate the possible beneficial effect of pre-pro-postbiotics and nutraceuticals or drugs, it is necessary to create a 2D monolayer of intestinal epithelial cells. Two-dimensional monolayers of intestinal organoids allow direct contact of the apical, adsorptive pole of the cells with the culture medium; this cannot occur in 3D culture as the apical sides of the cells are enclosed in the spherical organoids. A recent review demonstrated that intestinal epithelium was not only important as a target of proinflammatory stimuli, but it can also play a central role in the generation of inflammation and can induce and/or sustain an adaptive autoimmune response in several different settings¹⁹.

The first purpose of this thesis was the collection of intestinal organoids, from control subjects (CTR), Celiac Disease (CeD) patients at different stages of the disease, Eosinophilic Esophagitis (EoE)

subjects, and subjects with co-morbidities related to CeD, such as patients with Type 1 Diabetes (T1D) and Down Syndrome (DS) (Chapter 1).

Aims

To better characterised the role of intestinal organoids to study CeD patients the first aim of this thesis was to collect intestinal organoids from a cohort of paediatric patients, at different stages of celiac disease, its comorbidities respect to controls patients (Chapter 1).

Simultaneously, the activity of milk-based postbiotics from *Lactobacillus plantarum*, with and without the addition sources of linoleic acid, was tested on CaCo-2 cells to evaluate the possible effect in preventing inflammation induced by gliadin peptide, P31-43, and the possible reduction on the constitutive inflammation in intestinal organoids from patients with CeD (Chapter 2).

The third aim was to investigate the role of autophagy flux /mTOR pathway in intestinal biopsies and organoids from CeD patients in active and remission stages of the disease. Furthermore, the possible the crosstalk-between inflammation, delay of autophagy flux/the activation of the mTOR pathway was investigated (Chapter 3).

Lastly, in eosinophilic esophagitis patients in which one of the incriminating antigens is gluten, the fourth aim was to investigate the grade of inflammation in the duodenum, and their sensitivity to gliadin peptide, P31-43, in intestinal organoids of these patients in remission stage of the disease (Chapter 4).

CeD organoids and inflammation

Celiac Disease (CeD) is a chronic inflammatory disease with systemic manifestations caused by genetically predisposed individuals after the ingestion of gluten-containing foods. Although CeD is an enteropathy, there are systemic manifestations related to it. The genetic factors of CeD are associated with HLA (Human Leukocyte Antigen) DQ2/DQ8. These haplotypes are present in 30% of populations, but only 1-2% of them could be affected by CeD. For this reason, the genetic background, including HLA-DQ2/DQ8 or non-HLA genes, are not enough to explain all the pathogenetic mechanisms of the CeD. Environmental factors must be considered to understand all the faces of the pathogenetic events in this disease²⁰. The gliadin is the main environmental factor that generates the disease, but more recently, other factors such as other food components, among them Amylase/Trypsin Inhibitors (ATI), a non-healthy diet, together with infections and different innate immune mechanisms can have a role in CeD pathogenesis^{21,22}. As mentioned, the diet, and more in detail its components, could play a role in the development of CeD by modulating the microbiota composition and predisposing children to CeD. Gut microbiota has been shown to be associated with gluten metabolism, the immune response, and the regulation of intestinal permeability²³.

CeD is characterised by an enteropathy with an inflammatory response and structural changes that result in the remodelling of the small intestinal mucosa. Gluten ingestion activates an adaptive immune response, but the full range of the mechanisms activated by gluten is still unknown²⁴. The activation of the immune response in CeD patients causes alteration of the intestinal mucosa, with a higher level of lymphocytes, crypts hyperplasia and villus atrophy, which are evaluated together by MARSH classification that defines the histological lesion of the duodenal mucosa.

Although the role of the immune system has been studied for decades and associated with CeD, in recent years more focus has been given to the role of the intestinal epithelium. A recent study demonstrated that in the intestinal epithelium biopsies from biopsies of CeD patients' presented inflammation even before the onset of the epithelial damage²⁵ (Appendix 1). A deeper analysis on intestinal biopsies showed that inflammatory markers IL-1 β and IL-6 were increased in enterocytes even before the onset of the intestinal lesion. These inflammatory markers were increased not only in patients with villus atrophy (gluten-containing diet- CeD – GCD-CeD) but also in patients with gluten-free diet (GFD-CeD) with normal mucosal architecture and in potential celiac disease patients (POT-CeD) with positive serology (endomysia antibodies- EMA and antibodies to tissue transglutaminase -tTG) and normal architecture of intestinal mucosa²⁵ (Appendix 1).

Intestinal organoids derived from GCD-CeD reproduced the same alterations observed in GCD-CeD biopsies. Data published in Appendix 1 showed that GCD-CeD intestinal organoids were inflamed, with an increase of pNF- κ B (phosphorylation of nuclear factor kappa-light-chain-enhancer of activated B cells) and pERK (phosphorylation of endoplasmic reticulum kinase), together with an increase in pro-inflammatory cytokines (IL-1 β and IL-6) in the culture medium compared to control organoids. Moreover, the inflammation found in CeD organoids was persistent even after 12 weeks of cell culture, respect other inflammatory diseases, such as IBDs, that did not show any signs of inflammation after one/two weeks in culture. All this indicates that CeD organoids reproduce the inflammation present in CeD biopsies and that inflammation is persistent, representing a good model, at least for this readout, to study CeD²⁵ (Appendix 1).

CeD organoids, Protein Tyrosine Phosphatases (PTPs) and proliferation

Not only inflammation was found altered in CeD intestinal organoids, but also proliferation²⁶ (Appendix 2). The CeD intestinal biopsies are characterised by an inversion of the differentiation/proliferation program of the enterocytes, with an increase in the proliferative compartment and crypt hyperplasia, but the mechanisms that regulate the increased proliferation in CeD are today not completely understood. A previous study identified by genetic analysis an alteration of the Protein Tyrosine Phosphatase Receptor Type K (PTPRK) gene in CeD intestinal biopsies²⁷. This prompted us to study the role of PTPRK, a phosphatase that regulated EGFR (Epidermal Growth Factor Receptor) activation, in the proliferation of the enterocytes in CeD biopsies and GCD-CeD intestinal organoids respect to controls. WB analysis demonstrated that PTPRK levels were reduced in GCD-CeD and Pot-CeD biopsies whereas pEGFR, pERK and proliferation were increased respect to the controls. The GCD-CeD organoids reproduced these same alterations. Functional experiments were conducted to better understand the role of PTPRK in the regulation of epithelial proliferation in CeD intestinal organoids. Silencing of PTPRK in CTRs organoids increased pEGFR, pERK and proliferation while the overexpression of PTPRK in CeD organoids reduced pEGFR, pERK and proliferation.

All these data demonstrated that the modulation of PTPRK levels could reduce or increase proliferation in CeD or CTRs organoids. The CeD organoids can be a good model to study the mechanisms of epithelial proliferation in CeD²⁶ (Appendix 2).

These observations on PTPRK role in CeD proliferation prompted us to study the role of PTPs also in other inflammatory diseases, in which the intestine can play a central role, such as IBDs and T1D. PTPs are a family of enzymes essential for numerous cellular processes, such as cell proliferation, inflammation, differentiation, immune-mediated responses, and oncogenic transformation. The study of the recent literature unveiled the role of several PTPs—PTPN22, PTPN2, PTPN6, PTPN11, PTP σ , DUSP2, DUSP6 and PTPRK—at the level of the intestinal mucosa in IBD, CeD and T1D both in vitro and in vivo models. All these studies highlighted the alterations of different biological processes, including proliferation, autophagy, and inflammation mediated by PTPs, at the level of the intestinal mucosa of these diseases. PTPs are now actively studied to develop new drugs considering these nodal biological processes. Moreover, considering the availability of organoids as models to test new drugs in personalised ways, it is very likely that soon, these proteins will be the targets of useful drugs. In conclusion, this review of the recent literature revealed shared features between IBDs, T1D and CeD, previously underrated²⁸ (Appendix 3).

Model to test gluten toxicity in the intestinal epithelium: role of postbiotics

Several cellular models have been implemented to investigate gluten toxicity.

CaCo-2 cells, derived from colon adenocarcinoma, are an intestinal epithelial cell model used to investigate the effect of gliadin peptides. They can be differentiated in culture for about 20 days, resulting in an intestinal cell line with morphological and functional characteristics of the small intestine. Interestingly they are responsive to gluten treatment. In fact, by using this model it has been demonstrated that gliadin peptides were able to induce an increase of pNF-kB levels, compared to untreated cells^{29,30} (Appendix 4 and 5). The limit of this model is that they are secondary cells derived from a tumour, presumably not derived from a CeD patient.

Intestinal fibroblasts are mesenchymal-type cells, abundant in the mucosa, responsible for the formation of the extracellular matrix (ECM) and the rearrangement of the villi. In fibroblasts derived both from the skin and small intestine of CeD patients, alteration of proliferation mediated by pERK and pEGFR was observed with respect to CTRs, even in the absence of gluten treatment³¹. Moreover, in CeD fibroblasts derived both from the skin and the intestine, treatment with gliadin peptides induced inflammation, as shown by the NF-kB activation³². These models are indeed derived from CeD patients, but apparently, they do not represent the target cells, namely the intestinal cells.

Another model frequently used in CeD studies is the culture of intestinal biopsies. These cultures require a special bioreactor to keep the temperature and the CO₂ constant. They have been extensively used to show an increase in inflammatory markers and/or proliferation after gluten treatment in CeD^{31,32}. The limits of this model are the short life of the biopsy specimen, no more than 24-30 hours and they cannot be propagated or engineered.

The intestinal organoids derived from intestinal biopsies of CeD patients present several advantages with respect to intestinal biopsies. They can be propagated indefinitely and can also be engineered²⁵ (Appendix 1). More recently, intestinal organoids, derived from intestinal biopsies of CeD patients, showed a constitutive inflammation that increased after treatment with gliadin peptides, as shown by the activation of NF- κ B. Interestingly, intestinal organoids from CeD patients responded to gliadin peptide concentrations that were not able to induce an increase of inflammatory markers in controls, demonstrating their high sensitivity to gluten²⁵(Appendix 1).

In our study, we have used both CaCo-2 cells and CeD intestinal organoids to assess the effects of post-biotics on the gluten-induced inflammation in CaCo-2 cells and on the epithelial constitutive inflammation in organoids.

Gluten is the most important environmental factors that play a central role in CeD, and it is widely used in Western diet (WD). In Europe, the average consumption of gluten alone is about 20 grams per day, a very high amount considering the number of other substances ingested daily. The wheat represents one of the most common foods in WD, and in its protein fraction (8-15%), 95% is gluten. It is composed of gliadin combined with glutenin at the same ratio (1:1). Gluten, however, is not uniquely contained in wheat but also in other food components, in rye named secalin, in barley named hordein. However, all of them are collectively referred to as "gluten"³³. Gluten remains indigested after gastric, pancreatic, and intestinal endopeptidases. 33-mer and 25-mer (P31–55) containing the peptides P57–68 and P31–43, respectively, are very resistant to hydrolysis and are active in the intestine after the digestion process³⁴.

Previous studies have shown that peptide P31-43 has pleiotropic biological activity, being able to interfere with different molecular pathways. In different cellular models, this gliadin peptide has been associated with the alteration of vesicular trafficking, proliferation, and inflammation. It can induce a delay in early vesicular trafficking, remodelling of the actin filament, an increase of EGFR activity-dependent proliferation, upregulation of the level of IL-15 (interleukine-15) and INF- α (interferon- α) in vitro models and activation of the MAP kinase pathway with increased expression of pERK^{24,32}.

In-vitro studies have been conducted using postbiotics to prevent the inflammatory effects induced by gliadin peptides.

Postbiotics are soluble substances produced by live bacteria or released after their lysis. They are composed by components from the bacterial cell surface, lactic acid, short-chain fatty acids (SCFAs), bioactive peptides, and other metabolites. When provided in adequate quantities, postbiotics may promote host health, although their precise mechanisms of action are still unknown³⁵.

Postbiotics derived from *Lactobacillus paracasei* (LP) and *rhamnosus GG* (LGG) have been used on CaCo-2 cells to evaluate whether they could prevent gliadin peptide-induced effects. On CaCo-2 cells, inflammation induced by P31-43 was reduced if cells are pre-treated with both LP and LGG postbiotics. Moreover, the treatment with LP and LGG for three hours can reduce the constitutive inflammation in CeD organoids^{29,30} (Appendix 4 and 5).

For this reason, the second aim of the present thesis was to evaluate whether the milk-based postbiotics obtained from the fermentation of *Lactobacillus Plantarum* CECT 749 were able to reduce P31-43-induced inflammation in CaCo-2 cells and to reduce constitutive inflammation in CeD intestinal organoids. Milk-based postbiotics of *L. Plantarum* were grown in the presence of different matrices such as linoleic acid (LA), Spent Coffee Ground (SCG) and SCG oil to compare the efficiency of all of them in both CaCo-2 cells and CeD intestinal organoids (Chapter 2).

Autophagy in CeD

In addition to an increased state of inflammation, the presence of gliadin peptides can induce an alteration of autophagic flux in CaCo-2 cells. Autophagy is defined as a cellular degradation mechanism and recycling process that is highly conserved in all eukaryotes. There are three types of autophagy: micro, macro- and chaperone-mediated autophagy. Although these three, act differently, their ultimate purpose is to degrade and/or recycle substances through lysosomes³⁶.

Macroautophagy plays a central role not only in physiologic conditions, contributing to the correct functioning of the cell and degradation of non-functional and/or superfluous organelles, but also in stress conditions or energy production³⁷. The proper functioning of autophagic flux is necessary for the maintenance of cellular homeostasis. For this reason, autophagic dysfunction is linked to several human diseases, including lung, liver and heart disease, cancer, neurodegenerative and metabolic diseases such as diabetes^{36,38}. Autophagic flux can be modulated by the presence of amino acids;

when these are reduced, autophagy is stimulated. Amino acids play a crucial role in the mTOR pathway, stimulating its activity if they are present. These two processes are inversely related^{39–41}.

Little is known about autophagy flux and mTOR pathway in celiac disease. Sedda et al. have demonstrated that in CeD intestinal biopsies there was an alteration of mTOR1 pathway, with increased mTOR levels⁴². Moreover, the presence of gliadin peptides, both PTG (peptic-tryptic peptides) and P31-43, can modulate the mTOR pathway, respectively, in intestinal biopsies and in CaCo-2 cells. On the other hand, although the mTOR pathway has been studied on both biopsies and Caco-2 cells models, the autophagy flux has been investigated only in CaCo-2 cells^{29,30} (Appendix 4 and 5).

Considering the literature data, the third aim of this thesis was to investigate autophagic flux and the possible interplay between autophagy/mTOR activation and inflammation pathway in the intestinal biopsies from GCD-CeD, and by using intestinal organoids from celiac disease patients, both in active (GCD-CeD) and in remission (GFD-CeD) stage of the disease (Chapter 3).

Is gluten toxic also in other intestinal inflammatory diseases?

Many diseases, apart from CeD, are characterised by a high grade of inflammation in the intestine. It is the organ that represents the largest interface between the internal body and the external environment. Every condition that can modify intestinal homeostasis could be associated with the intestinal barrier. The increasing barrier permeability can play a central role in the progression of several disorders, not only related to the intestine, such as IBD, but also with other metabolic disorders, such as diabetes, both type 1 and 2⁴³. Obesity, which is a chronic inflammatory disease, exerts an effect on the gut. Adipose tissue accumulation at the level of the abdomen correlates closely with gut inflammation and mucosal barrier integrity⁴⁴.

Both in vitro and in vivo models studied the role of gluten in patients with T1D, indicating that it may be an important player in the pathogenesis of T1D, even suggesting that its elimination from the diet could modulate disease progression⁴⁵.

Gluten can be considered a pro-inflammatory food also in other conditions of an inflammatory nature that are localised in several gastrointestinal tract different from the duodenum⁴⁶. To date, it is not clear how the inflammation of one district can influence another. A typical example is oesophageal diseases that seem to be relegated to a specific district. In a random analysis of duodenal biopsies from EoE patients, we discovered that they presented inflammatory alterations of the duodenum. EoE is a

chronic immune-mediated food allergy disease of the oesophagus characterised by eosinophilic infiltration, that leads to an inflammatory response⁴⁷. These patients follow different types of therapy, such as an elimination diet with the exclusion of several antigens, including gluten, drug therapy, to reduce the count of eosinophils in the oesophagus and mechanical dilation. To date, no data in literature investigates the state of other gastrointestinal parts⁴⁶.

For these reasons, the last aim of this thesis was to investigate the duodenum of subjects with eosinophilic esophagitis in remission. Intestinal organoids were developed from subjects with eosinophilic esophagitis in remission state of the disease (REM-EoE) to investigate their inflammation markers respect to controls, and the effect of treatment with gliadin peptide P31-43. Lastly, REM-EoE intestinal organoids were used to investigate the autophagy flux and mTOR pathway with respect to organoids of control subjects (Chapter 4).

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Chapter 1

Collection of intestinal organoids from controls and CeD patients at different stage of the disease and its comorbidities

Abstract

Celiac disease (CeD) is a complex autoimmune condition triggered by gluten, with mechanisms that are yet to be fully understood. Animal models such as Irish Setters, non-human primates, and mice have been employed to study CeD, but none was helpful in mimicking all the mechanisms involved in CeD. Intestinal organoids replicate key CeD-related epithelial alterations, including proliferation, inflammation, and autophagy defects.

For this reason, the aim of this thesis was to collect intestinal organoids from children to investigate the role of intestinal epithelium cells in CeD patients in different stage of the disease, and its comorbidities, such as Type 1 Diabetes (T1D) and Down Syndrome (DS) compared to controls (CTRs).

Organoids were derived from intestinal biopsies, after EGD, to isolate crypts from all patients and controls.

To compare different groups intestinal organoids were classified based on disease stage, serological markers, and histological features (e.g., Marsh classification). The data collection allowed to identify differences between groups, but also to understand the differences intra-groups.

This work underscores the hypothesis to use intestinal organoids to model CeD pathophysiology, explore overlapping immune pathways in CeD and EoE and, possibly, to offers new tools for future research into these chronic immune-mediated diseases.

Introduction

To date a model that reproduces all the alterations associated with CeD is still not available, probably because of the complexity of the mechanisms related to CeD. In the last 40 years, different animal models were used to investigate the CeD, such as Irish setter dog, non-human primate, mice and rats^{1,2}.

The Irish Setters were used in the 1980s because they were naturally predisposed to gluten-sensitive enteropathy (GSE), which shared similarities with human CeD¹.

However, this model was first replaced by non-human primates, they provided high fidelity in mimicking human immune and digestive systems but were rarely used due to their elevated costs².

The animal that appeared to be most promising was the mouse because its genome lends itself to manipulation. Mice and Rats were used to investigate the role of HLA-DQ2 or HLA-DQ8 expressed in humans and to investigate gluten-specific T-cell responses mimicking only the immune component of CeD³. Moreover, mouse models revealed the role of IL-15 in the intestinal inflammation of CeD, reflecting the complex interplay between CeD and gluten⁴.

Mice can be transgenic and non-transgenic. In a study with transgenic mice, CD4⁺ T cells were isolated, and the mice were sensitised to gliadin via tail injections. These mice displayed changes in intestinal architecture, had altered adaptive immune responses and increased levels of IFN- γ and IL-7 in the duodenum. Therefore, they lacked autoimmune mechanisms, making them unsuitable as models for understanding the pathophysiology of CeD⁵.

Non-transgenic NOD (Non-Obese Diabetic) mice showed gluten sensitivity without the need for parenteral sensitization, marked by increased CD3⁺ intraepithelial lymphocytes (IELs) and reduced villus height after gluten consumption. A limitation of this model though was that the antibody response to tissue transglutaminase (tTG) appeared linked more to the inherent characteristics of NOD mice than to gluten exposure itself^{6,7}. However, all these models were not able to reproduce all the mechanisms related to CeD.

Organoids from CeD patients can be regarded as a good cellular model of CeD as they reproduce some of the nodal alterations present in the CeD intestinal epithelium, namely proliferation, inflammation, and alteration of the autophagy pathway⁸ (Appendix 1 and 2). Moreover, they allow to reveal the role of the epithelium in the pathogenesis of CeD.

This thesis aimed to collect intestinal organoids from control subjects (CTR), Celiac Disease (CeD) patients at different stages of the disease, EoE subjects, and subjects with co-morbidities related to CeD, such as patients with Type 1 Diabetes (T1D) and Down Syndrome (DS).

Results

Intestinal organoids from intestinal biopsies of children, with an age range between 1 year old and 18 years old, were collected. The biopsies were derived from CTRs, POT-CeD, GCD-CeD, GFD-CeD, EoE and patients with CeD comorbidities (T1D and Down Syndrome). One to two refused samples

from duodenal biopsies per individual, both from patients and controls, taken with esophagogastroduodenoscopy (EGD), were sufficient to isolate LGR5+ stem cells. The size of the biopsy fragment is not relevant, but the presence of crypts is necessary (Figure 1)

The isolated stem cells from crypts were plated in Matrigel and grown for 3-4 weeks. The presence of stimuli and growth factors allowed the crypt-derived stem cells to become, after 3-5 days of culture, organoids with a prevalence of intestinal epithelial cells (Figure 2).

All patients' and controls' data were inserted in an Excel database, and the following data were collected (Figure 3):

- **ID code**, the identification code this allowed them to be uniquely associated with that EGD preventing the same individual who had undergone several EGD procedures from having a single code despite being at different stages of the disease.
- **Personal Data**, each patient was identified with name, surname, date of birth.
- **EGD Data** prevents the same individual who had undergone several EGD from having a single code despite being at different stages of the disease.
- **Freezing Date**, samples can be frozen and stored and grown at different times from the time of the gastro stage, and it allows us to track the use of the sample over time.
- **Reason for EGD**, the reasons for which patients make an EGD are sometimes related to a simple suspicion of disease. Gastrointestinal symptoms may be common to several different pathological conditions, or sometimes they could be associated with functional disorders. These cannot be associated with diagnostic criteria, but catalogue samples are indicative of the type of symptoms and location within the different pathologies.
- **Type of disease**, this lane allowed, by acronyms, to distinguish different diseases, and possible subgroups within disease.
- **Marsh classification**, for each patient, not only for celiac disease patients, was done the histopathological evaluation and classified from T0 to T3 (divided into a, b and c).
- **Location** This last part of the database is associated with the position of freezing cells to make it easy to identify stored samples and give more details about cells' position in liquid nitrogen.

After this preliminary cataloguing, the organoids were grouped into subgroups as follows.

Control group (CTRs) of intestinal organoids included 75 subjects, all children that underwent to the EGD for gastrointestinal symptoms. As CTRs were used the organoids from subjects that had negative serological antibodies (EMA, anti-TtG IgA and IgG, calprotectin, and others), classified as Mash 0, and that did not present CD3, gamma delta, CDC25 or TTG deposits in the intestinal mucosa. The

final diagnosis of these patients was in almost all the cases “functional”, the patients that had a high BMI were anyway excluded (Figure 4).

Eosinophilic Esophagitis. At the beginning, these patients belonged to Control group, but these organoids showed an activation of inflammation markers higher than other controls for this reason they were analysed further to describe the duodenal inflammation and to be challenged by gluten peptides (Chapter 4).

The organoids from these patients were divided into 2 groups (active and remission) that were related to the number of eosinophils per field (eosinophils / High-power field) in the oesophagus (proximal, middle, and distal). The cut-off for defining disease status was 15 eos./HPF, which distinguished these subjects into active and remission stage of EoE. The collection of EoE included 19 organoids. They were divided into 2 subgroups Marsh T0 and Marsh T1 depending on the Marsh classification of the original biopsies. Of these 7 were in the remission phase of the disease (eos./HPF <15) and 3 were derived from EoE in the active phase of the disease (eos./HPF >15). Two organoids derived from patients in the remission phase of the disease were classified as T1 (Figure 4).

Gluten Containing Diet CeD (GCD-CeD) were patients with villous atrophy. The entire GCD-CeD group included 60 intestinal organoids, all of which were characterised by positive serology antibodies (EMA and anti-TtG IgA and IgG more than 9), these were divided into 3 subgroups according to the level of villus atrophy associated: Marsh's 3a with mild atrophy (11 organoids), 3b with a moderate villus (10 organoids) and 3c with total villus atrophy (39 organoids) (Figure 4).

Gluten Free Diet CeD (GFD-CeD) included CeD patients in the remission phase of the disease. Most of the GFD patients underwent to EGD because of gastrointestinal symptoms. Intestinal organoids derived from 7 GFD-CeD patients were collected, 4/7 were classified as T0 and 3/7 as T1, according to MARSH classification (Figure 4).

Potential CeD (POT-CeD), another group of the organoid collection, included patients that were characterised by the presence of a positive serology antibody (positive EMA and anti-TtG IgA and IgG more than 9) and a normal intestinal architecture (classified as T0 or T1). The total group collected was composed of 45 patients, 33/45 were T0 and 11/45 were T1 following MARSH classification. In previous studies, this category of patients has been followed up for several years and they had different “fates” either become celiac (43%) or did not develop atrophy independently from the presence of predisposing antibodies in the serum⁹.

Thirty-three organoids from POT-CeD were classified as T0, more than half of them were from patients who showed antibody persistence after 6 months and 1 year from the time of the biopsy, none of them was at GFD (Figure 4).

CeD and comorbidities

CeD has been associated with a higher risk of many other autoimmune diseases, such as T1D, liver disorders, a higher incidence of cancer, and malnutrition¹⁰. The incidence of CeD in T1D patients was 1-10%, and in some studies, it achieves 25%, probably caused by autoimmune predisposition. There were 9 intestinal organoids from T1D patients who had performed an EGD, after positive serology manifestation of EMA and anti-TtG IgA and IgA antibody. 2 out of 9 were classified as T0, and defined as POT-CeD with T1D, while the other 7 were in the active phase of the CeD and classified as T3 (Figure 4).

Interestingly patients with Down Syndrome (DS) have a risk ten times higher than of the non-Down population to manifest CeD. The prevalence of CeD in patients with Down syndrome was higher in the studies including only children with DS than in those including both adults and children and it is independent of the geographical location¹¹.

In our collection, a small subgroup (n=3) of intestinal organoids included patients with DS all of them presented gastrointestinal symptoms. They were derived from intestinal biopsies of DS patients with the following histology and serology: normal mucosa (a negative serology for CeD and normal architecture), celiac villus atrophy (positive serology for CeD and villus atrophy), not celiac villus atrophy (total villus atrophy in absence of serology antibody for CeD) (Figure 4).

EoE and CeD are both chronic immune-mediated gastrointestinal disorders, in which the mucosa is inflamed, and both are related to food antigens. The main antigen in CeD is gluten, for EoE there are several: milk, wheat, soy, eggs, tree nuts/peanuts, and fish/shellfish¹². Although they share at least an antigen, namely gluten, clinical and histopathological features are different¹³. CeD is characterised by villus atrophy in the duodenum, while EoE presents an increment of eosinophils in the oesophagus that are responsible for the formation of fibrotic tissue, but, by definition, no eosinophils are present in the duodenum of these patients.

The antigens elimination represents a possible strategy for both diseases. For CeD the antigen to be eliminated is gliadin, in the EoE several antigens have been identified. The elimination of several antigens is followed by periodic endoscopic examinations to identify one or more responsible foods¹³. Although there is not much data in literature demonstrating the relationship between EoE and CeD, and how they could influence each other, a review by Pelicano R et al. highlights that in CeD patients, mainly potentials that later, develop CeD, there can be high levels of eosinophils in the oesophagus.

They even suggest screening the oesophagus of these patients¹⁴. In our organoids collection, there was a REM- EoE patient who, after a few years developed CeD with Marsh T3c atrophy and positivity for celiac disease antibody in the serum. Another 3 organoids were from patients with GFD-CeD and EoE, 2/3 classified as T0 and the third one T1 (Figure 4). In the last two groups mentioned the number of organoids was limited as they were less frequent conditions.

In addition to the biobank of intestinal organoids from paediatric patients, we have also collected 6 organoids from adults with T1D. These included 5 men and only 1 woman, with an age range between 28 and 54. 2 out of 6 had atrophic gastritis, and other 2 out of 6 had CeD with T3 duodenal villus atrophy, one mild and one moderate grade (Figure 4).

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Figures

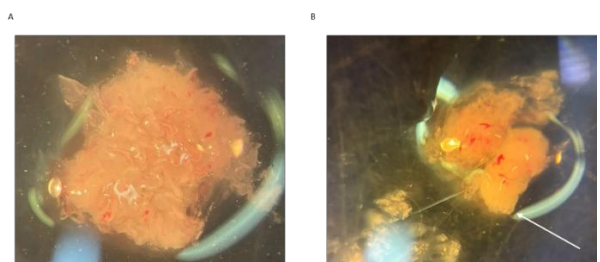


Figure 1. Representative images of two duodenal biopsies fragments from CTR and GCD-CeD observed under at stereoscopic microscope.

A. B. Villi are visible in firs image but absent in the second one.

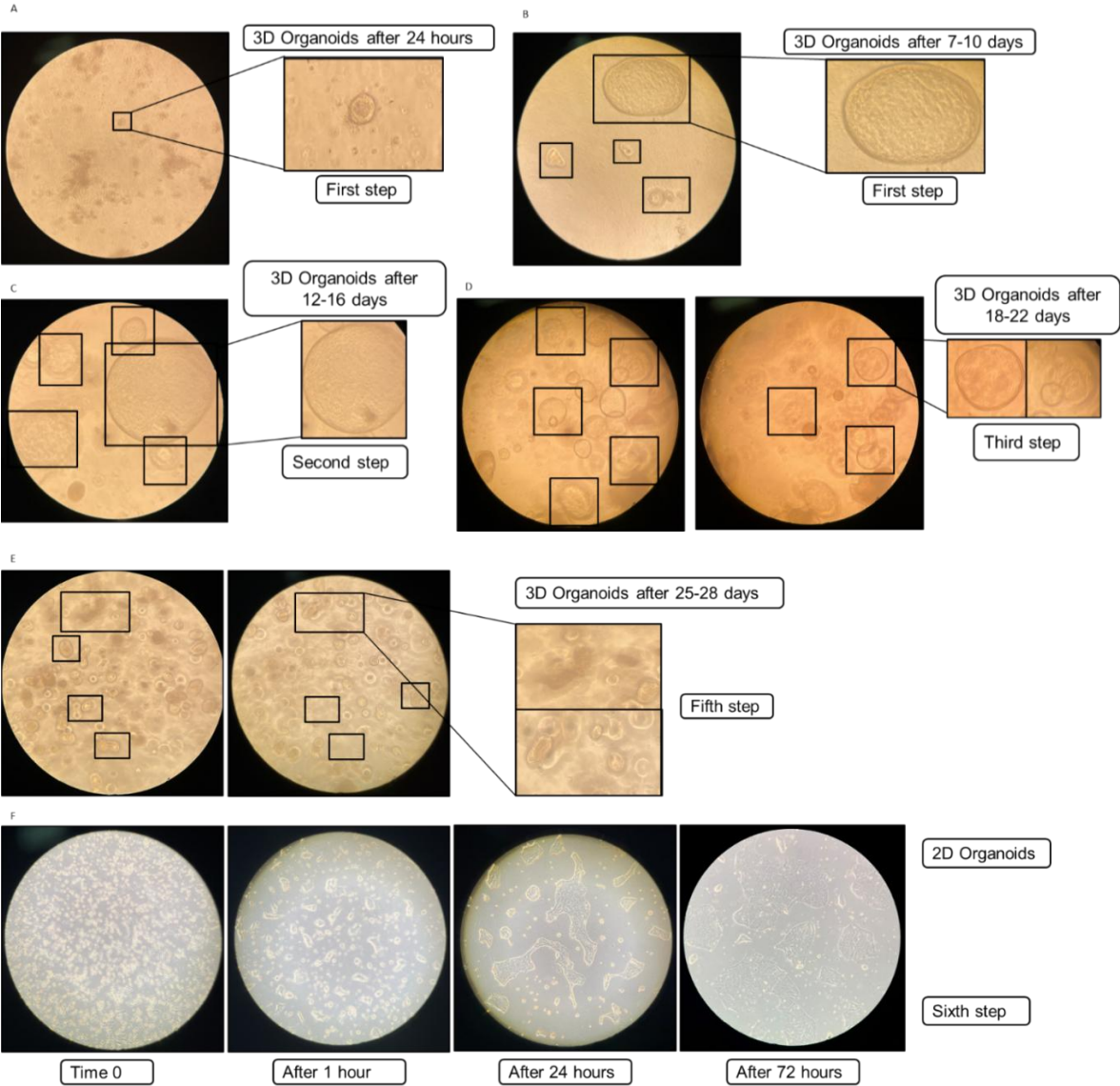


Figure 2. Optical microscope photographs were chosen to represent the confluence of intestinal organoids, both in 3D and in 2D, at different growth stages.

- (A) 3D intestinal organoids after 24 hours of plating crypts with LGR5+.
- (B) 3D intestinal organoids after 7-10 days of plating.
- (C) 3D intestinal organoids after 12-16 days of plating at second step.
- (D) 3D intestinal organoids after 18-22 days of plating at third step.
- (E) 3D intestinal organoids after 25-28 days of plating at fifth step.
- (F) 2D intestinal organoids at sixth step in different time of plating, at time 0, after 1h, 24 hours and 72 hours, immediately before the experiment.

A	B	C	D	E	F	G	H	I	J	K	L	M
ID Code	Surname	Name	Date of birth	EGD data	Freezing data	Reason of EGD	Type of disease	Marsh Classification	Location	BOX	Numbers of vials	Freezing passage

Figure 3. Schematic representation of database with all necessary information for intestinal organoids.

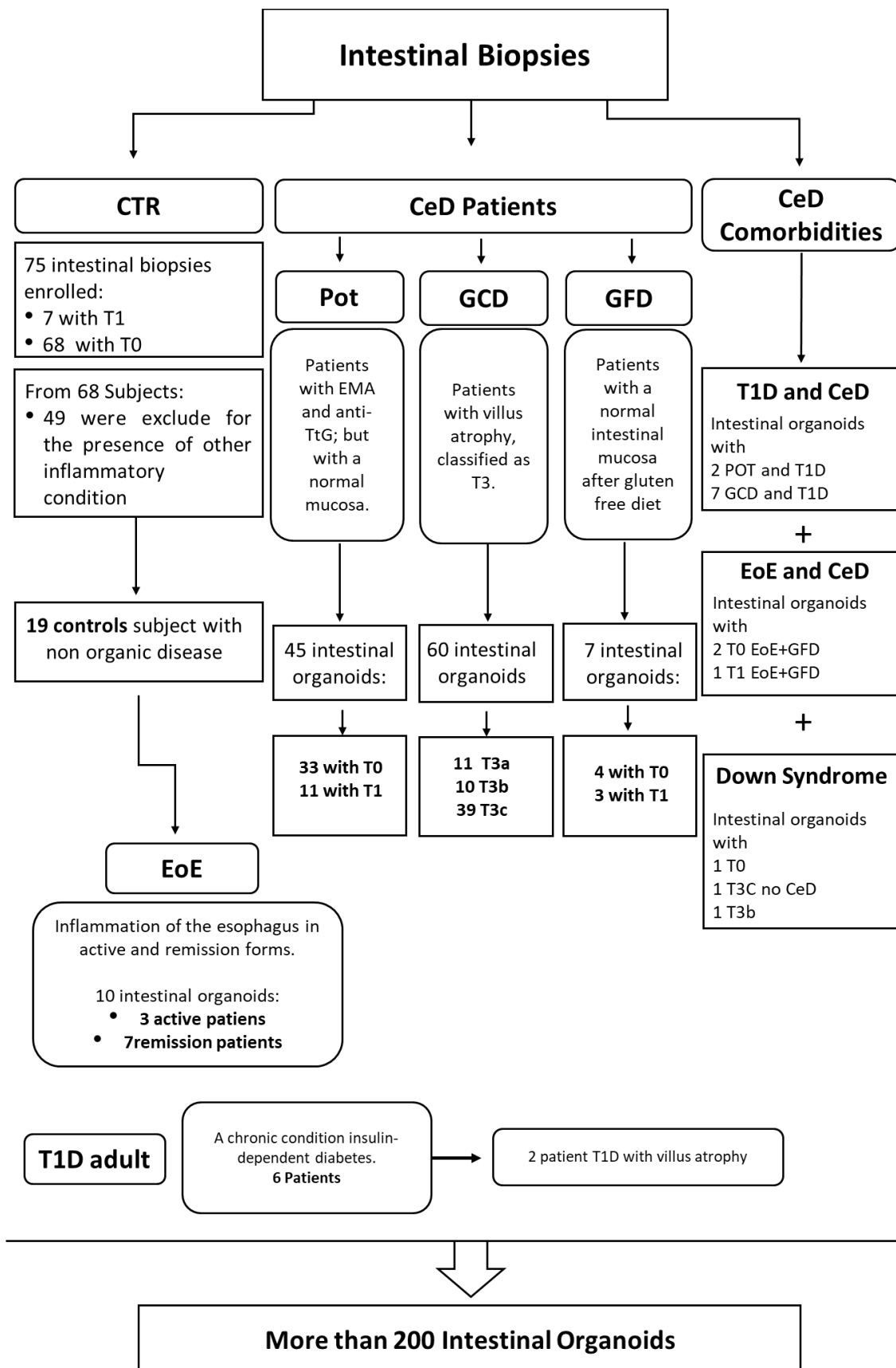


Figure 4. Schematic summary of intestinal organoids collection from subjects with inflammatory diseases and controls.

Chapter 2

Sustainable milk-based postbiotics beverage fermented by *L. plantarum*: an ally in combating inflammation

Claudia Bellomo^{1,2}, Federica Nigro³, Francesca Passananti³, Roberto Nigro³, Maria Vittoria Barone^{2*}, Merlin Nanayakkara^{2,4}

¹ Department of Translational Medical Science, Section of Pediatrics, University Federico II, Via S. Pansini 5, 80131 Naples, Italy.

² ELFID (European Laboratory for the Investigation of Food Induced Diseases), University Federico II, Via S. Pansini 5, 80131 Naples, Italy.

³ I.T.P. Innovation and Technology Provider s.r.l., Via Bisignano a Chiaia 68, 80121 Naples, Italy

⁴ Federico II University Hospital, 80131 Naples, Italy

* Correspondence:

Maria Vittoria Barone

mv.barone@unina.it;

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Abstract

Celiac disease (CeD) is an autoimmune disorder characterized by damage to the small intestine that occurs in genetically predisposed individuals after gluten consumption. The only treatment is a lifelong gluten-free diet. Gliadin, the main protein component of gluten, is found in wheat and other cereals and is poorly digested. One of its undigested peptides, P31-43, triggers several different processes, including inflammation. Intestinal organoids from CeD biopsies are good models for studying CeD inflammation. Postbiotics have been shown to modulate the effects of P31-43 in CaCo-2 cells and inflammation in CeD organoids. The aims of the present study were, on the one hand, to study the production of postbiotics from *L. plantarum* CECT 749-fermented milk enriched with LA (linoleic acid), SCGs (Spent Coffee Grounds) and SCG oil and, on the other hand, to study their anti-inflammatory effects in the presence of the gliadin peptide P31-43 in CaCo2 cells and constitutive inflammation in CeD organoids. The results showed that pretreatment with all milk-based postbiotics of *L. plantarum*, except for SCG oil, inhibited the activation of NF- κ B in the presence of the gliadin peptide in CaCo2 cells. The most efficient postbiotics, namely, milk-based postbiotics of *L. plantarum* with or without SCGs, could also reduce inflammation in intestinal organoids from CeD patients. In conclusion, milk-based postbiotics of *L. plantarum*, with or without SCGs, can prevent the proinflammatory effects of gliadin on CaCo-2 cells and constitutive inflammation in CeD intestinal organoids, independent of the CLA concentration.

1 Introduction

Celiac disease (CeD) is an immune-mediated enteropathy that primarily affects the small intestine in response to the ingestion of gluten, a protein present in wheat, barley, and rye. A gluten-free diet is the only therapy for these patients, who must eliminate gluten-containing cereals from their diet for life. CeD results from

interactions between genetic factors, which are characterized mainly by HLAs, Human Leukocyte Antigen, class I and II genes), and environmental factors, which contribute to the generation of inflammation in the intestine¹⁻³. Among the environmental factors that induce inflammation, nutrients, including gluten, play a central role. Nutrients may have two effects, proinflammatory or anti-inflammatory effects, and the microbiota may mediate their destiny⁴. However, how, when, and where inflammation is generated in CeD is still being determined⁵.

Gliadin, the main protein in gluten, is a protein that is difficult to digest by intestinal endopeptidases. Undigested gliadin peptides may have biological activity. Some of them can activate the adaptive immune response, as they are well presented to T cells. Other peptides, such as the p31–43 peptide, play key roles in the inflammatory/innate immune response to gliadin⁶. Specifically, this peptide has pleiotropic activity, including the induction of proliferation and inflammation with the activation of the nuclear transcription factor- κ B (NF- κ B) pathway⁷⁻⁹. NF- κ B is also a key protein in the regulation of inflammation in the intestine¹⁰. Moreover, the NF- κ B pathway has been found to be altered in intestinal biopsy samples from CeD patients, both at GCD and GFD^{11,12}, and in intestinal organoids¹². Interestingly, after gluten treatment, there was an increase in NF- κ B expression in CeD biopsy samples at both GCD and GFD^{11,13}.

Interestingly, in CeD biopsy samples and organoids, constitutive inflammation, characterized by an increase in IL6 and IL1 β , which are also part of the NF- κ B pathway, has been described even before the occurrence of the intestinal lesion^{12,14}. The constitutive inflammation of intestinal organoids from CeD patients is reduced in the presence of probiotics from *Lactobacillus paracasei* and *Lactobacillus rhamnosus* GG¹⁵⁻¹⁷.

Pro- and postbiotics and conjugated linoleic acids (CLAs) are helpful in treating inflammatory-related diseases¹⁸⁻²⁰. CLAs are polyunsaturated acids and are a mixture of positional and geometric isomers of linoleic acid, a polyunsaturated omega-6 fatty acid also known as cis, cis-9,12-octadecadienoic acid. Linoleic acid and CLA are essential fats that must be part of the diet. The concentration of CLAs can be increased naturally by the selection of ad hoc microorganisms and fermentation conditions. Linoleic acid is a precursor in the synthesis of CLA; it is often added to the matrix to be fermented in its pure form^{21,22} or by adding vegetable oils, such as safflower oil²³ and sunflower oil²⁴. Interestingly, the line guide written by Vitale and Giacco in 2020 defined roasted coffee waste, such as spent coffee grounds (SCGs), as foods which contains a high concentration of linoleic acid²⁵.

SCGs are the most abundant coffee byproduct²⁶, and in 2017, its worldwide production reached 9.6 million tons as described in International Coffee Organization (ICO). SCGs have been widely studied as a material for biodiesel production²⁷. However, in recent years, it has also been used for food applications.²⁶

The aims of the present work was both the sustainable production of a functional milk-based beverage fermented by *L. plantarum* CECT 749 that was enriched in CLAs after the addition of LA, SCGs and SCG oil, and the study of the potential anti-inflammatory effects of these compounds in the presence of P31-43 in CaCo-2 cells and CeD intestinal organoids.

2 Materials and methods

2.1 Fermentation experimental apparatus

The system consisted of a Pyrex batch reactor (20 cm , ID of 10 cm, 1.5 L) equipped with an external jacket to circulate a service fluid from a thermostatically controlled water bath. The mixing system consisted of a stainless-steel impeller equipped with three Rushton turbines. It was connected to a motor that adjusted the

stirring speed. The head plate was equipped with an input for the insertion of an In Pro 3100 probe (Mettler Toledo, Milan, Italy) that was connected to an M300 transmitter (Mettler Toledo, Milan, Italy), which is helpful for inline temperature/pH measurements, and an input connected to a tank containing a 4 M NaOH solution that was fed by a peristaltic pump for pH control.

2.2 Strain

L. plantarum CECT 749 (from the Spanish Type Culture Collection, CECT, Valencia Spain) is a gram-positive bacterium that has been studied for its antifungal activity²⁸ and ability to produce CLA^{21,23,29}. The strain was stored at -20 °C in cryovials containing 1 mL of a solution of animal-free broth (AFB; composition: 20 g/L Bacto Yeast Extract (BD Biosciences, Milan, Italy), 0.5 g/L MgSO₄ (Sigma–Aldrich, Milan, Italy), 50 g/L glucose (Sigma–Aldrich), 0.5 g/L citric acid (Sigma–Aldrich) and glycerol (20%, Sigma–Aldrich)). Before each fermentation, after thawing, the strain was revitalized by adding 9 mL of AFB and then incubated for 24 h at 37 °C. The revitalized strain had a bacterial concentration of 10⁸ CFU/mL, and the inoculum contained 1% v/v fermentation substrate.

2.3 Fermentation substrates and experimental conditions

The fermentation substrate was 1 L of skim milk (Granarolo® - UHT), which was purchased from a local market and contained 20 g/L glucose (Sigma–Aldrich, Milan, Italy). To test the microbial capacity in the production of CLA, different compounds, as CLA precursors, were used, allowing the following experimental conditions to be defined:

- Exp. Condition 1 (milk): Milk with only glucose as a negative control
- Exp. Condition 2 (milk + LA): Milk with glucose and 0.5 mg/mL free linoleic acid (LA, 99% purity; Sigma Aldrich, St. Louis, MO, USA), since a previous study suggested this concentration as the best concentration for maximum CLA production³⁰.
- Exp. Condition 3 (milk + SCG oil): Milk with glucose and 1.3 g of the oil extracted from *Passalacqua*® spent coffee grounds (*Passalacqua*®, Mexico blend type, 100% Arabica) to guarantee that the LA concentration was the same as that in Exp. Condition 2 (where the LA concentration in spent coffee grounds corresponds to 40% of the total fat in the spent coffee ground oil)³¹. The extraction method used to obtain the coffee oil is described below.
- Exp. Condition 4 (milk + SCG): Milk with glucose and 20 g of *Passalacqua*® spent coffee grounds; this amount was chosen since the literature reported that, to obtain the optimal LA concentration (0.5 mg/mL), 10 grams of dry waste would be needed. Wet spent coffee grounds contain an average of 50–60% water; therefore, to release the abovementioned amount of LA, two times the weight (20 grams) of spent coffee grounds was added³¹.

Glucose was added for each experimental condition to favour a more rapid fermentation process. The addition of linoleic acid, SCG oil, and raw SCGs to milk was followed by sonication to homogenize the matrix to be fermented and promote the dispersion and extraction of the fat phase in the dairy matrix.

For each condition tested, fermentation was carried out for 48 h at 37 °C. Fermented samples were withdrawn aseptically from the reactor at specific times (after inoculation (t₀) and after 24 (t₂₄) and 48 (t₄₈) hours).

2.4 SCG oil extraction method

SCG oil extraction was performed using a hydrofluorocarbon (HFC) solvent, 1,1,1,2-tetrafluoromethane (Norflurane, R134a), under subcritical conditions with a laboratory-scale system via a patented process (Garella

2012). Norflurane is a haloalkane refrigerant with a boiling point of 26.3 °C at atmospheric pressure and a vapour pressure of 6.61 bar at 25 °C (R Colucci Cante et al. 2020). Liquid Norflurane was percolated through a dry SCG matrix bed and placed in an extraction chamber at 8–10 bar to enrich its coffee oil content. The mixture was subsequently passed through an expansion vessel, where Norflurane was gasified at lower pressure (approximately 4–5 bar), and the oily solute was released at the bottom. Afterwards, the clean gaseous Norflurane was recompressed and recycled in liquid form to the extraction chamber, where the extraction phase was repeated.

2.5 Analytical methods

2.5.1 Microbiological analysis

A spread plate method was used to determine the vital bacterial load of the inoculum, the microbial concentration during the entire process, and the presence of contaminants. After serial dilutions, the sample was spread on MRS agar (Oxoid, Basingstoke, UK) for the enumeration of lactobacilli; MacConkey agar (Oxoid, Basingstoke, UK) and Gelatin peptone agar (Bioline, Milan, Italy) were used for the detection of eventual contaminants. All the plates were incubated at 37 °C for 48 h before being read. Anaerobic kits (Anaerogen Compact, Oxoid, Basingstoke, UK) were used for MRS plates to guarantee anaerobic growth conditions for *L. plantarum* CECT 749 during incubation.

2.5.2 Lactic acid quantification

The concentration of lactic acid was determined by high-performance liquid chromatography (HPLC) on an Agilent Technologies 1100 instrument equipped with an Agilent Synergi Hydro-RP C18 column (250 mm × 4.6 mm and a pore size of 4 µm) and a visible/UV detector. The mobile phase consisted of 0.27% KH₂PO₄ aqueous solution at a pH of 2 modified with H₃PO₄ (eluent A) and 100% methanol (eluent B), and a gradient consisting of 30% B in 2.6 min followed by 100% A in 2.9 min with a flow rate of 1 mL/min was used. The detection wavelength was set at 210 nm.

2.5.3 CLA analysis

Fat extraction was performed according to Stefanov et al.³². A total of 10 g of milk was mixed with 16 mL of previously prepared dichloromethane–ethanol (DM–E) solution (2:1 ratio, v/v) in a 40 mL centrifuge-grade glass test tube, vortexed for 90 s and then centrifuged for 8 min (2500 g at –4 °C, MPW Med. Instruments, MPW-352R, Warszawa, Polonia). The supernatant was discharged, and 10 mL of the DM–E solution was added to the test tube. The mixture was vortexed and then centrifuged for 6 min (2500 × g at –4 °C). The upper organic phase containing the milk fat was filtered (597½, 240 mm diameter, Schleicher & Schuell, Dassel, Germany), dichloromethane was removed by a reduced pressure rotary evaporator (Bibby Sterilin RE-100, Bibby Scharlau Italia S.r.l., Riozzo di Cerro al Lambro (MI) Italia), and the samples were evaporated until dryness was achieved. After extraction, the fats were transesterified according to the method of Ichihara et al. (Ichihara and Fukubayashi 2010). The lipid sample was dissolved in 0.20 mL of toluene, and then, 1.50 mL of methanol and 0.30 mL of the reagent mixture (consisting of 9.7 mL of HCl (35%, w/w) diluted with 41.5 mL of methanol) were added in this order. The tube was vortexed and then heated at 100 °C for one hour. After cooling, 1 mL of hexane and 1 mL of water were added to extract the methyl esters in the hexane phase. Finally, the samples were analysed via GC (Varian CP 3800 Gaschromatographer, Palo Alto, United States) equipped with an FID detector and a ZB WAX plus column (60 m 0.25 mm 0.2 µm, Phenomenex, Aschaffenburg, Germany), with helium used as the gas carrier (1.2 mL/min).

2.6 CaCo-2 cells and Treatment

Human colon adenocarcinoma-derived cells (CaCo-2) obtained from the Interlab Cell Line Collection (Centro di Biotecnologie Avanzate, Genoa, Italy) were grown in DMEM supplemented with 10% fetal calf serum, 100 units of penicillin–streptomycin/mL, and 1 mmol/L glutamine (Gibco Invitrogen, Milan, Italy). The cells were maintained in a humidified atmosphere (95% air and 5% CO₂) at 37 °C. The postbiotic milk-based beverages were obtained by thermal treatment (85 °C, 30 s). The postbiotic beverages were freeze-dried, resuspended in DMEM-free medium (2% w/v), and then centrifuged thrice at 1500 rpm for 5 minutes. The sequence of P31–43, an LPS-free synthetic peptide, was LGQQQPFPPQQPY (Caslo > 95% purity, MALDITOF analysis; DK-2800 Kongens Lyngby, Denmark), and it was used at 100 µg/mL^{15,16} (Appendix 4 and 5).

2.6.1 Cell viability and optimal concentrations of milk-based postbiotic.

For the cell viability experiment, more than 2x10⁶ cells were seeded in 60 mm culture dishes overnight for attachment. The next day, the cells were exposed in presence or not to milk-based probiotics and postbiotics supplemented with LA for 24 and 48 h of fermentation at concentrations between 0.1 and 10% for 1 h at 37 °C in DMEM-free medium. At the end of incubation, cell viability was assessed by a trypan blue dye exclusion assay based on the microscopic quantitation of live cells (unstained) and dead cells (blue cytoplasm). Cells were automatically counted using the Countess automated cell counter (Thermo Fisher Scientific, Milan, Italy). A high viability range was evident at concentrations of 0, 1%, 1%, and 2% (Supplementary Fig. 1 and Supplementary Fig. 2). As the CLA concentration in the matrix studied was the same after 24 and 48 h of fermentation and considering that the viability did not change at 0.1%, 1%, and 2%, we used the postbiotics after 24 h at concentrations of 1% and 2% weight/volume.

2.7 Intestinal Organoids

One to two duodenal biopsies per individual were taken with standard endoscopic EGD during routine gastroduodenoscopy from celiac disease patients with villus atrophy (MARSH T3c), and biopsy samples were placed in 10 mL of ice-cold PBS supplemented with 2 mM EDTA (cat. 15575020, Thermo Fisher, Milan, Italy) and 0.5 mM DDT (D0632, Sigma–Aldrich, Milan, Italy). After 60 min, Crypt units were isolated by using washing buffer (WB) containing penicillin (100 units/mL), streptomycin (0.1 mg/mL), l-glutamine (2 mM), and FBS (10%, v/v) in DMEM/F12 with HEPES on ice for 30 min. The digest was filtered through a 70 µm strainer (Falcon, Milan, Italy). Crypts were collected by centrifugation at 2800 rpm for 5 min. The supernatant was discarded, and the crypts were carefully resuspended in 40 µL of ice-cold Matrigel matrix (Corning 356231, Milan, Italy) to enable three-dimensional growth in 48-well plates; the plates were incubated in a cell culture incubator at 37 °C and 5% carbon dioxide for 10 min to allow the Matrigel to solidify. Afterwards, 300 µL of cell culture medium enriched with supplements (CM-S)¹² was added to each well and replaced every second day. For 2D organoids, organoids were seeded in six wells pretreated with Matrigel diluted at 1:40 in phosphate-buffered saline (PBS)^{12,15,16,33} (Appendix 1,2,4 and 5).

2.8 Western Blot

After treatment, the CaCo-2 cells were washed twice with cold PBS and resuspended in lysis buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 5 mM MgCl₂, 150 mM NaCl, 1% Triton, 1 mM PMSF, 1 mM VO₄, 100× Aprotinin, and 50× LAP, all of which were purchased from Sigma, Milan, Italy, except for LAP, which was obtained from Roche, Milan, Italy). The cell lysates were analysed using SDS-PAGE with a standard running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS) and transferred onto nitrocellulose membranes by a Trans-Blot Turbo transfer system (cat.1704158;Bio-Rad, Milan, Italy) in a stain free gel at 10% (#cat 4568033;Bio-Rad, Milan, Italy). The membranes were washed, blocked with 5% non-fat dry milk, and probed with onto nitrocellulose membranes (Whatman GmbH, Dassel, Germany). The membranes were blotted with rabbit anti-

pNF-kB (Elabscience, Microtech, Naples, Italy) and mouse anti-GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) (cat. G8795, Sigma–Aldrich). GAPDH or total protein intensity were used as loading controls. The bands were visualized using enhanced chemiluminescence (ECL) plus (Cat. 1705062 Bio-Rad Milan, Italy) with 2–10 min exposure times. The band intensity was evaluated by integrating all the pixels of a band after subtraction of the background to calculate the average of the pixels surrounding the band^{12,15,16,33} (Appendix 1,2,4 and 5).

2.9 Statistical analysis

Statistical analysis and graphics were obtained from Graph Pad Prism (San Diego, CA, USA). The means and standard deviations of the experimental data were calculated; their significance was evaluated by Student's *t* test and two way ANOVA multiple comparison, with only results that presented values of $p < 0.05$ considered significant.

3 Results

3.1 Spent coffee grounds (SCGs) improved bacterial growth and lactic acid production

We studied *Plantarum* CECT 749 bacterial growth under different conditions (Figure 1). In all cases, the initial concentration was approximately 10^6 CFU/mL, reaching $1.8 \times 10^8 \pm 2.6 \times 10^7$ CFU/mL after 24 h of fermentation in the case of the negative control (milk); $1.5 \times 10^7 \pm 7.1 \times 10^6$ CFU/mL when milk was supplemented with linoleic acid (milk + LA); $6.7 \times 10^7 \pm 2.3 \times 10^7$ CFU/mL when milk was supplemented with spent coffee ground oil (milk + SCGs oil); and $5.0 \times 10^8 \pm 7.1 \times 10^7$ CFU/mL when milk was supplemented with raw spent coffee grounds (milk + SCGs) (Figure 1A).

Milk supplemented with SCGs resulted in the best matrix for *L. plantarum* growth, reaching $6.6 \times 10^8 \pm$ CFU/mL at 48 h of fermentation, a value that was one log higher than that observed for milk fermented with LA ($5.8 \times 10^7 \pm 2.5 \times 10^7$ CFU/mL) and that observed for milk fermented with SCG oil ($6.2 \times 10^7 \pm 1.6 \times 10^7$ CFU/mL) (Figure 1A).

The amount of lactic acid produced in fermented milk (Figure 1B) in the presence of SCGs was 5.46 ± 0.05 g/L after 24 h ($p < .05$ respect to Milk, Milk with LA and Milk with SCG oil) and 12.66 ± 0.88 g/L after 48 h ($p < .05$ respect to Milk with LA) of fermentation. Milk fermented with only glucose (negative control) showed lactic acid production (lactic acid at 24 h: 1.46 ± 0.12 g/L; at 48 h: 3.94 ± 0.10 g/L) amounts that were similar to that measured in milk fermented with LA (lactic acid at 24 h: 0.98 ± 0.22 g/L; at 48 h: 2.05 ± 0.24 g/L) and with SCG oil (lactic acid at 24 h: 1.32 ± 0.13 g/L; at 48 h: 2.99 ± 0.15 g/L).

3.2 LA supplementation of milk promoted CLA production during fermentation with *L. plantarum*

The concentration of total CLAs found during fermentation is shown in Figure 2. SCG oil and SCGs, which were used as sources of linoleic acid, were tested for CLA production. CLAs were present at T0 in the samples containing SCG and SGC oil even before fermentation, with concentrations of approximately 0.5 g/L for both samples. The difference was statistically significant for both SCG and SGC oil respect to the sample milk added with LA ($p < .05$ for both matrix) (Figure 1C). Milk fermented alone is not represented in the graph because no CLAs were detected, as expected. Milk fermentation in the presence of LA was the only sample in which CLAs were produced, with concentrations of 1.52 ± 0.06 g/L ($p < .05$) and 1.29 ± 0.04 g/L ($p < .05$) after 24 h and 48 h, respectively.

3.3 Pretreatment of milk-based postbiotics with or without the addition of LA or SCGs prevented the P31–43-induced increase in pNF-kB

We tested the effects of milk-based postbiotics on CaCo-2 at concentrations of 1 and 2%. Interestingly, while a concentration of 1% (Supplementary Fig. 3) did not inhibit P31-43-induced inflammation, a relatively high concentration of 2% was very effective (Fig. 2A and B). Indicating that the concentration of the post biotic is an important factor for its activity. CaCo-2 cells were treated with milk-based postbiotics with the addition of LAs to understand whether CLAs exert different biological effects on inflammation pathways. CaCo-2 cells were pretreated with milk-based *L. plantarum* postbiotics at 2% w/v, with or without the addition of LA, for 30 minutes, and subsequently stimulated with P31-43. To assess inflammation, we evaluated the phosphorylation of NF- κ B by WB. Our data revealed an increase in pNF- κ B levels after stimulation with P31-43. Both milk-based postbiotics (with and without the addition of an LA source) reduced the level of pNF- κ B induced by P31-43. These findings indicated that milk-based postbiotics with free LA achieved the same pNF- κ B reduction effect as milk-based postbiotic with only *L. plantarum* (Fig. 2, A and B, $p < .05$ for both).

Additionally, we evaluated the effects of milk-based postbiotics added with SCGs and SCG oil in CaCo-2 cells in the presence of the gliadin peptide. For this purpose, CaCo-2 cells were pretreated with milk-based postbiotics at 2% w/v for 30 minutes and subsequently stimulated with P31-43, and the activation of pNF- κ B was evaluated via WB. Pretreatment with milk-based postbiotics combined with SCGs reduced in a statistically significant way the levels of pNF- κ B induced by P31-43 (Fig. 2, C and D; $p < .05$). However, milk-based postbiotics with SCG oil did not significantly inhibit the increase in pNF- κ B levels induced by P31-43 (Fig. 2, C and D).

The milk-based postbiotic with SCGs was more efficient than the milk-based postbiotic in preventing P31-43-induced activation of NF- κ B under the same conditions (Fig. 2, E and F). In fact, the mean phosphorylation of NF- κ B in CaCo-2 cells pretreated with the milk-based *L. plantarum* postbiotic with SCGs was half than that of NF- κ B in CaCo-2 cells pretreated with the milk-based *L. plantarum* postbiotic, although a significant difference was not detected between the two groups analysed.

3.4 In CeD intestinal organoids milk based postbiotic of *L. plantarum* with or without SCGs reduced pNF- κ B

The milk-based postbiotic activity of *L. plantarum* was tested in intestinal organoids derived from CeD patients with villus atrophy and compared with that of intestinal organoids derived from control subjects. To test postbiotic activity, intestinal organoids were opened in 2D on Matrigel pretreated plates, and treated with milk-based postbiotics from *L. plantarum* with or without SCGs for 3 hours. Compared with CTRs organoids, intestinal organoids from CeD patients were inflamed (Fig. 3, A and B; $p < .01$). In fact, CeD organoids presented increased NF- κ B phosphorylation levels, as measured by WB ¹²(Appendix 1). Compared with that of the untreated CeD intestinal organoid, the pNF- κ B of the milk-based *L. plantarum* postbiotic with or without the addition of SCGs was reduced by 4.63- and 5.42-fold, respectively (Fig. 3, A and B; $p < .05$ for both). Interestingly, the levels of pNF- κ B in intestinal organoids from CeD patients after treatment with milk-based *L. plantarum* postbiotics with and without SCGs were similar to the levels found in intestinal organoids from controls.

4. Discussion

We demonstrated that both the bacterial growth and lactic acid production of *L. plantarum* fermented in milk were negatively affected by the presence of LA and SCG oil; in contrast, the presence of SCGs in fermented milk promoted both bacterial growth and LA production with respect to *L. plantarum* grown without any additives. *L. plantarum* could convert free LA to conjugated linoleic acids (CLAs) as a source of LA. We used free LA, SCGs or SCG oil. The maximum amount of CLAs was produced after 24 h of fermentation in milk supplemented with LA, with a higher CLA concentration being achieved than other concentrations reports in the literature where the same fermentation conditions³⁰ or the same microorganisms²¹ were used. During fermentation in milk supplemented with SCGs or SCG oil, no CLA production was observed, probably because CLAs were already abundant in the matrix supplemented with the coffee byproducts.

The higher bacterial growth and lactic acid production observed when SCGs was added to milk could be due to the presence of some oligosaccharides³⁴, such as manno oligosaccharides (MOS) and galactomannans, for which previous works have already shown the prebiotic effect on lactic acid bacteria³⁵. SCGs has already been used as a substrate to be fermented by lactic acid bacteria to produce lactic acid^{36,37}, after preliminary chemical and enzymatic pretreatments. In the present work, *L. plantarum* was able to metabolize SCGs and to use it as prebiotic without any pretreatment, to the best of our knowledge this has been unreported before.

SCGs are a rich source of polysaccharides (more than 50% by dry weight), such as cellulose and hemicellulose; among the sugars, mannose, galactose, and glucose are the most abundant. Lignin is also present in significant amounts (close to 25%), and the total fibre content is 60%³⁸. Approximately 20% of SCGs consist of proteins. SCGs have antioxidant potential³⁸ because of the presence of phenolic compounds such as chlorogenic acid, caffeine, and flavonoids; considering its high fibre content, high concentration of linoleic acid and anti-inflammatory potential, it can be considered for the development of functional foods.

In the present study, we investigated the effects of milk-based postbiotics obtained from *L. plantarum* CECT 749 with and without free LA, SCGs or SCG oil on inflammation in CaCo-2 cells after treatment with the gliadin peptide P31–43 and in intestinal organoids from CeD patients. Intestinal epithelial cells derived from intestinal carcinoma, CaCo-2 cells, or staminal intestinal cells such as intestinal organoids were used to test the ability of milk-based postbiotics obtained from *L. plantarum* CECT 749 with and without free LA, SCGs or SCG oil to prevent gliadin peptide P31–43-induced inflammation.

CaCo-2 cells are widely used as an in vitro model to study the intestinal barrier and intestinal absorption in patients with celiac disease and gluten sensitivity. After exposure to gluten peptides, CaCo-2 cells exhibit increased intestinal permeability, and several other pathways, such as inflammation are altered⁸. Conte et al. demonstrated that in the presence of both P31–43 and PTG, CaCo-2 cells were inflamed and exhibited increased pNF- κ B levels¹⁵. In the same model, postbiotics from both *L. paracasei* and *L. rhamnosus* GG prevented the proinflammatory effects induced by gliadin peptides on CaCo-2 cells^{15,16} (Appendix 4 and 5).

We investigated the effects of milk-based postbiotics obtained by the fermentation of *L. plantarum* alone and after the addition of free LA, SCGs, and SCG oil on P31–43-induced inflammation in CaCo-2 cells. The effect was observed only with 2% fermented products, indicating that their activity is concentration dependent and that *L. plantarum* with added LA was able to prevent the effects of P31–43 on NF- κ B activation. Although *L. plantarum* with SCGs was more efficient than *L. plantarum* alone in preventing P31–43 activity, the difference was not statistically significant. *L. plantarum* with SCG oil alone was not able to prevent the effects of P31–43 on CaCo2 cells. Considering that only *L. plantarum* with free LA was able to increase the amount of CLAs produced, it is very likely that the effects on P31–43-induced inflammation do not depend on CLA production. We do not know why *L. plantarum* lost its activity after treatment with SCG oil; very likely, the high-fat matrix was not ideal for the production of one or more metabolites needed for the anti-inflammatory effect.

Intestinal organoids, which mimic miniature of the intestine, are a cell model used to study pathogenic agents and mechanisms related to celiac disease, such as inflammation^{39,40}. They generally develop from staminal cells extracted from intestinal biopsy samples. They generally grow in 3D structures embedded in a matrix containing fibronectin, and the spherical shape mimics the polarized epithelial monolayer, with the apical part of the epithelial cells looking towards the centre of the spheroid. To treat them, they have to be opened in 2D, providing simpler, high-throughput models for studying cell interactions and barrier function^{41,42}. Organoids are good models for studying some pathogenetic mechanisms of CeD, such as proliferation and constitutive inflammation, because they reproduce these alterations found in CeD biopsy samples^{12,33} (Appendix 1 and Appendix 2). Moreover, other studies demonstrated that treatment with *L. paracasei* and *L. rhamnosus* GG was able to reduce constitutive inflammation in CeD intestinal organoids^{15,16} (Appendix 4 and Appendix 5). In

this study, we show that milk-based postbiotics of *L. plantarum*, with or without SGCs, can reduce the inflammation found in CeD organoids with comparable efficiency.

The mechanism by which milk-based postbiotics obtained by the fermentation of *L. plantarum*, with and without the addition of LA or SGCs, exert their biological effects after treatment with P31–43 in CaCo-2 cells and CeD intestinal organoids still needs to be clarified. The possible ability of milk-based postbiotics of *L. plantarum* with SGCs to prevent P31–43-induced inflammation could be associated with the presence of molecules with antioxidant activity and anti-inflammatory effects in this waste product⁴³. This observation confirms the possibility of reusing food waste from coffee processing to produce functional foods with improved beneficial properties. This enhanced effect of SGCs cannot be described on the constitutive inflammation of CeD organoids. This is probably because different inflammatory pathways are engaged in intestinal organoids.

In conclusion, milk-based postbiotics of *L. plantarum*, with or without SGCs, can prevent P31–43-induced inflammation in CaCo-2 cells and reduce constitutive inflammation in CeD organoids.

These preclinical studies provide a good basis for initiating clinical trials in celiac patients to prevent the proinflammatory effects of gliadin peptides. In particular, it would be interesting to test the ability of postbiotics to prevent disease in potential patients who have anti-transglutaminase antibodies but have not yet developed the intestinal lesions typical of celiac disease. However, more studies are needed to investigate the related safety parameters and biological activity of postbiotics in vivo. Despite patients' best efforts, some subjects can experience continuous exposure due to cross-contamination or traces of gluten in food. These risks could, in some cases, compromise the health and quality of life of these patients. Therefore, it is generally useful to study compounds that can prevent the inflammatory effects of gliadin with the hope of reducing the burden of living with celiac disease and improving long-term health outcomes.

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5. Figures

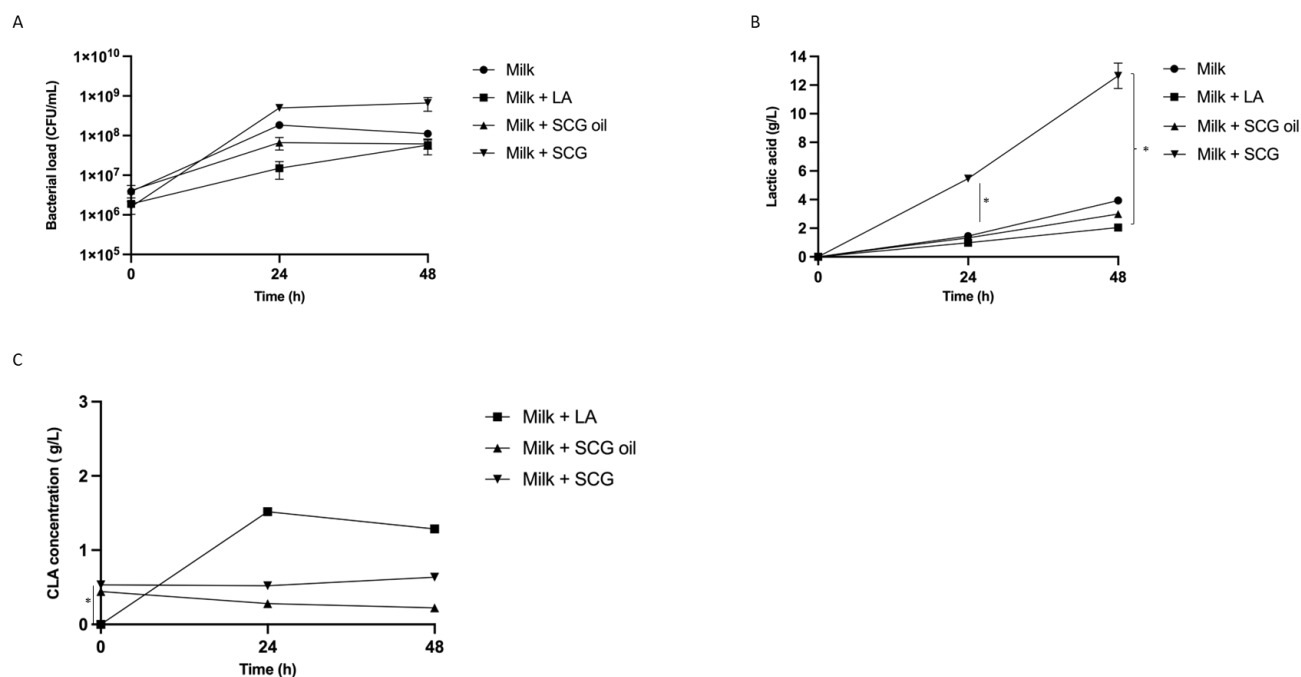


Figure 1. Bacterial growth, lactic acid production and CLAs concentration in milk-based postbiotics with linoleic acid (LA), Spent Coffee Grounds (SCGs) and SCG oil.

(A) Bacterial growth (CFU/mL) observed after 0, 24 and 48 hours of fermentation in milk alone (milk, circles), milk fermented with linoleic acid (milk + LA; squares), milk fermented with spent coffee ground oil (milk + SCG oil; triangles) and milk fermented with raw spent coffee grounds (milk + SCGs; inverted triangles). The bars represent the standard deviations of three independent experiments. Different letters indicate significant differences. ANOVA test: $*=p<.05$.

(B) Lactic acid production observed after 0, 24 and 48 hours of fermentation in milk alone (milk, circles), milk fermented with linoleic acid (milk + LA; squares), milk fermented with spent coffee ground oil (milk + SCG oil;

triangles) and milk fermented with raw spent coffee grounds (milk + SCGs; inverted triangles). The bars represent the standard deviations of three independent experiments. Different letters indicate significant differences ANOVA test: $*=p<.05$.

(C) Concentration of total CLAs (g/L) observed after 0, 24 and 48 hours of fermentation in milk fermented with linoleic acid (Milk + LA; squares), milk fermented with spent coffee ground oil (milk + SCG oil; triangles) and milk fermented with raw spent coffee grounds (milk + SCGs; inverted triangles). The bars represent the standard deviations of three independent experiments. Different letters indicate significant differences ANOVA test: $*=p<.05$.

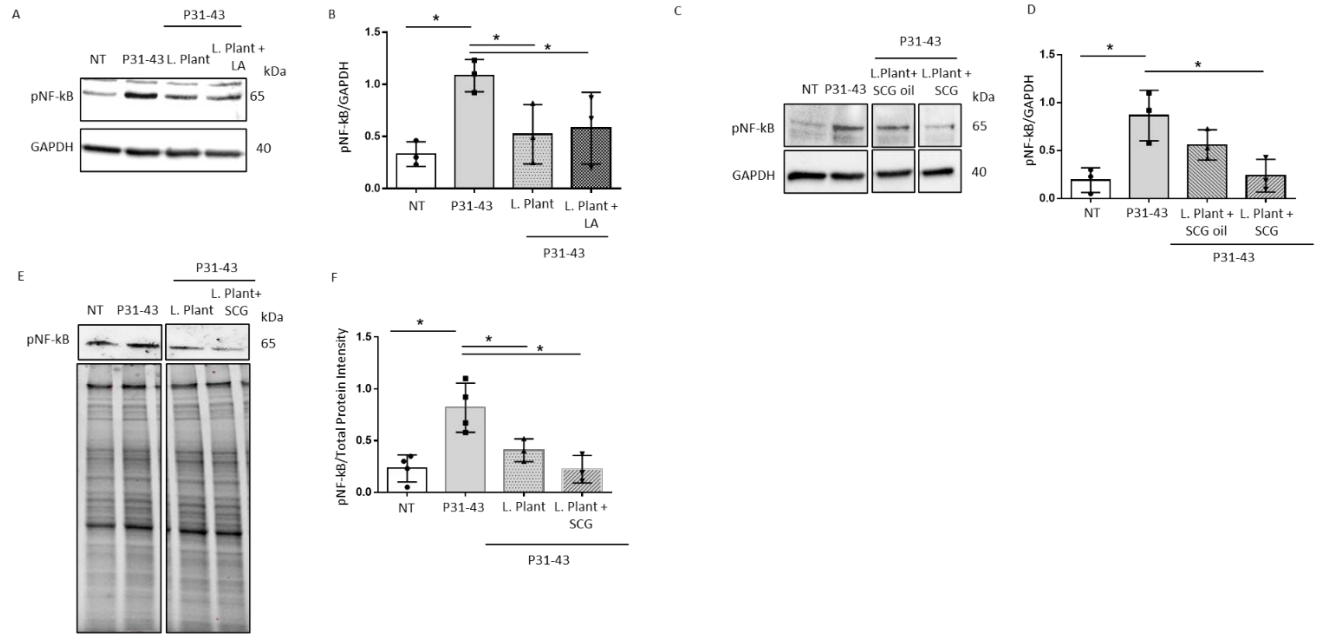


Figure 2. Effects of milk based postbiotics of *L. Plantarum* CECT 749, with or without the addition of LA, SCG oil or SCGs at 2% concentration, after 24 hours of fermentation on CaCo-2 cells after treatment with P31-43.

(A) Western blot analysis of protein lysates from untreated CaCo-2 cells (NT), those treated with P31-43 for one hour, and those pretreated with milk based postbiotic of *L. Plantarum* CECT 749 after 24 hours of fermentation with the addition of LA, blotted with antibodies against pNF-kB. GAPDH was used as a loading control. The immunoblotting analysis was representative of three independent experiments.

(B) Densitometric analysis of bands from the WB shown in A. Columns represent the mean, and bars represent the standard deviation of the relative intensity of pNF-kB with respect to the total GAPDH protein. Student's *t* test: $*=p<.05$.

(C) Western blot analysis of protein lysates from untreated CaCo-2 cells (NT), those treated with P31-43 for one hour, and those pretreated with milk-based postbiotic of *L. plantarum* CECT 749 after 24 hours of fermentation with the addition of SCG oil and SCGs, were blotted with antibodies against pNF-kB. GAPDH was used as a loading control. The immunoblotting analysis was representative of three independent experiments.

(D) Densitometric analysis of bands from the WB shown in A. Columns represent the mean, and bars represent the standard deviation of the relative intensity of pNF-kB with respect to the total GAPDH protein. Student's *t* test: $*=p<.05$.

(E) Western blot analysis of protein lysates from untreated CaCo-2 cells (NT), those treated with P31-43 for one hour, and those pretreated with milk based postbiotic of *L. plantarum* CECT 749 after 24 hours of fermentation,

and alone and with the addition of SCGs, were blotted with antibodies against pNF-kB. Stain-free gel was used as a loading control. The immunoblotting analysis was representative of three independent experiments. (F) Densitometric analysis of the bands from the WB shown in A. Columns represent the mean, and bars represent the standard deviation of the relative intensity of pNF-kB with respect to the total protein intensity. Student's t test: * = $p < .05$.

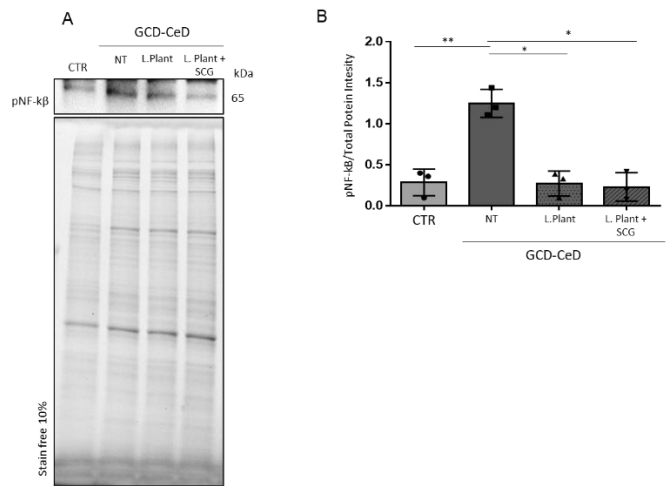


Figure 3. Effects of the milk-based postbiotic of *L. plantarum* CECT 749 and milk-based postbiotic of *L. plantarum* alone or with the addition of SCGs in intestinal organoids of CeD patients. (A) Western blot analysis of protein lysates from organoids from CeD patients, untreated and treated with milk-based postbiotic of *L. plantarum* alone or with the addition of SCGs for 3 hours, were blotted with antibodies against pNF-kB. (B) Densitometric analysis of the bands from the WB shown in A. Columns represent the mean, and bars represent the standard deviation of the relative intensity of pNF-kB with respect to the total protein intensity. Student's t test: * = $p < .05$.

6. Tables

Patients	Age range (Years)	Sex	Biopsy (marsh classification*)	Serum antiTG2 (U/mL)	Anti-endomysial antibody (EMA)
CTR	4-15	M=2 F=1	3=T0	Negative	Negative
GCD-CeD	3-17	M=2 F=1	3=T3c	>100	Positive

Table 1. Organoids characteristics.

T3: Flat destructive lesion (c: total).

504 **7. Supplementary Figure**

505

	[p/v]	Vitality	Total	Live	Death
Untreated		99%	2.5x10 ⁶	2.47 x10 ⁶	2.5x10 ⁴
Probiotic + LA (24h)	10%	5%	1.3x10 ⁶	6.5x10 ⁴	1.23x10 ⁶
	5%	34%	1.2x10 ⁶	4.08x10 ⁵	7.9x10 ⁵
	2%	93%	1.4x10 ⁶	1.3x10 ⁶	1x10 ⁵
	1%	98%	9.8x10 ⁵	9.6x10 ⁵	2x10 ⁴
	0.1%	98%	1.5x10 ⁶	1.47x10 ⁶	3x10 ⁴
Postbiotic + LA (24h)	10%	9%	1.2x10 ⁶	1.08x10 ⁵	1.09x10 ⁶
	5%	37%	1.7x10 ⁶	6,20x10 ⁵	1.08x10 ⁶
	2%	94%	1.3x10 ⁶	1,22x10 ⁶	8x10 ⁴
	1%	98%	1.0x10 ⁶	9,8x10 ⁵	2x10 ⁴
	0.1%	97%	1.6x10 ⁶	1.55x10 ⁶	5x10 ⁴

506 **Supplementary Figure 1. Caco-2 cell survival evaluated after treatment, for one hour, with different**
507 **concentration of milk based probiotic and postbiotics, of L. Plantarum CECT 749 after 24 hours of**
508 **fermentation with the addition of LA.**

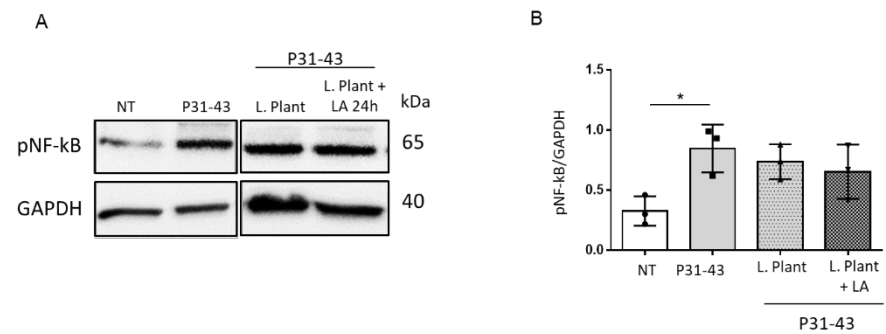
509 Cell viability was assessed by trypan blue staining after one hour of exposure to the postbiotics in five different
510 concentration from 10 to 0,1 [% p/v]. Untreated cells were used as negative controls.

511

	[p/v]	Vitality	Total	Live	Death
Untreated		99%	2.5x10 ⁶	2.47 x10 ⁶	2.5x10 ⁴
Probiotic + LA (48h)	10%	5%	1.3x10 ⁶	6.5x10 ⁴	1.23x10 ⁶
	5%	34%	1.2x10 ⁶	4.08x10 ⁵	7.9x10 ⁵
	2%	93%	1.4x10 ⁶	1.3x10 ⁶	1x10 ⁵
	1%	98%	9.8x10 ⁵	9.6x10 ⁵	2x10 ⁴
	0.1%	98%	1.5x10 ⁶	1.47x10 ⁶	3x10 ⁴
Postbiotic + LA (48h)	10%	9%	1.2x10 ⁶	1.08x10 ⁵	1.09x10 ⁶
	5%	37%	1.7x10 ⁶	6,20x10 ⁵	1.08x10 ⁶
	2%	94%	1.3x10 ⁶	1,22x10 ⁶	8x10 ⁴
	1%	98%	1.0x10 ⁶	9,8x10 ⁵	2x10 ⁴
	0.1%	97%	1.6x10 ⁶	1.55x10 ⁶	5x10 ⁴

512 **Supplementary figure 2. Caco-2 cell survival evaluated after treatment for one hour with different**
513 **concentration of milk based probiotic and postbiotics, of L. Plantarum CECT 749 after 48 hours of**
514 **fermentation with the addition of LA.**

Cell viability was assessed by trypan blue staining after one hour of exposure to the postbiotics in five different concentration from 10 to 0,1 [% p/v]. Untreated cells were used as negative controls.



Supplementary figure 3. Effects of milk based postbiotic of *L. Plantarum* CECT 749 with and without the addition of LA in CaCo2 cells in presence of P31-43 at 1% of concentration.

(A)Western blot analysis of protein lysates from Caco-2 cells untreated (NT), treated with P31-43 for one hour, and pretreated with milk based postbiotic of *L. Plantarum* with and without the addition of LA were blotted with antibodies against pNF-kB. GAPDH was used as a loading control. The immunoblotting analysis was representative of three independent experiments. (B) Densitometric analysis of bands from WB as in A. Columns represent the mean, bars the standard deviation of the relative intensity of pNF-kB respect to total GAPDH protein. Student's t-test = * $p < 0.05$, ** $p < 0.01$.

Chapter 3

Crosstalk between inflammation and autophagy in CeD organoids

Claudia Bellomo^{1,2}, Francesca Furone^{1,2}, Renata Auricchio^{1,2}, Vittoria D'Esposito^{3,4}, Pietro Formisano^{3,4}, Genoveffa D'Aniello², Roberta Rotondo⁵, Maria Vittoria Barone^{1,2} and Merlin Nanayakkara^{2,6}

1. Department of Translational Medical Science, Section of Pediatrics, University Federico II, Via S. Pansini 5, 80131 Naples, Italy.
2. ELFID (European Laboratory for the Investigation of Food Induced Diseases), University Federico II, Via S. Pansini 5, 80131 Naples, Italy.
3. National Council of Research, URT of the Institute of Experimental Endocrinology and Oncology "G. Salvatore" Naples, Italy.
4. Department of Translational Medicine, Federico II University of Naples, Via S. Pansini 5, 80131 Naples, Italy.
5. Department of Medicine and Surgery, University of Parma, Parma, Italy.
6. Federico II University Hospital, 80131 Naples, Italy

*Corresponding author: Maria Vittoria Barone

e-mail: mv.barone@unina.it

Tel: 0817464569

Abstract

Celiac disease (CeD) is a small bowel disorder characterised by mucosal inflammation, villous atrophy, and crypt hyperplasia, induced by gliadin peptides in genetically predisposed individuals. In CeD intestinal epithelium alterations of inflammation, intracellular vesicular trafficking, mTOR signalling and autophagy have been demonstrated. Our aim was to investigate the interplay between autophagy and inflammation in intestinal biopsies and organoids from CeD patients. Intestinal biopsies from gluten-containing-diet-CeD (GCD-CeD) p62(p<.05), pE4-BP1 (eIF4E-binding-protein/phosphorylated; p<.01) and pNF-kB (nuclear-factor-kappa-light-chain-enhancer of activated-B/cells phosphorylated; p<.05) protein levels were increased respect to controls biopsies (CTRs) by Western Blot analysis (WB). In intestinal organoids from GCD-CeD and gluten-free-diet-CeD (GFD-CeD) biopsies, p62(p<.05), pE4-BP1(p<.01) and pNF-kB(p<.05) protein levels were increased respect to CTRs. Immunofluorescence staining confirmed the increase of p62 in CeD patients. Bafilomycin and rapamycin were used respectively to block and induce the autophagy flux. Bafilomycin increased p62, pE4-BP1 and pNF-kB in CTRs organoids while rapamycin reduced p62, pE4-BP1 and pNF-kB levels in CeD organoids. Bio-Plex analysis showed the increase of 22/27 cytokines in culture media (CM) from GCD-CeD organoids and 7/27 in CM from GFD-CeD organoids. CTRs organoids treated with CM from GCD-CeD organoids presented reduction of autophagic flux and increase of inflammation respect to the untreated organoids. In conclusion, a

crosstalk between autophagy and inflammation is present in both CeD biopsies and intestinal organoids.

Keywords: Celiac disease, autophagy, mTOR pathway, inflammation, intestinal organoids, cytokinome, p62, pE4BP, pNF-kB.

1. Introduction

Celiac disease (CeD) is a multifactorial chronic disease induced by food. Wheat proteins and related prolamins from cereals induce an immune-mediated enteropathy in genetically susceptible individuals. CeD is also characterized by a variety of gluten-induced symptoms and the generation of CeD-associated auto-antibodies¹. The HLA-restricted gliadin specific intestinal T cell response plays a central role in the pathogenesis of CeD². The therapy consists in a lifelong diet without gluten (GFD).

Data from the literature indicates that intracellular vesicular trafficking is altered in CeD cells. A delay of the endocytic trafficking from the early to the late vesicles is also present at the level of the intestinal epithelial cells³. Vesicular trafficking is a sort of crossroad for cell signalling and can regulate several different pathways including proliferation and inflammation. Both these pathways have been found altered in CeD intestinal biopsies and, at the level of the intestinal epithelium¹.

The recent literature describes in CeD a meeting of several different factors such as cellular vulnerability, pro-inflammatory effects of gluten and other wheat proteins, western diet, and environmental triggers, such as viruses, that prepare, and /or amplify, the T cell-mediated response to gluten⁴. Multiple endogenous and exogenous factors can create a pro-inflammatory environment in the CeD intestine, leading to an expansion of gliadin-specific T cells in genetically susceptible individuals and further shifting them towards a pro-inflammatory phenotype⁴. The natural history of CeD, studied prospectively in subjects at risk, revealed an early inflammatory phase, even pre-gluten, which precedes the autoimmune response to gluten mediated by T-cells⁵. A CeD cellular phenotype has also been identified, most likely genetically/epigenetically determined, which is characterized by constitutive cellular alterations, including inflammation, that could generate a greater sensitivity of the celiac cells to some pro-inflammatory environmental factors (foods and gluten itself, infections etc.)^{6,7}. Most of the alterations found in the inflammatory pathways have been described at the level of the intestinal epithelium, both analysed in the biopsies and in intestinal organoids⁸ (Appendix 1). Interestingly inflammatory markers such as pNF-kB (phosphorylation of nuclear factor kappa-light-chain-enhancer of activated B cells), IL-6 (interleukine-6) and IL-1 β have been found altered in

intestinal biopsies of so-called potential patients, that present predisposing HLA, TTg autoantibodies, but intact intestinal mucosa, even before the onset of the intestinal lesion⁸ (Appendix 1).

Activation of the mTOR pathway is present in intestinal biopsies from CeD patients⁹. Activation of the mTORC1 pathway has been observed together with the increase of mTOR at the level of the intestinal epithelium of CeD patients⁹. The mTORC1 pathway is linked to activation of cell growth and proliferation in the presence of nutrients, growth factors, energy and stress¹⁰. Moreover, gliadin peptides can induce mTOR phosphorylation^{9,11,12} (Appendix 4 and 5). Autophagy also has been found altered in CeD biopsies in genetic¹³ and expression studies, in blood, in intestinal biopsies¹⁴ and in skin fibroblasts from CeD patients¹⁵. Interestingly, gliadin peptides can reduce the autophagic flux in Caco-2 cells^{11,12} (Appendix 4 and 5).

The aim of this manuscript was to investigate the autophagic flux and its link to inflammation in the intestinal epithelium of intestinal biopsies and organoids from CeD patients both at gluten-containing diet (GCD) and at GFD.

2. Results

2.1 In intestinal biopsies from CeD patients the autophagic flux was reduced and inflammation was increased.

In intestinal biopsies from CeD patients in the acute phase of the disease (GCD-CeD) and controls' (CTRs) we have analysed p62 protein levels by western blot (WB) to study the autophagic flux to lysosomes. The level of p62 protein was increased in a statistically significant way (Figure 1 A and B; $p < .05$).

Immunofluorescence staining of GCD-CeD and CTRs biopsies confirmed the results obtained by WB on p62 protein levels (Figure 1 C and D; $p < .001$). These data indicate that the autophagic flux is impaired in intestinal biopsies from CeD patients. Moreover, the levels of p4E-BP1 (eIF4E-binding-protein/phosphorylated) protein, a downstream effector of the mTOR pathway, was increased in a statistically significant way in GCD-CeD biopsies respect to controls (Figure 1 A and E; $p < .01$). This confirming an observation already present in the literature⁹.

Next, we analysed the levels of pNF- κ B, as a marker of inflammation, in the same biopsies from GCD-CeD and CTRs. The levels of pNF- κ B are increased in GCD-CeD biopsies respect to CTRs as analysed by WB (Figure 1 A and F; $p < .05$). Indicating that defects of the autophagic flux, mTOR pathway activation and inflammation were present in GCD-CeD biopsies.

2.2 In intestinal organoids from GCD- and GFD-CeD patients autophagic flux was reduced, mTOR pathway was activated and inflammation was present.

Intestinal organoids were derived from intestinal biopsies from GCD-CeD, GFD-CeD and CTRs. They represent mainly the epithelial compartment of the intestinal biopsies from which they are derived⁸ (Appendix 1). In GCD- and GFD-CeD intestinal organoids p62 protein levels were analysed both by WB and by immunofluorescence to study the autophagy flux (Figure 2). The levels of p62 (Figure 2 A and B) were increased in a statistically significant way, as analysed by WB in GCD-CeD ($p<.05$). Interestingly, organoids from GFD-CeD patients also showed a significant alteration of the autophagic flux (Figure A and B, $p<.05$).

Immunofluorescence staining of organoids from GCD-CeD, GFD-CeD and CTRs confirmed the results obtained by WB on p62 protein levels (Figure 2). In fact, p62 staining was increased in a statistically significant way in both GCD- (Figure 2 C and D; $p<.01$) and GFD-CeD (Figure 2 C and D; $p<.05$). These data indicate that intestinal organoids from GCD-CeD reproduced the impairment of the autophagic flux described in intestinal biopsies and that GFD-CeD organoids presented the same alteration.

pE4-BP1 protein was increased in a statistically significant way both in GCD- and GFD-CeD patients respect to controls as analysed by WB (Figure 2 A and E; $p<.01$ for both GFD- and GCD-CeD).

The levels of pNF- κ B were increased in a statistically significant way in GCD- and GFD-CeD organoids (Figure 2 A and F; $p<.05$ for both) respect to CTRs analysed by WB analysis. This indicates that the defects of the autophagic flux, mTOR pathway and inflammation previously described in CeD biopsies were also present in CeD intestinal organoids.

2.3 In CTRs organoids bafilomycin blocked the autophagic flux and induced inflammation

To understand the relationship between delay of the autophagy and inflammation we have blocked autophagy in CTRs organoids by treating them with bafilomycin, a drug that blocks autophagy by preventing the formation of autophagosomes¹⁶. In Figure 3 (A and B) we showed by WB analysis, a statistically significant increase of p62 levels in CTR organoids treated with bafilomycin at the concentrations of .5 μ M ($p<.01$). and 1 μ M ($p<.05$). As expected in the presence of bafilomycin the autophagy flux was reduced¹⁶. This observation was confirmed by immunofluorescence (Figure 3 C

and D) after treatment with bafilomycin at the concentration of .5 μ M there was an increase of p62 staining that was statistically significant ($p < .01$). The block of autophagy induced by bafilomycin treatment also produced an increase of the pE4-BP1 levels (Figure 3 A and E; $p < .05$ for both concentrations of bafilomycin). This indicates that because of the autophagy block, there was also an activation of the mTOR pathway.

In these same conditions also the levels of pNF-kB were increased in a statistically significant way as shown in Figure 3 (F and G; $p < .05$ at both concentrations of bafilomycin used). Rapamycin, reduced p62, pE4BP and pNF-kB in CTRs organoids although the reduction was not statistically significant in our experimental conditions (Figure 3). Together, these data indicated that blocking the autophagic flux in CTRs organoids was able to induce inflammation.

2.4 In CeD intestinal organoids at GCD and GFD rapamycin induced the autophagic flux and reduced inflammation

To understand the interplay between autophagy and inflammation in CeD organoids from patients at GCD and GFD, we have induced the autophagic flux by treating them with rapamycin, a drug able to induce autophagy by mTOR dephosphorylation. Organoids from GCD-CeD and GFD-CeD treated with different concentrations of rapamycin (25nM, 50nM, 100nM) were shown in Figures 4 and 5 respectively. Rapamycin was able to reduce p62 levels both in GCD- (Figure 4 A and B) and GFD-CeD (Figure 5 A and B) organoids in a statistically significant way ($p < .05$ at all the concentrations used in both GCD- and GFD-CeD patients' organoids) by WB analysis. This increase was confirmed by immunofluorescence analysis of the p62 staining of GCD- (Figure 4 C and D; $p < .01$) and GFD-CeD (Figure 5 C and D; $p < .01$) treated with 25nM of rapamycin.

After treatment with rapamycin a statistically significant reduction of pE4-BP1 was present in organoids from both GCD- (Figure 4 A and E; $p < .05$ at all concentrations of rapamycin used) and GFD-CeD (Figure 5 A and E; $p < .05$ for 50nM of rapamycin). Moreover, rapamycin was able to reduce in a statistically significant way pNF-kB levels both in GCD- (Figure 4 F and G; $p < .05$ for 25 nM and $p < .01$ for 100nM of rapamycin used) and GFD-CeD (Figure 5 F and G; $p < .05$ for all the concentrations used) intestinal organoids. Interestingly, bafilomycin, an inhibitor of the autophagy pathway, was able to increase further p62 in a statistically significant way in GCD-CeD organoids (Figure 4 A and B; $p < .01$ for .5 and 1 μ M concentrations of bafilomycin used) but did not affect pE4-BP1 or pNF-kB levels, in our experimental conditions (Figure 4). Similarly, bafilomycin was able to increase p62 (Figure 5 A and B), pE4-BP1 (Figure 5 A and E) and pNF-kB (Figure 5 F and G) levels in GFD-CeD organoids, but the increases were not statistically significant, probably due to both the

small number of organoids evaluated and the variability of the expression of the markers used. These data indicate that rapamycin-induced autophagic flux blocked mTOR pathway and reduced the inflammation both in GCD- and GFD-CeD organoids.

2.5 Both GCD-and GFD-CeD intestinal organoids produced inflammatory cytokines, chemokines, and growth factors

The analysis by Bio-Plex assay of 27 pro-inflammatory cytokines, chemokines, and growth factors was done on the cultures media (CMs) from CeD organoids derived from patients at GCD -and GFD-CeD and from CTRs (Figure 6).

This analysis showed that respect to CMs from CTRs organoids in the GCD-CeD organoids, 22 pro-inflammatory cytokines, chemokines, and growth factors out of 27 analysed were increased in a statistically significant way (Figure 6 A and B).

Interestingly 6 of them were increased in a statistically significant way also in CMs from GFD-CeD organoids (Figure 6 A). In CMs from both GCD- and GFD-CeD, IL-5 ($p<.05$ and $p<.001$), IL-8 ($p<.01$ for both), IL-13 ($p<.05$ for both), INF- γ ($p<.01$ and $p<.05$), MCP-M ($p<.0001$ and $p<.01$) and VEGF ($p<.05$ and $p<.001$) were increased in a statistically significant way. All these cytokines, chemokines, and growth factors found altered in CMs from CeD organoids have been described to have a role at the level of the intestinal epithelium (Table 1).

Sixteen pro-inflammatory cytokines, chemokine and growth factors were increased in a statistically significant way only in CMs from GCD-CeD organoids. Namely the cytokines: IL-1 β ($p<.01$), IL-1Ra ($p<.05$), IL-4 ($p<.001$), IL-15 ($p<.05$), IL-17 ($p<.01$), IL-7 ($p<.01$) IL-9 ($p<.01$), IL-10 ($p<.05$), IL-12p70 ($p<.01$); the chemokines: MIP-1 α ($p<.01$), MIP-1 β ($p<.05$), IP-10 ($p<.05$), RANTES ($p<.01$); the growth factors: PDGF ($p<.0001$), GM-CSF ($p<.001$) and G-CSF ($p<.01$) (Figure 6B). TNF- α was the only cytokine that was increased in a statistically significant way ($p<.0001$) in CMs from GFD-CeD organoids (Figure 6 C), but not in GCD-CeD. Interestingly, this cytokine is also involved in wound healing. Only IL-2, IL-6, Eotaxin and FGF-basic did not show any differences in the CMs from CeD organoids respect to the CMs from CTR organoids (Figure 6 D).

2.5 Cultures media from GCD-CeD organoids were able to induce reduction of the autophagic flux, activation of the mTOR pathway and inflammation in CTR's organoids

CMs conditioned by GCD-CeD organoids previously analysed by Bio-Plex assay were used to treat CTRs organoids to test their ability to induce block of the autophagic flux, activation of the mTOR pathway and inflammation in CTRs (Figure 7). The CMs from 4 GCD-CeD organoids were used to treat the organoids from 3 CTRs and then analysed by WB to test the levels of p62 (Figure 7A-D), pE4-BP1 (Figure 7A-C and E) and pNF-kB (Figure 7A-C and F). Figure 7 showed a statistically significant increase in CTRs organoids after treatment with CMs from GCD-CeD of p62 (Figure 7 D; $p<.01$), pE4-BP1 (Figure 7 E; $p<.05$) and pNF-kB (Figure 7 F; $p<.05$). One of the controls (CTR 3) was treated with CMs from two different GCD-CeD organoids, both CMs (pz-CM 3 and 4) were able to induce the increase of all the markers tested (Figure 7 C-F). Moreover, to confirm the inflammatory state of CTRs organoids, 3 of them were treated with CMs produced by 3 different GCD-CeD organoids to test by ELISA assay the production of IL-1 β in the CMs. In Figure 7 G we showed a statistically significant increase of IL-1 β in CTRs organoids ($p<.05$) only after overnight (O.N.) treatment with CMs from GCD-CeD organoids. This confirms the inflammatory state of the CTRs organoids after treatment with CMs from GCD-CeD organoids.

3. Discussion

In this manuscript, we have shown that both in intestinal biopsies and organoids from GCD-CeD there was a delay of the autophagic flux, an activation of the mTOR pathway and an increase of inflammatory markers. Interestingly, such alterations were present also in GFD-CeD organoids. To understand the relationship between the delay of the autophagic flux and activation of the mTOR pathway with the inflammation, we have induced a delay of the autophagic flux by bafilomycin treatment in CTRs organoids and observed an increase of both mTOR pathway activation and inflammation. The opposite result was obtained when we induced the autophagic flux by rapamycin treatment of both GCD- and GFD-CeD organoids. The intestinal organoids from CeD patients not only were inflamed, but also produced inflammatory cytokines, chemokines, and growth factors all of them related to inflammation and most of them with autophagy (Table 1). Moreover, the culture media conditioned by the growth of GCD-CeD intestinal organoids were able to induce inflammations together with a delay of the autophagic flux and the activation of the mTOR pathway in CTRs organoids. All this shows that in CeD organoids there is a crosstalk between autophagy/mTOR pathway activation and inflammation.

The mTOR pathway has already been described as altered in intestinal biopsies from GCD-CeD⁹. Sedda et al. demonstrated the activation of mTOR pathway, in intestinal biopsies from GCD-CeD, using pE4-BP1 as a downstream effector of mTOR⁹. Autophagy also has been described in CeD fibroblasts where it has been shown that skin fibroblasts from GFD-CeD patients' low levels of Ca²⁺

were present in the cytosol and the ER lumen, consequently, TG2 was less activated and there was a defective autophagy¹⁵. Moreover, gliadin peptides can induce activation of the mTOR pathway in GFD-CeD intestinal biopsies in culture⁹ and, also, activation of the mTOR pathway and reduction of the autophagic flux in intestinal cells in culture^{11,12} (Appendix 4 and 5).

The mTOR pathway is activated in presence of nutrients, growth factors and stress^{17,18}. In the absence of nutrients and/or in the presence of rapamycin, autophagy is activated. Basically, the two biological processes are alternative¹⁹. In intestinal organoids from CeD patients, we confirmed this observation, showing that when autophagy was delayed, the mTOR pathway was activated. What generates both the activation of the mTOR pathway and the reduction of autophagic flux in CeD biopsies and fibroblasts is not clear. Genetic and expression studies have shown a reduction of ULK3 (Unc-51-like autophagy-activating kinases 3) in CeD intestinal biopsies^{13,20,21}. ULK3 is an important protein in the regulation of autophagy in mammalian cells, homologous to ATG1 in yeast, it is needed in the early steps of autophagosome biogenesis²². This protein has been involved in the pathogenesis of Crohn's disease as well^{22,23}.

Taken together, these observations could sustain the hypothesis that the reduction of autophagy-regulating genes, such as ULK3, in CeD intestine could lead to reduced autophagic flux with the consequent activation of the mTOR pathway and inflammation. On the other side, our experiments conducted on the CTRs organoids showed that inflammatory cytokines, chemokines, and growth factors in CMs produced by GCD-CeD can induce delay of the autophagic flux and induce activation of the mTOR pathway in CTRs organoids, indicating that inflammation can have a role in these processes. Inflammatory proteins can be present in the serum of CeD patients before the onset of the disease and even before the introduction of gluten in infants²⁴. Inflammation is also present in intestinal biopsies from CeD not only at GCD, but also in patients at GFD, with normal intestinal mucosa and without autoantibodies in the serum⁸ (Appendix 1) or in potential-CeD subjects at GCD, with the predisposing genetics and autoantibodies in the serum, but with normal intestinal mucosa⁸ (Appendix 1). All this indicates that inflammation can be present very early in the natural history of the disease.

Reduction of the autophagic flux and activation of the mTOR pathway have been linked to inflammation also in other systems²⁵. In fact, autophagy plays critical roles in inflammation, sustaining the development, homeostasis, and survival of inflammatory cells with the consequent production of several cytokines that can modulate autophagy flux²⁵.

In this manuscript we have shown that crosstalk between autophagy/activation of the mTOR pathway and inflammation was present in the intestinal epithelium of CeD patients not only in the acute phase of the disease, at GCD, but also in patients at GFD, with normal mucosa. They not only were inflamed, but produced inflammatory cytokines, chemokines, and growth factors, all related to inflammation and activation of NF- κ B (Table 1). Interestingly most of these cytokines, chemokines, and growth factors have been described to have a role in the regulation of autophagy (Table 1), confirming that these two phenomena are strictly related. The 6 cytokines, chemokines, and growth factors that were increased in both GCD- and GFD-CeD (Table 1) were all involved in the regulation of the autophagy and are related to the NF- κ B pathway. Moreover, other 16 cytokines, chemokines and growth factors were increased in CMs from GCD-CeD, all related with NF- κ B pathway and several of them with autophagy. The cytokine tumour necrosis factor- α (TNF- α) was increased only in CMs from GFD-CeD organoids. This cytokine is a modulator of cell death or proliferation²⁶; it plays a critical role in the pathogenesis of inflammatory bowel disease (IBD), infectious diseases, intestinal wound healing, and tumour formation²⁶. Moreover, it is an enhancer of TG2, an enzyme that has a nodal role in the CeD pathogenesis²⁷. TNF- α is also a key mediator of intestinal inflammation and causes an increase in intestinal epithelial tight junction²⁸.

In conclusion, autophagy/activation of the mTOR pathway and inflammation are strictly connected in an interplay that ultimately may render the epithelial cells of CeD subjects more prone to pro-inflammatory stimuli from the environment, including gliadin peptides that can insist on the same pathways already altered in CeD intestinal epithelium. All this opens new hypothesis of intervention in CeD prevention and therapy.

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4. Materials and Methods

4.1 Biopsies

Duodenal biopsies were obtained from patients with GCD-CeD presenting with villous atrophy (5 patients, mean age of 10 years), patients with GFD-CD (4 subjects, mean age of 11 years) and controls affected by gastroesophageal reflux (5 subjects, mean age of 13 years) or other functional disorders. The age range for all patients was 2–16 years. Patients with villous atrophy (Marsh T3c) had positive serology (anti-tTg antibodies >50 U/ml and EMA positive). Anti-tTg antibody titers were measured using Eurospital kit EU-tTG (Table 1). Specimens were harvested, snap-frozen in liquid nitrogen, embedded in OCT and stored at –80 °C until required for further analyses (Table 2).

4.2 Intestinal organoids

One duodenal biopsy from per individual from CeD patients and controls was taken with standard endoscopic EGDS during routine gastroduodenoscopy (Table 3) and placed in 10 mL of cold PBS with the addition of 2 mM EDTA (cat.15575020, Thermo Fisher, Milan, Italy) and 0.5 mM DDT (D0632, Sigma-Aldrich, Milan, Italy). After 50 minutes of agitation at 4 °C, the biopsy samples were washed with PBS, filtered through a 70 µm strainer (Falcon, NY, USA) and centrifugated at 2800 rpm for 5 min at 4°C, then the crypts were resuspended in 40 µL of ice-cold Matrigel matrix (cat.35623, Corning, Milan, Italy) to permit three-dimensional growth in 48 well plates. Afterwards, 300 µL cell culture medium enriched with supplements (CM-S) was added to each well (Appendix 2). To pass from a 3D to a 2D structure, organoids were openly seeded on 35 mm multi-wells pre-treated with Matrigel (1:40 in PBS). We generally processed the 2D organoids within 24–72h after seeding (Appendix 1). The human spheroids of GCD-CeD, GFD-CeD and CTRs were seeded in six multi-wells (Corning, Milan, Italy) coated with Matrigel diluted 1:40 in phosphate-buffered saline (PBS) for 3 days. After they were stimulated with bafilomycin (SML1661, Sigma-Aldrich, Milan, Italy), at .5 µM and 1 µM concentrations, and rapamycin (cat. PHZ1235, Life Technologies, Milan, Italy), at a concentration of 25nM, 50nM, 100nM, for 3h at 37 °C (Table 3)^{8,29} (Appendix 1 and 2).

4.3 Western Blot

Intestinal organoids cultivated for 4 weeks and opened in 2D from duodenum obtained from CeD patients and CTRs were processed. After removing Matrigel by Cell Recovery Solution (cat. 354270, Corning Milan Italy), organoids were homogenized in 40-50 µL tissue homogenization buffer^{8,29} (Appendix 1 and 2). The cell lysates (10 µg/mL) were analysed by SDS-PAGE and transferred onto

nitrocellulose membranes by Trans Blot Turbo (cat. 1704158, Bio-Rad, Milan, Italy). The membranes were blocked with 5% non-fat dry milk and probed with anti-pNF-kB (cat. FNab10840, Fine Biotech Co., Hubei, China), anti-p62/SQSTM1 (cat. P0067, Sigma Aldrich, St. Louis, Missouri, USA), and anti-pE4-BP1 (Y1068) (cat.3777, Cell Signalling, Euroclone, Milan, Italy) were used as loading controls stain-free gel (10% cat.#4568033 BioRad, Milan, Italy) (4-15% cat.#4568086 Biorad, Milan, Italy). Then, 1-8 minutes exposure using ECL-plus (Cat. 1705062 Bio-Rad, USA) allowed to visualise the bands of interest. The bands intensity was evaluated by integrating all the pixels of the band after subtraction of the background to calculate the average of the pixels surrounding the band^{8,29}(Appendix 1 and Appendix 2).

4.4 Cytokine Analysis

Intestinal organoids were grown in 2D to investigate the levels of cytokines, chemokines and growth factors in cultures medium (CMs). Culture medium of 2D organoids was collected in 1,5ml Eppendorf after 3 hours of experiment and stored at -80°C. CMs were screened for the concentration of interleukin (IL)-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17A, basic fibroblast growth factor (FGF), eotaxin, granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon- γ (IFN- γ), interferon- γ inducible protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 (MIP-1) α , MIP-1 β , C-C motif chemokine ligand 5 (CCL5)/RANTES, TNF- α , platelet-derived growth factor (PDGF-BB), and vascular endothelial growth factor (VEGF) using the Bio-Plex Multiplex Human Cytokine, Chemokine, and Growth Factor Kit (cat. n. M500KCAF0Y, Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol, as previously described³⁰. The magnetic bead-based assay was performed on a Bio-Plex 200 System (Bio-Rad, Hercules, CA, USA). All the values obtained were included within the detection limits indicated by the manufacturer (bio-rad.com/Bio-Plex/Analyte Guide)³¹.

4.5 Immunofluorescence staining of biopsies

We used immunofluorescence staining to evaluate p62 levels. The 5- μ m cryostat sections from biopsies were air dried, fixed with acetone (10 min), and stained with rabbit anti-p62 (cat. P0067, Sigma Aldrich, St. Louis, Missouri, USA) antibody for 1 h at room temperature, after blocking with 1% bovine serum albumin (BSA) in PBS for 15 min at room temperature (RT). Then, the sections were washed (3 times, 5 min each) with PBS at RT, and Alexa 488 anti-rabbit (1:50; cat.11001 Milan, Italy) was applied for 45 min at room temperature in a dark box to avoid bleaching. After washes with PBS, sections were mounted with Mowiol (Sigma-Aldrich, Milan, Italy) and observed under a Zeiss

LSM510 or Zeiss LSM700 confocal microscope (laser scanning microscope) (Germany). Images were obtained with a 63× objective unless differently stated.

4.6 Immunofluorescence staining of organoids

The human spheroids were seeded in 15 multi-wells (IBIDI) coated with Matrigel diluted 1:40 in phosphate-buffered saline (PBS) for 2 days. After a day the organoids in 2D were fixed with 4% paraformaldehyde (PFA) for 10 min, The cells were washed 3 times with 1X PBS for 10 min each time. Afterwards, they were permeated with 0.2 % Triton/1X PBS for 10 min at room temperature. The cells were washed 3 times with 1X PBS for 10 min each time. The slides were blocked with 5% BSA/1X PBS (blocking solution) for 1 h at room temperature. The primary antibody anti-rabbit p62 was diluted in a blocking solution overnight at 4 °C in a humidified chamber. Then the cells were washed 3 times with 1X PBS for 10 min each time. Alexa Fluor 546 donkey anti-rabbit secondary antibody (A10040 Invitrogen, Milan, Italy) was added to the organoids. The cells were then washed 3 times with 1X PBS for 10 min each time, mounted with Mowiol (cat. 475904-M Sigma-Aldrich, Milan, Italy) and observed under a confocal microscope (Zeiss LSM 510, Jena, Germany).

4.7 ELISA

The levels of IL-1 β and were measured using commercial test kits (IL-1 β cat.850.006.192, IL-6 cat.950.030.192, Diaclone, Besancon Cedex, France) on cell culture supernatants.

4.8 Statistical Analysis

Statistical analyses and graphical representations were performed using GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA). Differences between groups were analysed using Student's t-tests, with statistical significance set at $p < 0.05$. All analyses were two-tailed.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the University of Naples Federico II, Protocol number: 115/09/ESPROT).

5. Figures

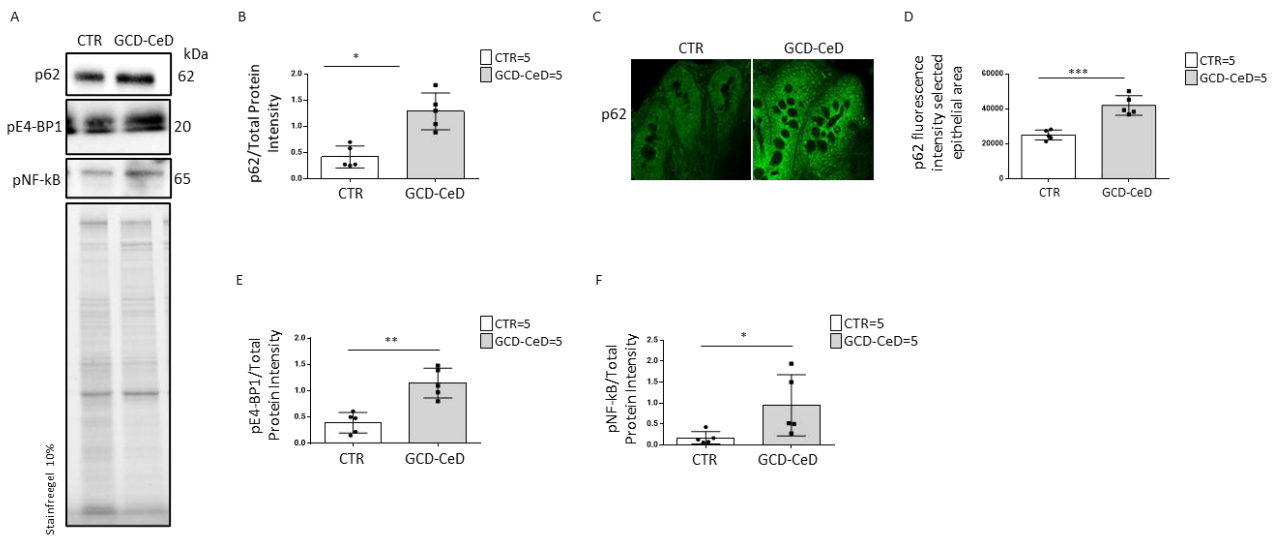


Figure 1. In intestinal biopsies from celiac disease (CeD) patients autophagic flux was reduced, mTOR pathway was activated and inflammation was present.

A. Western blot analysis of total protein lysates of intestinal biopsies from controls (CTR) and CeD patients fed a gluten-containing diet (GCD-CeD). Upper line was blotted with an antibody against p62. The second line was blotted, with an antibody against pE4-BP1 and the last line with an antibody against pNF-kB. As a loading control was used stain free gel at 10%. Representative images were selected.

B. Densitometric analysis of the p62/stain free gel at 10% bands from CTR and GCD-CeD. The numbers of biopsies analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t* test: * = $p < .05$.

C. and D. Images of immunofluorescence staining for p62 in GCD-CeD patients' biopsies compared to CTRs. The number of biopsies analysed is indicated. Representative fields. Scale bar = 20 μ m. The columns represent the mean, and bars represent the standard deviation. Student's *t* test: *** = $p < .001$.

E. Densitometric analysis of the pE4-BP1/stain free gel at 10% bands from CTR and GCD-CeD. The numbers of biopsies analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t* test: ** = $p < .01$.

F. Densitometric analysis of the pNF-kB/stain free gel at 10% bands from CTR and GCD-CeD. The numbers of biopsies analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t* test: * = $p < .05$.

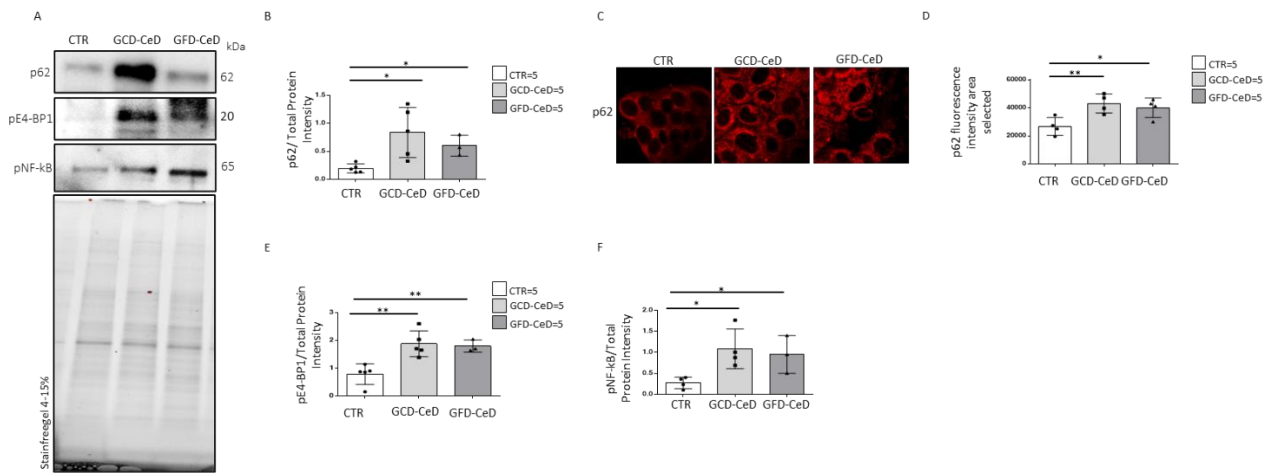


Figure 2. In CeD patient's organoids the autophagic flux was reduced and inflammation was increased respect to CTRs.

A. Western blot analysis of total protein lysates of intestinal organoids from CTRs, GCD-CeD and GFD-CeD. Upper line was blotted with an antibody against p62. Middle line was blotted with an antibody against pE4-BP1 and the last line was blotted with an antibody against pNF-kB. As a loading control was used stain free gel 4-15%. Representative images were selected.

B. Densitometric analysis of the p62/stain free gel 4-15% bands from CTR, GCD-CeD and GFD-CeD organoids. The numbers of organoids analysed are indicated. Columns represent the mean, and bars represent the standard deviation, Student's *t* test: * = $p < .05$.

C. and D. Images of immunofluorescence staining for p62 in GCD-and GFD-CeD patients' organoids compared to CTRs. The number of intestinal organoids analysed is indicated. Representative fields. Scale bar = 20 μ m. The columns represent the mean, and bars represent the standard deviation. Student's *t* test: * = $p < .05$ ** = $p < .01$.

E. Densitometric analysis of the pE4-BP1/stain free gel 4-15% bands from CTR, GCD-CeD and GFD-CeD organoids. The numbers of organoids analysed are indicated. Columns represent the mean, and bars represent the standard deviation, Student's *t* test: ** = $p < .01$.

F. Densitometric analysis of the pNF-kB/stain free gel 4-15% bands from CTR, GCD-CeD and GFD-CeD organoids. The numbers of organoids analysed are indicated. Columns represent the mean, and bars represent the standard deviation, Student's *t* test: * = $p < .05$.

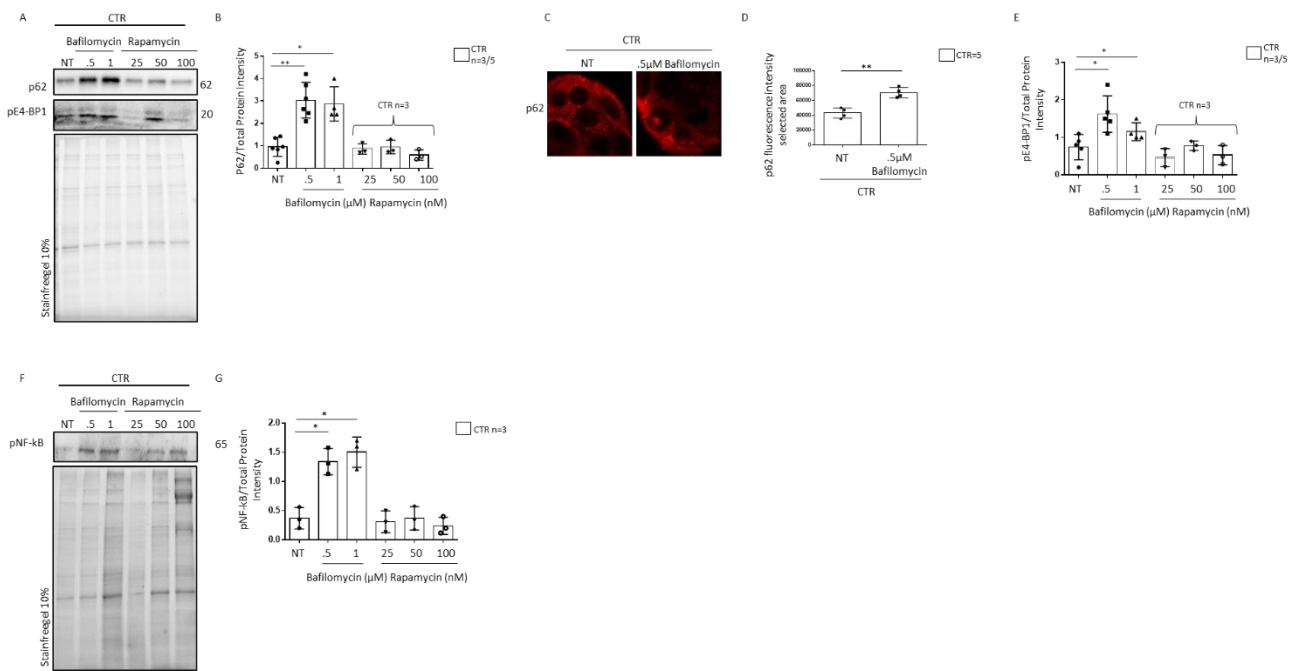


Figure 3. Bafilomycin was able to increase p62, pE4-BP1 and pNF-kB levels in CTRs organoids.

A. Western blot analysis of total protein lysates of intestinal organoids from controls (CTRs) treated and not with bafilomycin at the concentrations of .5 and 1 μ M and with rapamycin at the concentrations of 25, 50 and 100 nM for 3 hours. Upper line was blotted with an antibody against p62, bottom line with antibodies against pE4-BP1. As a loading control was used stain free gel 10%. Representative images were selected.

B. Densitometric analysis of the p62/stain free gel at 10% band from CTR organoids treated and not treated with bafilomycin and rapamycin at different concentrations for 3 hours. The numbers of organoids analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t* test: * $=p < .05$, ** $=p < .01$.

C. and D. Images of immunofluorescence staining for p62 in CTR organoids treated with bafilomycin at the concentration of .5 μ M for 3 hours. The number of intestinal organoids analysed is indicated. Representative fields. Scale bar = 20 μ m. The columns represent the mean, and bars represent the standard deviation. Student's *t* test: ** $=p < .01$

E. Densitometric analysis of the pE4-BP1/stain free gel at 10% band from CTR organoids treated and not treated with bafilomycin and rapamycin at different concentrations for 3 hours. The numbers of organoids analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t* test: * $=p < .05$.

F. Western blot analysis of total protein lysates of intestinal organoids from controls (CTRs) treated and not with bafilomycin and rapamycin at different concentration for 3 hours. Upper line was blotted with an antibody against pNF-kB, lower gel showed stain free gel 10% used as a loading control. Representative images were selected.

G. Densitometric analysis of the pNF-kB/stain free gel at 10% band from CTR organoids treated and not treated with bafilomycin and rapamycin at different concentrations for 3 hours. The numbers of organoids analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t* test: * = $p < .05$.

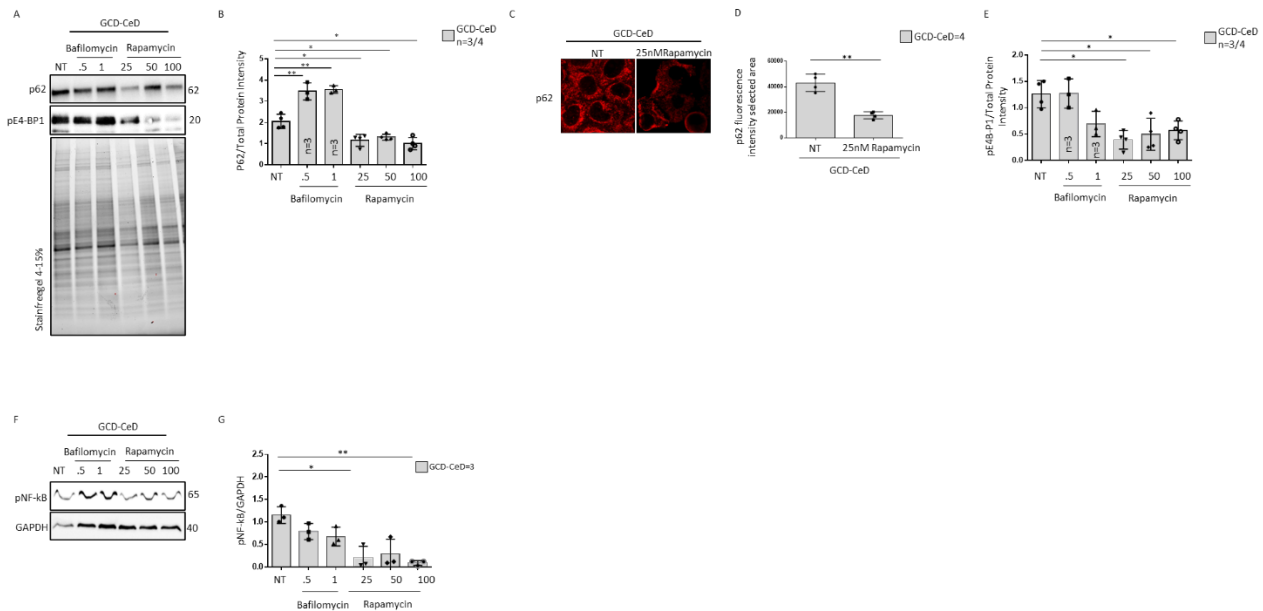


Figure 4. Rapamycin was able to decrease p62, pE4-BP1 and pNF-kB levels in GCD-CeD organoids.

A. Western blot analysis of total protein lysates of intestinal organoids from GCD-CeD treated and not with bafilomycin at the concentrations of .5 and 1 μ M and with rapamycin at the concentrations of 25, 50 and 100 nM for 3 hours. Upper line was blotted with an antibody against p62 and bottom line with an antibody against pE4-BP1. As a loading control was used stain free gel 4-15%. Representative images were selected.

B. Densitometric analysis of the p62/stain free gel at 4-15% bands from GCD-CeD organoids treated and not treated with bafilomycin and rapamycin at different concentrations. The numbers of organoids analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t* test: * = $p < .05$; ** = $p < .01$.

C. and D. Images of immunofluorescence staining for p62 in GCD-CeD organoids treated with rapamycin at the concentration of 25 nM for 3 hours. The number of intestinal organoids analyzed is indicated. Representative fields. Scale bar = 20 μ m. The columns represent the mean, and bars represent the standard deviation. Student's *t* test: ** = $p < .01$.

E. Densitometric analysis of the pE4-BP1/stain free gel at 4-15% band from GCD-CeD organoids treated and not treated with rapamycin at the concentration of 25 nM, 50 nM and 100 nM for 3 hours.

The numbers of organoids analyzed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t test*: *= $p<.05$.

F. Western blot analysis of total protein lysates of intestinal organoids from GCD-CeD treated and not with bafilomycin and with rapamycin at different concentrations for 3 hours. Upper line was blotted with an antibody against pNF-kB and bottom line with an antibody against GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) as a loading control. Representative images were selected.

G. Densitometric analysis of the pNF-kB/GAPDH bands from GCD-CeD organoids treated and not treated with bafilomycin and rapamycin at different concentrations. The numbers of organoids analyzed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t test*: *= $p<.05$; **= $p<.01$.

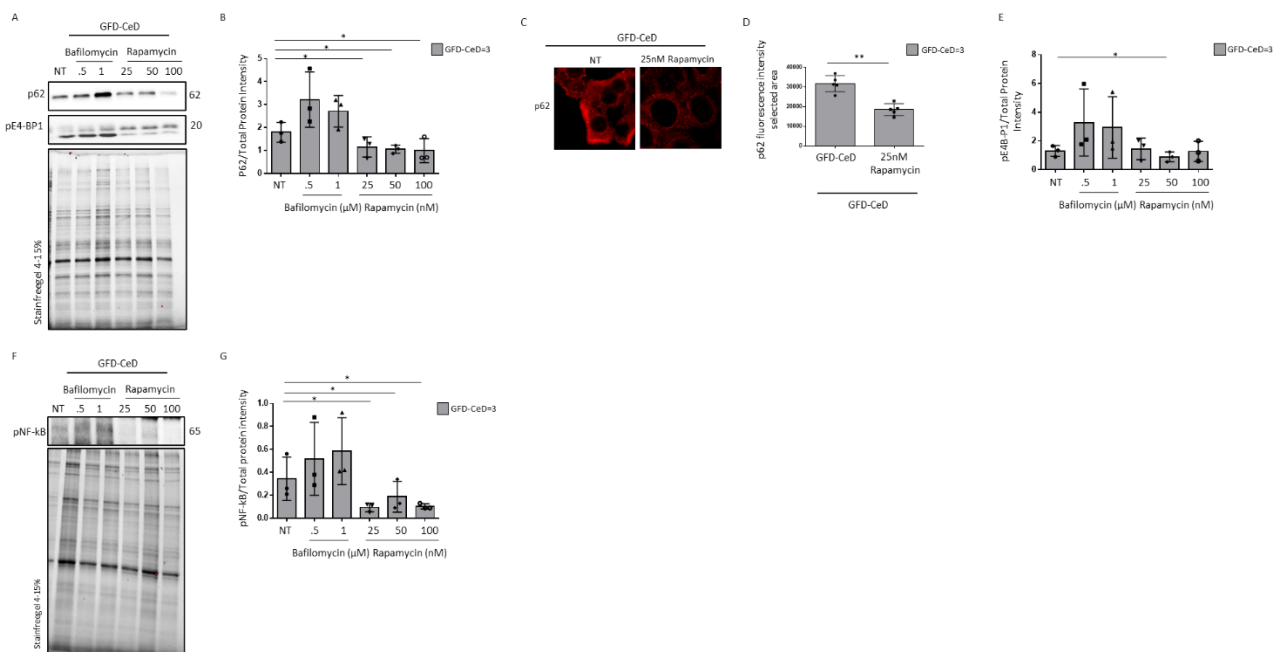


Figure 5. Rapamycin was able to decrease p62, pE4-BP1 and pNF-kB levels in GFD-CeD organoids.

A. Western blot analysis of total protein lysates of intestinal organoids from GFD-CeD treated and not with bafilomycin at the concentrations of .5 and 1 μ M and with rapamycin at the concentrations of 25, 50 and 100 nM for 3 hours. Upper line was blotted with an antibody against p62 and bottom line with an antibody against pE4-BP1. As a loading control was used stain free gel 4-15%. Representative images were selected.

B. Densitometric analysis of the p62/stain free gel at 4-15% bands from GFD-CeD organoids treated and not treated with bafilomycin and rapamycin at different concentrations. The numbers of organoids analyzed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t test*: $*=p<.05$

C. and D. Images of immunofluorescence staining for p62 in GFD-CeD organoids treated with rapamycin at the concentration of 25 nM for 3 hours. The number of intestinal organoids analyzed is indicated. Representative fields. Scale bar=20 μ m. The columns represent the mean, and bars represent the standard deviation. Student's *t test*: $**=p<.01$.

E. Densitometric analysis of the pE4-BP1/stain free gel at 4-15% band from GFD-CeD organoids treated and not treated with bafilomycin and rapamycin at different concentrations for 3 hours. The numbers of organoids analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t test*: $*=p<.05$.

F. Western blot analysis of total protein lysates of intestinal organoids from GFD-CeD treated and not with bafilomycin and rapamycin at different concentrations for 3 hours. Upper line was blotted with an antibody against pNF-kB, lower gel showed stain free gel 4-15% used as a loading control. Representative images were selected.

G. Densitometric analysis of the pNF-kB/stain free gel at 4-15% band from GFD-CeD organoids treated and not treated with bafilomycin and rapamycin at different concentrations for 3 hours. The numbers of organoids analyzed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t test*: $*=p<.05$.

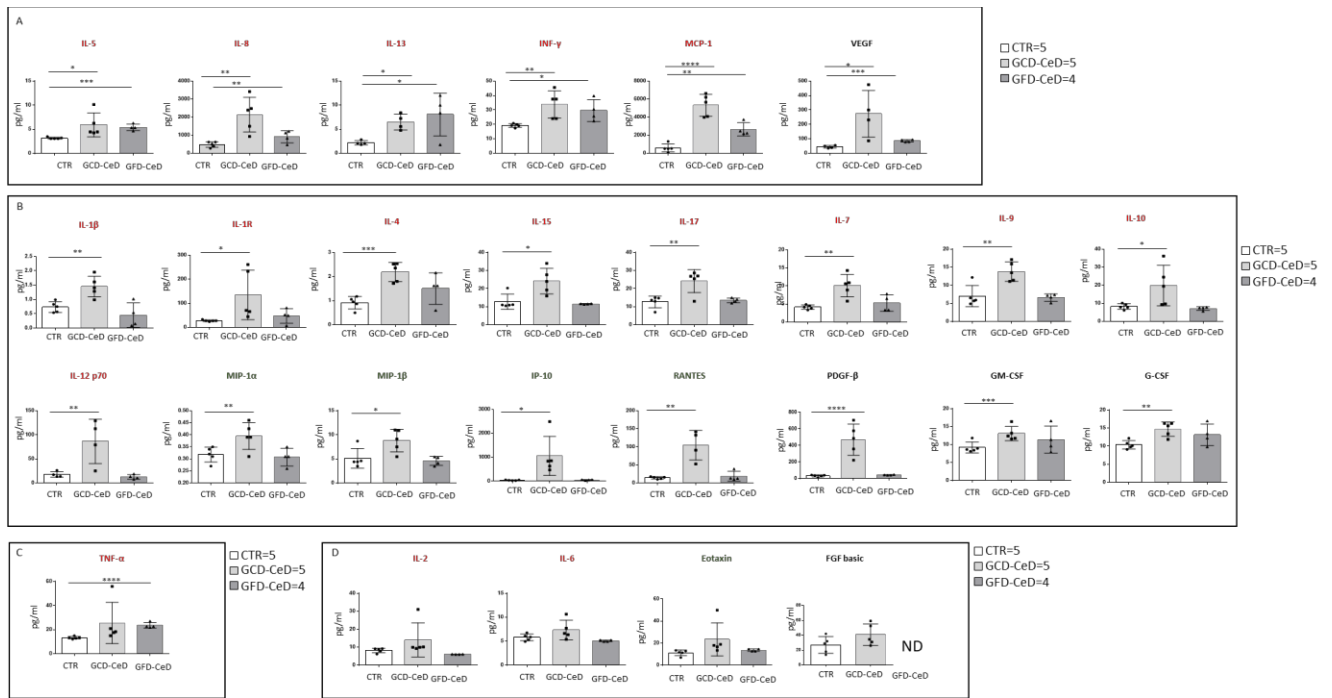


Figure 6. Cytokine analysis of 27 pro-inflammatory cytokines, chemokines and growth factors of culture media from GCD-, GFD-CeD and CTR.

Bar chart for cytokines, chemokines, and growth factors, expressed as pg/ml, in CMs from GCD- and GFD-CeD intestinal organoids respect to CTRs, cytokines were indicated in red, chemokines in green and growth factors in blue.

A. Cytokines, chemokines, and growth factors, statistically significant in both GCD- and GFD-CeD respect to controls. Columns represent the mean, and bars represent the standard deviation. Student's t test: $*=p<.05$; $**=p<.01$; $***=p<.001$; $**** = p < .0001$.

B. Cytokines, chemokine, and growth factors, statistically significant in GCD-CeD respect to controls. Columns represent the mean, and bars represent the standard deviation. Student's t test: $*=p<.05$; $**=p<.01$; $***=p<.001$; $**** = p < .0001$.

C. TNF-alpha was statistically significant only in GFD-CeD respect to controls. Columns represent the mean, and bars represent the standard deviation. Student's t test: $**** = p < .0001$.

D. Cytokines, chemokines, and growth factors that were not statistically significant in GCD- and GFD-CeD respect to controls. Columns represent the mean, and bars represent the standard deviation.

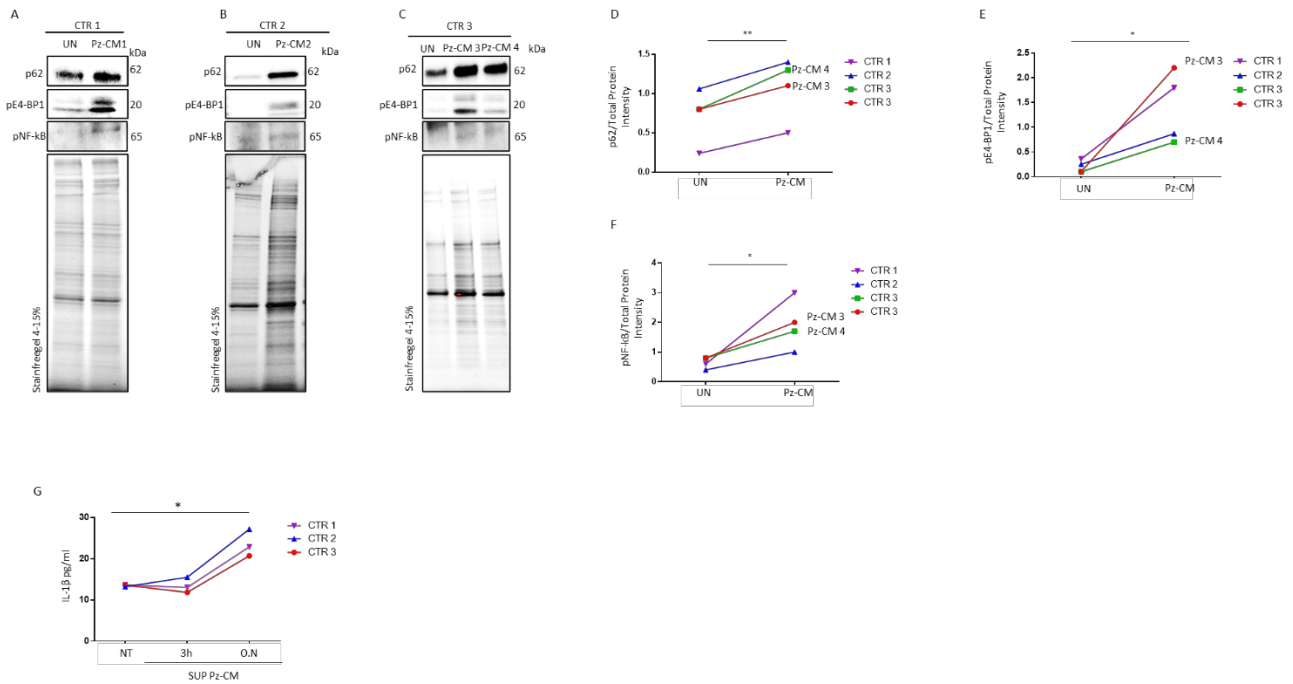


Figure 7. Inflammatory cytokines, chemokines and growth factors produced in the cultures media by GCD-CeD organoids can increase p62, pE4-BP1, pNF-kB and IL-1 β levels in CTRs.

A. B. and C. Western blot analysis of total protein lysates of intestinal organoids from 3 CTRs treated and not with cultures media (CMs) from 4 GCD-CeD organoids for 3 hours. Upper line was blotted with an antibody against p62, middle lane with an antibody against pE4-BP1 and bottom line with an antibody against pNF-kB. Stain free gel at 4-15% was used as loading controls. Representative images were selected.

D. Densitometric analysis of the p62/stain free gel at 4-15% bands from CTR organoids treated and not treated (UN) with CMs from GCD-CeD for 3 hours. The numbers of organoids analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t* test: **= $p < .01$.

E. Densitometric analysis of the pE4-BP1/stain free gel at 4-15% band from CTR organoids treated and not treated (UN) with CMs from GCD-CeD for 3 hours. The numbers of organoids analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t* test: *= $p < .05$.

F. Densitometric analysis of the pNF-kB/stain free gel at 4-15% band from CTR organoids treated and not treated (UN) with CMs from GCD-CeD for 3 hours. The numbers of organoids analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t* test: *= $p < .05$.

G. IL-1 β levels tested by ELISA assay performed on the CM of CTRs treated and not (UN) with CM from GCD-CeD for 3h and O.N (over-night). Student's *t* test: *= $p < .05$.

6.Tables

Cytokines, Chemokines, Growing factors	Role in intestinal mucosa	Role in autophagy	Relate to NF-kB pathway	References of role in intestinal mucosa	References of autophagy role	References of relationship with NF-kB pathway
IL-5	Eosinophils are attracted to the intestinal tissue by IL-5 in ulcerative colitis.	IL-5 induces autophagy to promote the production of eosinophil cationic protein and eosinophil airway inflammation	Yes, as enhancer.	32	33	34
IL-8	This cytokine is the most abundant inflammatory cytokine produced by the intestinal epithelium. It's increased in both IBD and CeD intestinal biopsies. Moreover, PT-gliadin is a potent agonist of IL-8.	Autophagy positively regulates the transcription and secretion of IL-8.	Yes, as effector.	35–37	38	39
IL 13 (Interleukin-13)	Disruption of intestinal epithelium by recruitment of NKT cells. The production of IL-13 by duodenal biopsies is low in GCD-CeD patients.	IL-13 inhibits autophagy.	Yes.	40,41	42	39,43,44
MCP-1 also named CCL-2 (C-C Motif Chemokine Ligand 2)	The most abundant inflammatory cytokine produced by the intestinal epithelium, that responds to intestinal permeability increasing. This plays an important role in regulating gut inflammation and insulin tolerance in vivo.	Yes.	Yes, is a modulator of NF-kB pathway.	35	45	46,47

IFN-γ (Interferon-γ)	It has a role in intestinal epithelial barrier function and increase intestinal permeability. IFN γ , a dominant cytokine in intestinal mucosa in active CeD. Moreover, it is the most potent inducer of TG2, and synergism with TNF- α may contribute to exacerbate the pathogenic mechanism of CeD.	IFN- γ induces autophagy.	Yes, as enhancer.	48–50	38	51,52
VEGF	It is essential for both physiological and pathological angiogenesis. It is also involved in early development of intestinal epithelium and in maintaining homeostasis after mucosal damage.	VEGF induces autophagy.	Yes, as effector.	53	54,55	56,57
IL-1β	IL-1 β has pluripotent pro-inflammatory effects associated to several inflammatory diseases. It also induces an increasing of intestinal permeability and modulate the epithelial barrier, by the activation of the NF- κ B pathway.	Autophagy limits IL-1 β activation or release. Moreover, it can also inhibit autophagy flux.	Yes, as effector.	58,59	42	60
IL-1ra	In physiological condition IECs express and secrete IL-1ra. However, in inflamed mucosa the secretion of IL-1ra by IECs is slightly increased but could be not enough to antagonise the greatly increased production of pro-inflammatory cytokines, from both lamina propria mononuclear cells and the IECs.	IL1ra induces autophagy activation.	Yes, as inhibitor of IL-1.	61	62	63
IL-4	It plays a role in the regulation of intestinal mucosal barrier functions. Its stimulation increased the permeability of human colonic epithelial monolayers. IL-4 is increased in food allergy and in early Crohn's disease	IL-4 induced autophagy in B cells.	Yes	64–66	67	104

IL-7	High levels of IL-7 promote the T cells activation; T cells activated mediate inflammation, not only in the intestine, but also in other organs.	IL-7 can both promote or inhibit autophagy flux.	Yes, as enhancer	68	69,70	71
IL-9	IL-9 is involved in the barrier function of the intestinal epithelium through regulation of Claudin-2, a mediator of leaky gut barrier. It is overexpressed in intestinal biopsies of UC patients and in serum of patients with a severe prognosis of IBD.	NO.	Yes, as effector of Th9 cells.	72		73
IL-10	IL-10 is critical to maintaining healthy epithelial homeostasis through supporting cellular function, the barrier integrity through regulating intercellular junctions, and mucosal tolerance. It supports the maturation, function of differentiated IECs, such as Paneth cells and goblet cells, while preserving stem cell renewal. IL-10 sustains and supports cell repair; exerts antiapoptotic effects on IECs by acting on IEC gene expression and extrinsic apoptosis-inducing factors.	IL-10 inhibits autophagy.	Yes, as inhibitor of IKK-phosphorylation.	74	42	75
IL-15	IL-15 is produced by IECs. Intestinal IL-15 is required in development and maintenance of IELs, it is also critical for the survival and function of NK-cells. IL-15 and IL-15R complex are involved in the pathogenesis of IBD as mediators in activation of innate and adaptive immunity in intestinal mucosa. It is the major mediator of the innate immune response and cooperates with active epidermal growth factor	IL-15 stimulates autophagy pathway.	Yes, as enhancer.	76,77	78	79

	(EGF) to alter vesicular trafficking in CeD.					
IL-17	It plays a critical role in chronic intestinal inflammation. Although IL-17 and TH17 cells could drive inflammation that damages the gut mucosa, IL-17 and IL-22 also play protective roles in limiting infection of the gut.	IL-17 can both induce or inhibit autophagy.	Yes, as enhancer.	80	81	82
IL-12P70	Unclear	IL-12 can induce autophagy.	Yes, as effector.		83	84
MIP-1α (macrophage inflammatory protein 1a)	In necrotizing enterocolitis, CCL3 induced M1 macrophage polarization in intestinal mucosa, promoting inflammatory injury of intestinal tissue.	NO.	Yes, as effector.	85		71
MIP-1β (macrophage inflammatory protein 1b)	In study with colitis mice identify in MIP-1b an enhancer for develop of large transmural ulcers.	NO.	Yes, as effector.	86		71
IP 10 (Interferon (IFN)-γ inducible protein 10)	This cytokine is permanently over-expressed in IBD patients. Block of it in mice prevents the acute colitis, suggesting its role in IBD pathogenesis.	IP-10 plays an important role in impaired autophagy.	Yes, as effector.	87	88	87,89
CCL-5 (C-C Motif Chemokine Ligand 5)	An overabundance of CCL5 may attract extra inflammatory components, thereby amplifying the inflammatory response of the gut mucosa, increased generation of CCL5 may lead to excessive expression of CCR5. The CCL5/CCR5 axis will attract white blood cells to aggregate around the lesion area, amplifying the local inflammatory response.	NO.	One of the primary downstream pathways of CCL5/CCR5 is NF-kB.	90		90

PDGF-BB (Platelet Derived Growth Factor Subunit B)	PDGF-BB is highly expressed in IBD, suggesting its role in inflammatory response. PDGF-BB plays a protective role against intestinal inflammatory reaction.	It is a regulator and enhancer of autophagy in smooth muscle cells.	Yes, as enhancer.	91	92	93,94
GM-CSF	GM-CSF is a critical regulator of macrophage activation in intestinal inflammation. It is able to induce anti-microbial inflammatory M1 macrophages while suppressing a tissue-reparative macrophage phenotype.	Decreasing of autophagy by activation of mTOR; GM-CSF helps Beclin1 to stimulate autophagy.	Yes, as enhancer.	95	96	97
G-CSF	G-CSF can determine a pro or anti-inflammatory response by influencing interaction between T cell and dendritic cell. It is a modulator of intestinal immune system and assist in mucosal healing. Increased G-CSF antibodies levels are associated with alterations of epithelial barrier function, permeability, bacterial translocation and epithelial cell survival and proliferation.	G-CSF activates autophagy.	Yes, as effector.	98	99	100
TNF-α (Tumor Necrosis Factor α)	It's a modulator of cell death or proliferation, a key mediator of intestinal inflammation, causes an increase in intestinal epithelial tight junction. It plays a critical role for the pathogenesis of IBD, infectious diseases, intestinal wound healing and tumor formation. It is an enhancer of TG2 as like as IFN- γ . TNF- α causes damage to the intestinal mucosa by triggering the death of intestinal epithelial cells.	TNF- α positively regulates autophagy.	Yes, as enhancer.	101,102	103,104	101

Table 1: List of the cytokines, chemokines and growth factors increased in culture media from GCD- and GFD-CeD organoids respect to CTRs. The first six cytokines, chemokines and growth factors were increased both in GCD- and GFD-CeD; the followings 16 were increased only in GCD-CeD and the last cytokine, TNF-alpha was increased only in GFD-CeD. Their role in the intestinal epithelium and/or in the regulation of the autophagy and/or their relation to the NF-kB pathway was highlighted.

Table 2.

Patients	Range Age (Years)	Sex	Biopsy (Marsh Classification *)	Serum AntiTG2 (U/mL)	Anti- Endomysial Antibody (EMA)
Controls (N = 5)	9 – 17	M = 1 F = 4	5 = T0	0 – 1.7	Negative
GCD–CeD (N = 5)	4 – 16	M = 2 F = 3	4 = T3c 1 = T3 a/b	> 50	Positive

Patients' characteristics.* T0: Normal; T3: Flat destructive lesion (a: mild, b: moderate, c: total).

Table 3.

Patients	Range Age (Years)	Sex	Biopsy (Marsh Classification *)	Serum AntiTG2 (U/mL)	Anti- Endomysial Antibody (EMA)
Controls (N = 5)	9 – 18	M = 3 F = 3	4 = T0 1 = T1	0-0,9	Negative
GCD–CeD (N = 5)	2 – 15	M = 2 F = 3	5 = T3c	2 > 50 3 ≥ 100	Positive
GFD–CeD (N = 5)	2 – 16	M = 2 F = 3	5 = T0	0-2	Negative

Organoids characteristics. * T0: Normal; T1: infiltrative lesion; T3: Flat destructive lesion (c: total).

Chapter 4

Inflammation and delay of the autophagy in intestinal organoids from remission Eosinophilic Esophagitis patients (REM-EoE)

Bellomo C.^{1,2}, Strisciuglio C.³, Miele E.¹, Furone F.^{1,2}, D'Aniello G.², D'Esposito V.^{3,4}, Formisano P.^{3,4}, Maglio M.², Nanayakkara M.¹, Barone M. V.^{1,2}

¹. Department of Translational Medical Science, Section of Pediatrics, University Federico II, Naples, Italy.

². ELFID (European Laboratory for the Investigation of Food Induced Diseases), University Federico II,, Naples, Italy

³. Department of Woman, Child, General and Specialistic Surgery, University of Campania "Luigi Vanvitelli", Naples, Italy.

⁴. National Council of Research, URT of the Institute of Experimental Endocrinology and Oncology "G. Salvatore", Naples, Italy.

⁵. Department of Translational Medicine, Federico II University of Naples,, Naples, Italy.

Corresponding author: M.V. Barone

Tel: 0817464569

Abstract:

Eosinophilic Esophagitis is a chronic, immune-mediated oesophageal disease characterised by eosinophilic infiltration and inflammation in response to allergens, primarily contained in food. It is characterised by dysphagia and other gastrointestinal symptoms and is associated with atopic diseases. Diagnosis is made by oesophageal endoscopic evaluation when infiltration of over fifteen eosinophils per high-power field (HPF) and basal cell hyperplasia is present. Dietary modifications, including the exclusion of gluten from the diet, pharmacotherapy, and sometimes mechanical dilation are the current therapeutic treatments that can lead to remission of the diseases. Ongoing research aims to improve understanding and management of EoE, enhancing patient outcomes and quality of life.

The aim of the present study was to evaluate the inflammation and autophagy/ mTOR activation in intestinal organoids from EoE subjects in the remission phase of the disease and the effects of pro-inflammatory stimuli such as gliadin peptide, P31-43.

To this aim, we isolated intestinal organoids from duodenal biopsies from 7 patients in the remission phase of the disease (REM-EoE) (with less than 15 eosinophils HPF), 3 non-eosinophilic oesophageal

gastrointestinal disease (ne-EGID) and 4 controls (CTR). pNF-kB was used as marker of inflammation, p62 and pE4BP as markers of autophagy and mTOR pathway respectively, they were evaluated by WB before and after gliadin peptide treatment. Twenty-seven inflammatory cytokines, chemokines and growth factors were evaluated by Bio-plex, in the organoids cell medium (CM) before and after gliadin peptide treatment.

Intestinal organoids from REM-EoE were inflamed significantly respect controls as shown by pNF-kB increase ($p < .0001$). In organoids culture medium, as analysed by Bio-plex, there was a statistically significant increase of 7 cytokines, 3 chemokines and 3 growth factors all related to inflammation and autophagy. Gliadin peptide treatment, in 3 patients out of 6 organoids, from REM-EoE increased pNF-kB respect to untreated organoids, as evaluated by WB. Bio-plex analysis after gliadin peptide, P31-43, treatment showed increase of several cytokines mostly related to the NF-kB pathway. Autophagy flux was delayed and mTOR pathway activated.

These data demonstrated that intestinal organoids derived from REM-EoE patients were inflamed, presented delay of the autophagic flux and activation of the mTOR pathway independently of the presence of eosinophils in the oesophagus. Moreover, half of REM-EoE patients' organoids were sensitive gliadin peptide pro-inflammatory stimuli, indicating that intestinal organoids could be a tool for precision medicine in this disease. Duodenal inflammation and gliadin peptide effects could play a still uncovered role in EoE pathogenesis.

1. Introduction

Eosinophilic oesophagitis (EoE), a chronic immune-mediated oesophageal disease, is characterized by eosinophil-predominant inflammation with symptoms related to oesophageal dysfunction^{1,2}.

EoE is defined as a pathology with multifactorial aetiology in which genetic and environmental factors contribute to the pathogenesis. Genetic variants associated with epithelial barrier dysfunction and a Th-2 immune response have been identified. Environmental factors such as inflammatory mediators, atopic conditions, certain dietary triggers, and intestinal microbiota are added to genetic factors^{3,4}.

The symptoms in patients with EoE are often related to strictures in the oesophagus. In these patients' oesophageal inflammation involves the entire thickness of the wall, with consequent subepithelial fibrosis and tissue remodelling which can lead to oesophageal stricture⁴⁻⁶.

For the diagnosis of EoE, the gold standard is based on oesophageal biopsies that shows increased intraepithelial eosinophils, with no eosinophilic infiltration in the stomach or duodenum⁷.

EoE is becoming, in the last decade, a common disorder with an incidence of 0.5-1 cases per 1.000 inhabitants. As it can cause significant restrictions to quality of life, it requires a fast diagnosis and specific therapy^{8,9}.

Dietary therapy with the exclusion of several allergens including gluten, potentially combined with intermittent use of topical steroids, and sometimes mechanical dilatation are currently the therapeutic intervention for these patients¹⁰.

Gliadin is the main protein contained in gluten. Gliadin peptides are difficult to digest by intestinal endopeptidases and the undigested peptides may have biological activity. Some of them, such as 25-mer, can activate the adaptive immune response, as it is well presented to T cells. The p31–43 peptide, play key roles in the inflammatory/innate immune response to gliadin¹¹. P31-43 gliadin peptide has pleiotropic activity, such as the induction of proliferation and inflammation, with the activation of the nuclear transcription factor- κ B (NF- κ B) pathway^{11–13}, a key protein in the regulation of inflammation in the intestine¹³.

Autophagy has been described in EoE oesophageal biopsies probably associated with a cytoprotective mechanism in oesophageal epithelial cells. In murine oesophageal organoids the autophagy mechanisms were essential to control basal cell hyperplasia¹⁴. Moreover, in EoE children's biopsies there was increased autophagic vesicle content respect controls, and an alteration of autophagy was observed by flow cytometric analysis¹⁴. These data support the role of autophagy in the oesophageal epithelium in maintaining epithelial balance and homeostasis in the stress condition associated with EoE¹⁴.

Why these patients can improve by the exclusion of gluten from their diet is not clear. Moreover, in these patients the focus of the studies available in literature is on the oesophagus, the state of the duodenal mucosa is generally not evaluated, except for the eosinophil count^{15–18} (Appendix 1 and 2). For these reasons, the present study aimed to analyse inflammation and autophagy in intestinal organoids from the duodenum of EoE patients in the remission phase of the disease and to use them to test the pro-inflammatory effects of gliadin peptide, P31-43.

2. Results

2.1 The levels of pNF- κ B were significantly increased in intestinal organoids from REM-EoE respect to controls

Crypts isolation from intestinal biopsies of 7 patients REM-EoE, 3 non-eosinophilic oesophageal gastrointestinal disease (ne-EGID) and 4 controls (CTR) were used to develop intestinal organoids. They were derived from intestinal staminal cells and in our experimental conditions were composed

mainly of enterocytes. Organoids are spheroids grown in a tri-dimensional matrix with the apical side of the enterocyte enclosed within the spherical organoids. As in these conditions, the apical part of the epithelial cells is inaccessible to treatment, for our experiments we have opened them in 2D on a matrix bed^{16,17} (Appendix 1 and 2). Phosphorylation of nuclear factor kappa-light-chain-enhancer of activated B cells (pNF-kB) was used as a marker of inflammation. pNF-kB levels were increased in a statistically significant way ($p<.0001$) in REM-EoE organoids respect to CTR organoids by WB analysis (Figure 1 A and B). Controls organoids derived both from ne-EGID and from patients with functional disorders not related to the oesophagus (CTR) did not present any alteration of the pNF-kB levels (Fig 1 C and D). This indicates that the increase in the inflammation was specific of the REM-EoE organoids.

2.2 Cytokines, Chemokines and Growth factors were altered in intestinal organoids of EoE remission patients respect to controls

The culture media (CM), from the same intestinal organoids of REM- EoE, ne-EGID and CTR tested before for NF-kB activity, were used to evaluate the levels of 27 pro-inflammatory cytokines, chemokines, and growth factors by Bio-Plex assay. The levels of IL-1 β ($p<.001$), IL-5 ($p<.01$), IL-7 ($p<.001$), IL-9 ($p<.05$), IL-15 ($p<.05$), IL-17 ($p<.05$), TNF- α ($p<.01$), MIP-1 α ($p<.05$), MIP-1 β ($p<.05$), Eotaxin ($p<.001$), FGF-basic ($p<.01$), G-CSF ($p<.05$), and VEGF ($p<.001$) were increased in a statistically significant way in REM-EoE organoids respect to CTR (Fig. 1E). Moreover, MCP-1 was increased over the maximum read level of the assay, in the CM of REM-EoE organoids respect to controls (Fig. 1E). Most of the cytokines that were increased in EoE patients respect to CTR had a role at the level of the intestine and were relate to the NF-kB pathway (Table 1). Interleukin 10 and IL-1Ra also resulted increased but did not reach the statical significance (Fig. 1E; $p<.069$ and $p<.08$ respectively). IL-4, IL-6, IL-8, IL-13, PDGF- β and GM- CSF means were increased more than twice in REM-EoE respect to CTR, but did not reach the statistical significance, indicating that there was a certain level of variability in patients with REM-EoE for the expression of these cytokines and growth factors (Fig. 1F). IL-13, the most studied cytokine in EoE was increased only in CM from three patients' organoids (pz#4, #5, # 6), in a statistically significant way (Figure 1 G; $p<.001$). Interestingly, IL-1 β , IL-5, IL-7, IL-15, IL-17, FGF-basic, G-CSF, TNF- α , VEGF, MCP-1 and IL-1Ra, all of them statistically significantly increased in REM-EoE respect to CTR, had also a role in the regulation of the autophagy at the level of the intestine (Table 1).

2.3 Intestinal organoids from REM-EoE patients were used to test sensitivity to gliadin peptide P31-43

Intestinal organoids from CTR, ne-EGID and REM- EoE were treated with gliadin peptides for 3 hours at a concentration that did not affect CTR organoids (Figure 2) and the level of pNF-kB was evaluated by WB¹⁶ (Appendix 1). As expected, in CTR the level of pNF-kB did not increase after gliadin peptides treatment at the concentration of 10 micrograms/ml (Figure 2 A and B). In the same conditions the treatment with gliadin peptides was not able to increase pNF-kB levels in ne-EGID (Figure 2 C and D). On the other side, 6 patients with REM-EoE were treated with gliadin peptides at the concentration tested in CTR and ne-EGID. As shown in figure 3, three REM-EoE patients out of six (Figure 2 E-H) responded to the treatment with gliadin peptides by increasing pNF-kB. The increment was, respectively, for patient #1 of 2.29 times (Figure 2 E); for patient #2 of 3 times (Figure 2 F) and for patient #3 of 10 times (Figure 2 G) respect to untreated organoids. The increase of pNF-kB was statistically significant (Figure 2 H; $p<.05$) considering the three REM-EoE organoids responsive to the treatment with gliadin peptides (#1, #2, #3). The other three patients, #4, #5 and #6 did not respond to the gliadin peptides treatment (Figure 2 I-N).

When all six REM-EoE patients organoids were considered together, the levels of pNF-kB did not increase in a statistically significant way (Figure 2 O).

2.4 Cytokines profile analysis of cell medium from intestinal organoids of EoE remission patients after gliadin peptides treatment

The CM of REM-EoE organoids untreated and treated with gliadin peptides were tested to evaluate the levels of inflammatory cytokines, chemokines, and growth factors. Bio-plex analysis of the CM of six REM-EoE patients' organoids, after treatment with gliadin peptide P31-43, showed a statistically significant increase only of TNF- α levels, respect the untreated patients (Fig. 3 A, $p<.05$). The levels of IL-9 ($p=.06$), VEGF ($p=.08$) and PDGFB ($p=.07$) were increased, although they did not reach the statistical significance (Figure 3 B). The other 23 cytokines, chemokines and growth factors evaluated did not show statistically significant differences after gliadin peptide treatment (Supplementary Figure 1A).

Organoids from REM-EoE patients #1, #2 and #3, that responded to gliadin peptide, P31-43, by increasing pNF-kB as evaluated by WB (Figure 2 E- H) increased in the CM only IL2 in a statistically significant way, evaluated by Bio-Plex analysis (Figure 3 C; $p<.05$). Interleukin-5, IL-6, TNF- α , MIP-1 β and PDGF- β did not reach statistical significance although they showed an increment after treatment with gliadin peptides (Figure 3 D). All this indicates that these organoids are not only inflamed but can also produce inflammatory cytokines after gliadin peptides treatment.

Interestingly the CM of patients' organoids with ne-EGID, did not show any increase of cytokines, chemokines, and growth factors after treatment with gliadin peptides, indicating that the effect of gliadin was specific for REM-EoE organoids (Figure 4).

2.5 In intestinal organoids from REM-EoE patients' the mTOR pathway is activated and the autophagic flux is reduced

Eleven out of 17 cytokines, chemokines and growth factors found altered in CM from REM-EoE organoids were also correlate with autophagy flux. For this reason, we have evaluated the autophagic flux and the activation of the mTOR pathway in intestinal organoids from REM-EoE and CTR. p62 and pE4-BP1 protein levels were analysed by WB to study the autophagic flux and the mTOR pathway respectively. The levels of p62 (Figure 5 A and B; $p<.05$) and pE4-BP1 (Figure 5 A and C; $p<.05$) were significantly increased in REM-EoE intestinal organoids respect to controls, as analysed by WB analysis (Fig 5 A, B, C). All these data indicated an alteration of the autophagic flux in intestinal organoids from patients in remission phase of EoE respect to controls.

3. Discussion

In this manuscript, we have described inflammation, delay of the autophagic flux and activation of the mTOR pathway at the level of the intestinal epithelium in organoids from REM-EoE patients. In fact, markers of inflammation, such as pNF-kB, of autophagy, such as p62 and of activation of the mTOR pathway, such as pE4-BP1, were altered in organoids from REM-EoE patients respect to controls. Organoids from REM-EoE patients not only were inflamed, but they also produced cytokines, chemokines, and growth factors in culture media. All of them were related to NF-kB pathway and 11 out of 17 were associated with the regulation of the autophagic pathway. We have also verified the hypothesis that intestinal organoids could be used to test gliadin sensitivity in these patients.

Inflammation, at the level of the oesophagus epithelium, plays a crucial role in EoE^{19,20}. Chronic inflammation and epithelial barrier disruption have been demonstrated by genomic and transcriptomic studies to have a role in EoE. Inflammation in EoE can activate several cell types, including eosinophils, dendritic cells, effector Th2 cells, mast cells, and basophils. The initial trigger in this disease is usually the exposure to food or aeroallergens, causing the release of oesophagus epithelial alarmins and type 2-specific cytokines²¹. It has been also suggested that the alteration of the oesophageal epithelial permeability could lead to a permissive environment that allows contact between antigens and the immune system²².

Several pro-inflammatory cytokines have been found to increase in both oesophageal epithelium and serum in EoE, of mice model^{23,24} and patients^{25–28}. In EoE there is an important production of IL-13 at the level of oesophagus²⁵. Three-dimensional organoid and air-liquid interface cultures of esophageal epithelial of EoE have been cultivated also in the presence of IL-13²⁹ showing that there was an increase of barrier permeability dependent on this cytokine. Esophageal organoids from EoE have been attempted, although, in theory, they are a good cellular model of the disease, they tend to lose differentiation while in culture. Probably the optimal culture conditions have not been found yet³⁰. Interestingly, in our experiments on CM from REM-EoE duodenal organoids, IL-13 did not increase in a statistically significant way respect to CTR.

The exclusion of six common food triggers of oesophageal injury—milk, egg, wheat, soy, fish and nuts—substantially reduced signs and symptoms of EoE^{31,32}. Eventually, these triggering foods are introduced one at a time after EGD to check the reduction of eosinophils in the oesophagus. Apparently together with milk, also wheat/gliadin are the antigens' food more frequently excluded in EoE to induce remission³². The diagnostic pathway to find the allergens responsible for the eosinophils increase in EoE can be taxing, mainly in children^{33,34}. The patients enrolled in this study were following different dietary exclusions and drug therapy (Table 3).

Duodenal alteration in REM-EoE has been described before in only one study that investigated the possible relationship between EoE and alteration of duodenal track suggesting that duodenum could play a role in EoE pathophysiology²⁸. In this paper, we showed that intestinal organoids from the duodenum of REM-EoE are inflamed and produce inflammatory cytokines. We tested, then, the hypothesis that they could be used to investigate the effect of gluten in REM-EoE. The gluten response described in organoids from REM-EoE patients enrolled was independent of any alterations related to Celiac Disease i.e. lymphocytic infiltrate in duodenal biopsies and antibody anti-tTG (transglutaminase) and EMA (endomysial antibodies) in the serum or Marsh classification (Table 4). Moreover, gliadin peptide P31-43 did not have any effect on the pNF- κ B levels of organoids derived from patients with oesophageal disease not related to eosinophils (ne-EGID) showing a specificity of the effects in REM-EoE organoids. Then we investigated the inflammatory marker pNF- κ B by WB analysis of REM-EoE organoids. We found that only three of them responded to gliadin treatment by increasing further this marker. Showing that only half of our intestinal organoids derived from REM-EoE patients responded to gliadin peptides. In the culture media of all organoids from REM-EoE patients, the only cytokine that increased after treatment with gliadin peptide P31-43 was TNF- α , one of the main activators of canonical NF- κ B signalling, also a mediator of inflammation and allergy in the gut^{35,36}. In the media of the patients that increased pNF- κ B after gliadin peptide treatment only IL-2 was increased in a statistically significant way. This cytokine has a role in intestinal epithelial

cells (IEC), by inducing cell migration³⁷. Moreover, it can activate peripheral blood eosinophils to release granule mediators (EPO) and several cytokines. It is an important mediator in allergic inflammation and a possible target for pharmacological modulation³⁸. In the CM analysed by Bio-plex, gliadin peptide P31-43 did not have any effect on organoids derived from patients with esophageal disease not related to eosinophils (ne-EGID) confirming a specificity of the effects of the gliadin peptide P31-43 in REM-EoE organoids. Interleukin 13 did not increase in a statistically significant way after gliadin peptide, treatment when considering all patients together. Interestingly the three patients (pz#3, #4, #5), that after gliadin peptide P31-43 did not increase pNF-kB, showed an increase, statistically significant, of this cytokine, respect to CTR.

All the patients enrolled in this study were eating gluten at the time of the biopsies from which organoids were derived. Three out of 7 patients had reintroduced gluten in the diet. One of these patients (pz#2), 2 years after the biopsy from which the organoid was generated, developed non-celiac villus atrophy in the duodenum. Interestingly, the organoid from this patient responded to gliadin peptides treatment increasing pNF-kB and cytokines in the CM. All this indicates that at least in some patients, gliadin can have inflammatory effects on REM-EoE organoids from duodenum. What is the link between non-celiac atrophy and gliadin is not clear, yet, and non-celiac atrophy itself is a new entity still not fully investigated. Moreover, there is no indication of management of these patients.

Autophagy has been described in EoE oesophageal biopsies probably associated with a cytoprotective mechanism in oesophageal epithelial cells. In murine oesophageal organoids, the autophagy mechanisms were essential to control basal cell hyperplasia¹⁴. Moreover, in EoE children's biopsies, there was increased autophagic vesicle content respect to controls, and an alteration of autophagy was observed by flow cytometric analysis supporting a role of autophagy in the oesophageal epithelium to maintain epithelial balance and homeostasis in EoE¹⁴.

In this paper, we show that in intestinal organoids from all REM-EoE patients analysed there was a delay of autophagy flux and, as expected, an increase of pE4-BP1, a downstream effector of mTOR. In our experimental condition, it's not possible to understand whether the autophagy/mTOR pathway is primarily or secondarily altered in organoids from REM-EoE patients. But both the alteration of autophagy and the increase of mTOR pathway confirm the metabolic stress of these epithelial cells. What is the role of the inflammation and/or autophagy delay and/or mTOR pathway activation in the duodenum, a region far away from the oesophagus, of REM-EoE patients, needs to be further investigated. The main question that remains unanswered is whether this inflammation and/or autophagy delay and/or mTOR pathway activation precedes or follows the oesophagus alteration. In fact, it could be either the result of the oesophagus inflammation, or even, the cause. Either way, the inflammatory alteration of intestinal organoids from REM-EoE patients could be used to forecast

food sensitivity in these patients in a personalised way. Considering that duodenal organoids are easy to develop, they could be a precious tool in precision medicine to test not only gluten but also other allergens avoiding, altogether, the need for several EGD in these patients.

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4. Material and Methods

4.1 Patients characteristics

Duodenal biopsies were obtained from 7 patients at the remission stage of eosinophilic esophagitis (REM-EoE), 3 non-eosinophilic esophageal gastrointestinal disease (ne-EGID) and 4 controls (CTR) affected by functional dyspepsia. The age range for all of them was 2-16 years old. The group of patients with REM-EoE and the CTR group had negative serology for celiac disease (anti-tTg antibodies and EMA). Moreover, all the controls were on a free diet and were not taking any drugs. Eosinophils per HPF indicated for REM-EoE patients represented the average number of eosinophils present in the proximal, middle and distal oesophagus (Table 2). On the other hand, the patients with eosinophilic esophagitis followed different treatments, as described in the table (Table 3).

4.2 Immunohistochemical Evaluation

One fragment from duodenal biopsy was added to an optimal cutting temperature compound (Killik; BioOptica, Milan, Italy), stored at -80°C , and used for immunohistochemical staining for CD3⁺, TCR $\gamma\delta$ ⁺, and CD25⁺ cells, as previously reported. The number of stained cells per millimetres of epithelium determined the density of the cells expressing CD3 and TCR $\gamma\delta$ in the intraepithelial compartment. The cut-off values for CD3⁺ and TCR $\gamma\delta$ ⁺ cells were 34 mm and 3.4 mm per epithelium, respectively. On the other hand, the number of cells expressing CD25 in the lamina propria was evaluated within a total area of 1 mm². The cut-off value for CD25⁺ cells was 4 per mm² of the lamina propria. To determine the cut-off values, 100 children with untreated celiac disease and 50 non-celiac disease control children were studied. The percentiles were obtained using the Statistical Package for the Social Sciences SPSS software (IBM, Chicago, IL, USA). The cut-off values represented the 90th percentile of control patients (Table 4).

4.3 Intestinal deposits of anti-TG2

From 4 out of 7 controls and from 4 out of 7 REM-EoE patients if present one fragment more, it was frozen in optimal cutting temperature compound (OCT-Tissue-Tek, Sakura, Finetek, Nederland) and stored in liquid nitrogen. Duodenal biopsies from all patients were investigated for the presence of intestinal deposits of anti-TG2 IgA antibodies. Briefly, fixed in acetone, 5 μm frozen sections of each patient were examined by double immunofluorescence. After a 15-minute preincubation with rabbit normal serum (1:100, Dako, Milan, Italy), the sections were covered with a monoclonal mouse antibody against guinea pig TG2 (CUB 7402) (1:200, NeoMarkers, Frimont, CA, USA) for 1 h at room temperature in a humid chamber. The sections were washed in PBS and incubated with a

mixture of fluorescein isothiocyanate (FITC)-labelled rabbit antibody against human IgA (1:100, Dako), to detect (in green) IgA deposits, and R-phycoerythrin (RPE)-labelled rabbit anti-mouse antibody (1:40, Dako), to detect (in red) TG2, for 30 min in the dark. Finally, the section was washed several times in PBS and mounted by glycerol/PBS (1:10). The colocalization images of IgA mucosal deposits and TG2 that resulted in yellow were analysed by a confocal microscopy (LSM510, Zeiss, Milan, Italy). Five sections from each sample were evaluated by two blinded investigators who had no knowledge of the patient's clinical or laboratory findings. Intra-observer and inter-observer variations were each 98%. The evaluation of anti-TG2 IgA mucosal deposits was performed considering the pattern and the intensity of the staining. It was graded semiquantitative as follows: negative (1), very weak (2), weak with patchy distribution (3), strong with patchy distribution (4) and strong with a homogeneous distribution (5) (Table 4).

4.4 Intestinal Organoids

One duodenal biopsy from REM-EoE patients and controls was taken during routine gastro-duodenoscopy (Table 2) and placed in 10 mL of cold PBS with the addition of 2 Mm EDTA (cat.15575020, Thermo Fisher, Milan, Italy) and 0.5 Mm DDT (D0632, Sigma-Aldrich, Milan, Italy). After 50 minutes of agitation at 4 °C, the biopsy samples were washed with PBS, filtered through a 70 µm strainer (Falcon, NY, USA) and centrifugated at 2800 rpm for 5 min at 4°C, then the crypts were resuspended in 40 µL of ice-cold Matrigel matrix (cat.35623, Corning, Milan, Italy) to permit three-dimensional growth in 48 well plates. Afterwards, 300 µL cell culture medium enriched with supplements (CM-S) was added to each well¹⁷ (Appendix 2). To pass from a 3D to a 2D structure, organoids were openly seeded on 35 mm multi-wells pre-treated with Matrigel (1:40 in PBS). We generally processed the 2D organoids within 24–72 h after seeding^{16–17} (Appendix 1 and 2).

4.5 Western Blot

Intestinal organoids cultivated for 4 weeks and opened in 2D from duodenum obtained from REM-EoE and CTR were processed. After they were stimulated with P31-43 (Caslo, Kongens Lyngby, Denmark) at 10µg/mL for 3 hours at 37 °C, removing Matrigel by Cell Recovery Solution (cat. 354270, Corning Milan Italy), organoids were homogenized in 40-50 µL tissue homogenization buffer^{16,17} (Appendix 1 and 2). The cell lysates (10 µg/mL) were analysed by SDS-PAGE and transferred onto nitrocellulose membranes by Trans Blot Turbo (cat. 1704158, Bio-Rad, Milan, Italy). The membranes were blocked with 5% non-fat dry milk and probed with anti-pNF-kB (cat. FNab10840, FineTest, Hubei, China) and anti-p62/SQSTM1 (cat. P0067, Sigma Aldrich, St. Louis, Missouri, USA), anti-pE4-BP1 (Y1068) (cat.3777, Cell Signalling, Euroclone, Milan, Italy) and

mouse anti-GAPDH, and stain free gel at 10% (cat. #4568035, Bio-Rad, USA) were used as loading controls organoids. Then, two 2-5 minutes exposure using ECL-plus (Cat. 1705062 Bio-Rad, USA) allowed to visualise the bands of interest. The bands intensity was evaluated by integrating all the pixels of the band after subtraction of the background to calculate the average of the pixels surrounding the band¹⁶⁻¹⁷ (Appendix 1 and Appendix 2).

4.6 Cytokine Analysis

Intestinal organoids were grown in 2D to investigate the levels of cytokines, chemokines and growth factors in culture medium with or without presence of P31-43 gliadin peptide (Caslo > 95% purity, MALDITOF analysis; DK-2800 Kongens Lyngby, Denmark) at 10 µg/ml. Culture medium of 2D organoids were collected in 1,5 ml Eppendorf after 3 hours of experiment and stored at -80°C. Culture medium were screened for the concentration of interleukin (IL)-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17A, basic fibroblast growth factor (FGF), eotaxin, granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon- γ (IFN- γ), interferon- γ inducible protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 (MIP-1) α , MIP-1 β , C-C motif chemokine ligand 5 (CCL5)/RANTES, TNF- α , platelet-derived growth factor (PDGF-BB), and vascular endothelial growth factor (VEGF) using the Bio-Plex Multiplex Human Cytokine, Chemokine, and Growth Factor Kit (cat. n. M500KCAF0Y, Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol, as previously described³⁹. The magnetic bead-based assay was performed on a Bio-Plex 200 System (Bio-Rad, Hercules, CA, USA). All the values obtained were included within the detection limits indicated by the manufacturer (bio-rad.com/Bio-Plex/AnalyteGuide)⁴⁰.

4.7 Statistical Analysis

Statistical analyses and graphical representations were performed using GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA). Differences between groups were analyzed using Student's t-tests, with statistical significance set at $p < .05$. All analyses were two-tailed.

4.8 Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the University of Naples Federico II, Protocol number: 115/09/ESPROT).

5. Figures

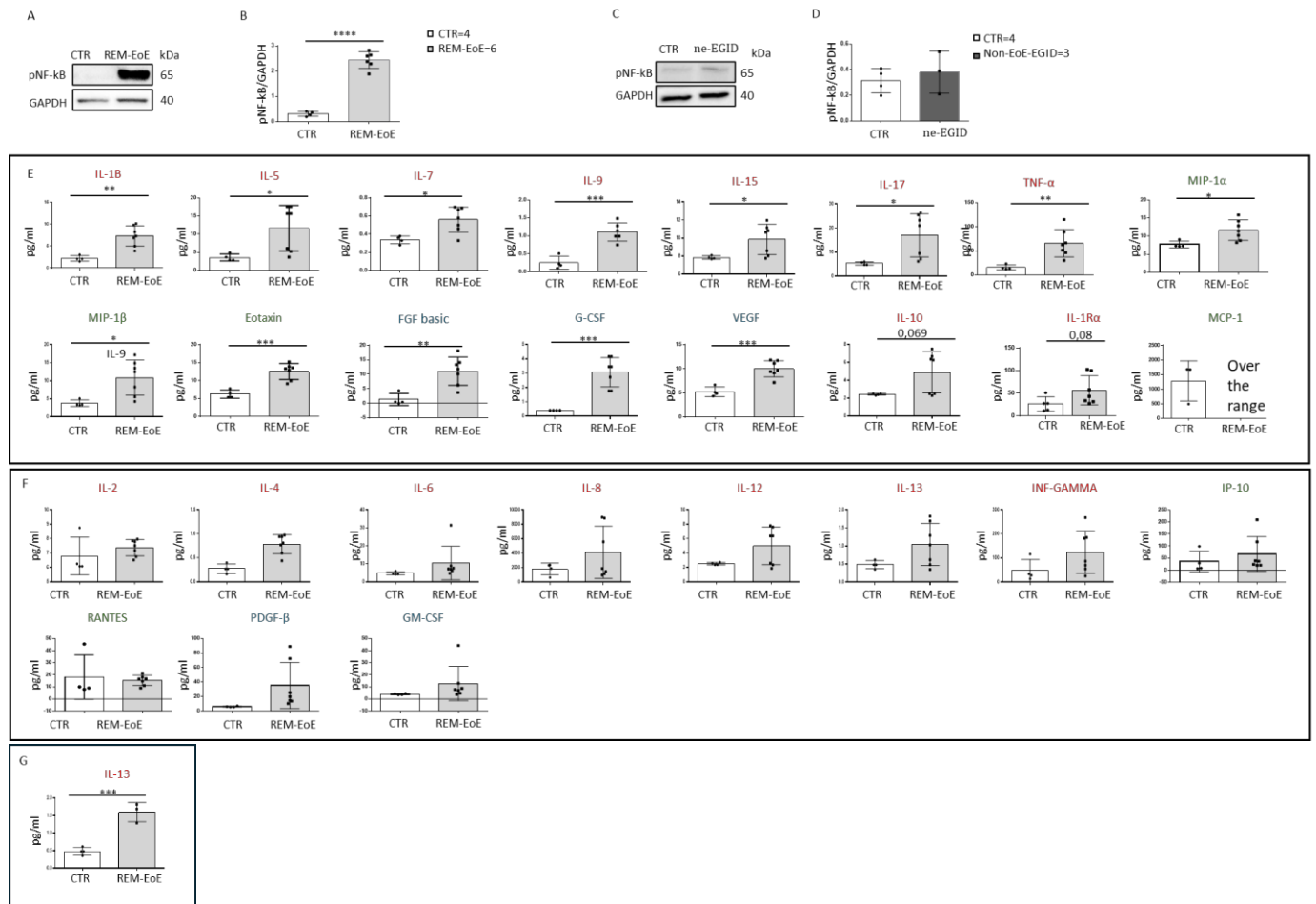


Figure 1. Activation of NF-kB and production of cytokine, chemokine and growth factors in REM-EoE patients respect to control's intestinal organoids

A. Western blot analysis of total protein lysates of organoids from controls (CTR) and patients with EoE remission (REM-EoE). Upper line was blotted with an antibody against the phosphorylated form of NF-kB (pNF-kB). Bottom line was blotted with anti-GAPDH antibody as a loading control. Representative images were selected.

B. Densitometric analysis of the pNF-kB/GAPDH bands from CTR and REM-EoE. The numbers of organoids analyzed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's t test: **** = $p < .0001$.

C. Western blot analysis of total protein lysates of organoids from controls (CTR) and non-eosinophilic oesophageal gastrointestinal disease (ne-EGID). Upper line was blotted with an antibody against the phosphorylated form of NF-kB (pNF-kB). Bottom line was blotted with anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody as a loading control. Representative images were selected.

D. Densitometric analysis of the pNF-kB/GAPDH bands from CTR and ne-EGPD. The numbers of organoids analysed are indicated. Columns represent the mean, and bars represent the standard deviation.

E. F. Bar chart for cytokines, chemokines, and growth factors, expressed as pg/ml, in CM from REM-EoE intestinal organoids respect to CTR, cytokines were indicated in red, chemokine in green and growth factors in blue. Columns represent the mean, and bars represent the standard deviation. Student's t test: $*=p<.05$; $**=p<.01$; $***=p<.001$; $****=p<.0001$.

G. Bar chart for IL-13, expressed as pg/ml, in CM from REM-EoE intestinal organoids (pz#4, #5 and #6) respect to CTR.

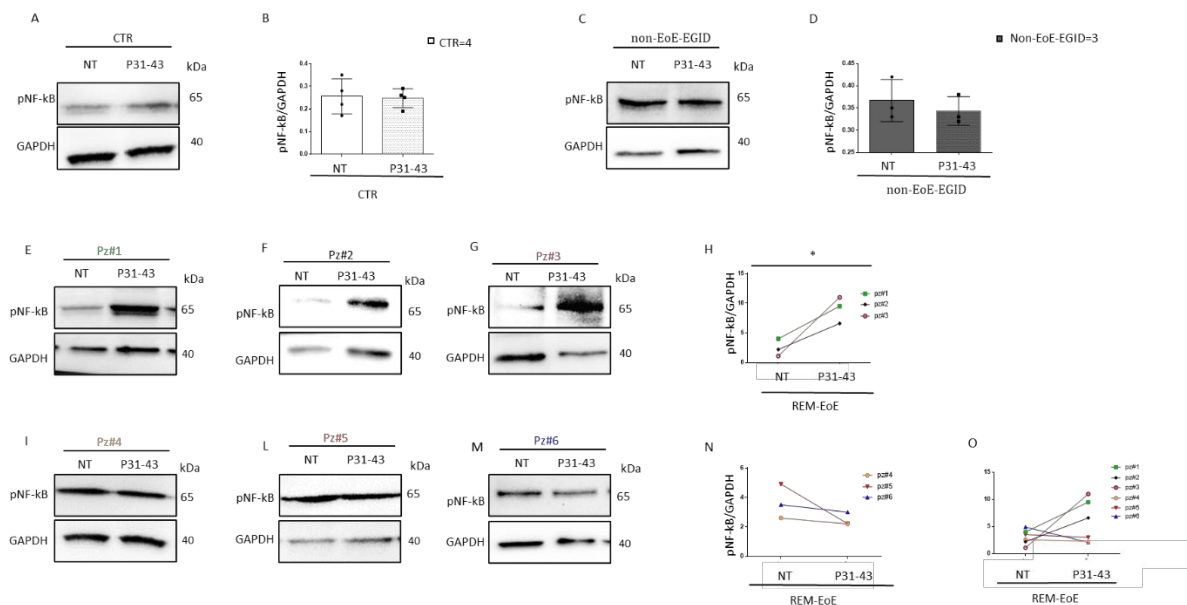


Figure 2. Three out of 6 REM-EoE intestinal organoids responded to gliadin peptides increasing pNF-kB levels at concentrations that did not affect CTR and ne-EGED

A. Western blot analysis of total protein lysates of organoids from controls (CTR) treated with and without gliadin peptide, P31-43. Upper line was blotted with an antibody against the phosphorylated form of NF-kB (pNF-kB). Bottom line was blotted with anti-GAPDH antibody as a loading control. Representative images were selected.

B. Densitometric analysis of the pNF-kB/GAPDH bands from CTR, with and without gliadin peptide. The numbers of organoids analysed are indicated. Columns represent the mean, and bars represent the standard deviation.

C. Western blot analysis of total protein lysates of organoids from non-eosinophilic oesophageal gastrointestinal disease (ne-EGID) treated with and without gliadin peptide, P31-43. Upper line was

blotted with an antibody against the pNF-kB. Bottom line was blotted with anti-GAPDH antibody as a loading control. Representative images were selected.

D. Densitometric analysis of the pNF-kB/GAPDH bands from ne-EGID, with and without gliadin peptide. The numbers of organoids analysed are indicated. Columns represent the mean, and bars represent the standard deviation.

E. F. G. Western blot analysis of total protein lysates of organoids from REM-EoE respectively from pz #1, #2 and #3 treated with and without gliadin peptide, P31-43. Upper line was blotted with an antibody against the pNF-kB. Bottom line was blotted with anti-GAPDH antibody as a loading control.

H. Densitometric analysis of the pNF-kB/GAPDH bands from REM-EoE respectively from pz #1, #2 and #3 with and without gliadin peptide. Each line represented the response after treatment with gliadin peptide. Student t test $\ast = p < .05$

I. L. M. Western blot analysis of total protein lysates of organoids from REM-EoE respectively from pz #4, #5 and #6 treated with and without gliadin peptide, P31-43. Upper line was blotted with an antibody against pNF-kB. Bottom line was blotted with anti-GAPDH antibody as a loading control.

N. Densitometric analysis of the pNF-kB/GAPDH bands from REM-EoE respectively from pz #4, #5 and #6 with and without gliadin peptide. Each line represented the response after treatment with gliadin peptide.

O. Densitometric analysis of the pNF-kB/GAPDH bands from total REM-EoE intestinal organoids treated with and without gliadin peptide. Superimposed symbols with connecting line represented the response of all intestinal organoids after treatment with gliadin peptide.

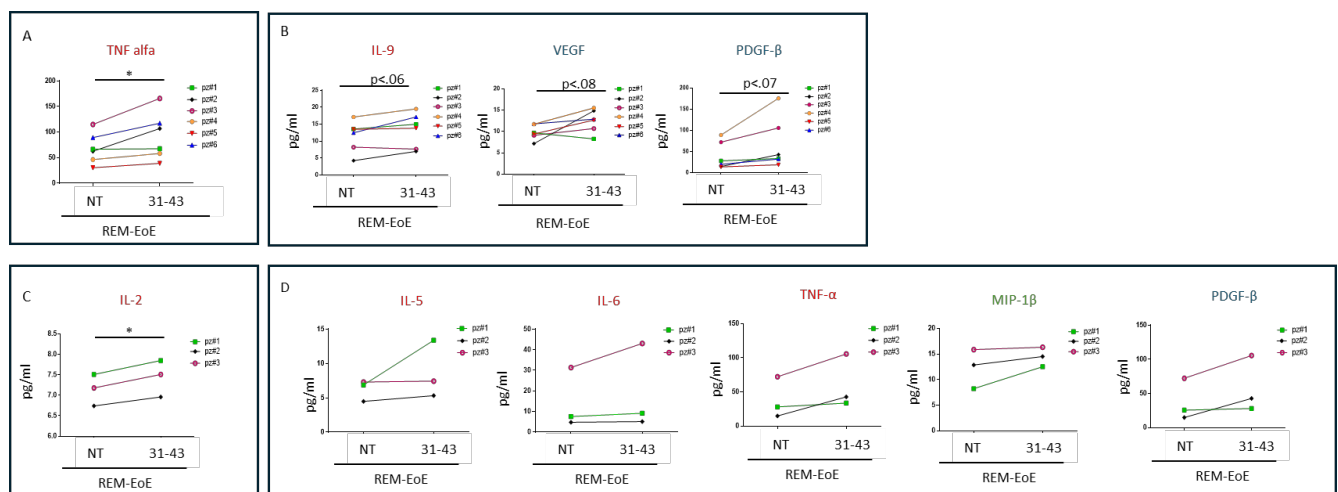


Figure 3. Gliadin peptides P31-43 increased pro-inflammatory cytokines, chemokines and growth factors in CM of REM-EoE intestinal organoids

A. Superimposed symbols with connecting line represent TNF- α concentration, expressed as pg/ml, in cell medium of all REM-EoE intestinal organoids treated with and without gliadin peptide, P31-43. Each line represented the TNF- α production of each organoid. Student's t test: * = $p < 0.05$

C. D. Graphical representation of IL-2, TNF- α , PDGF- β , IL-5, IL-6 and MIP-1 β production, expressed as pg/ml, in cell medium of REM-EoE intestinal organoids pz#1, #2 and #3, who were sensible to gliadin peptide, P31-43. Student's t test: * = $p < .05$

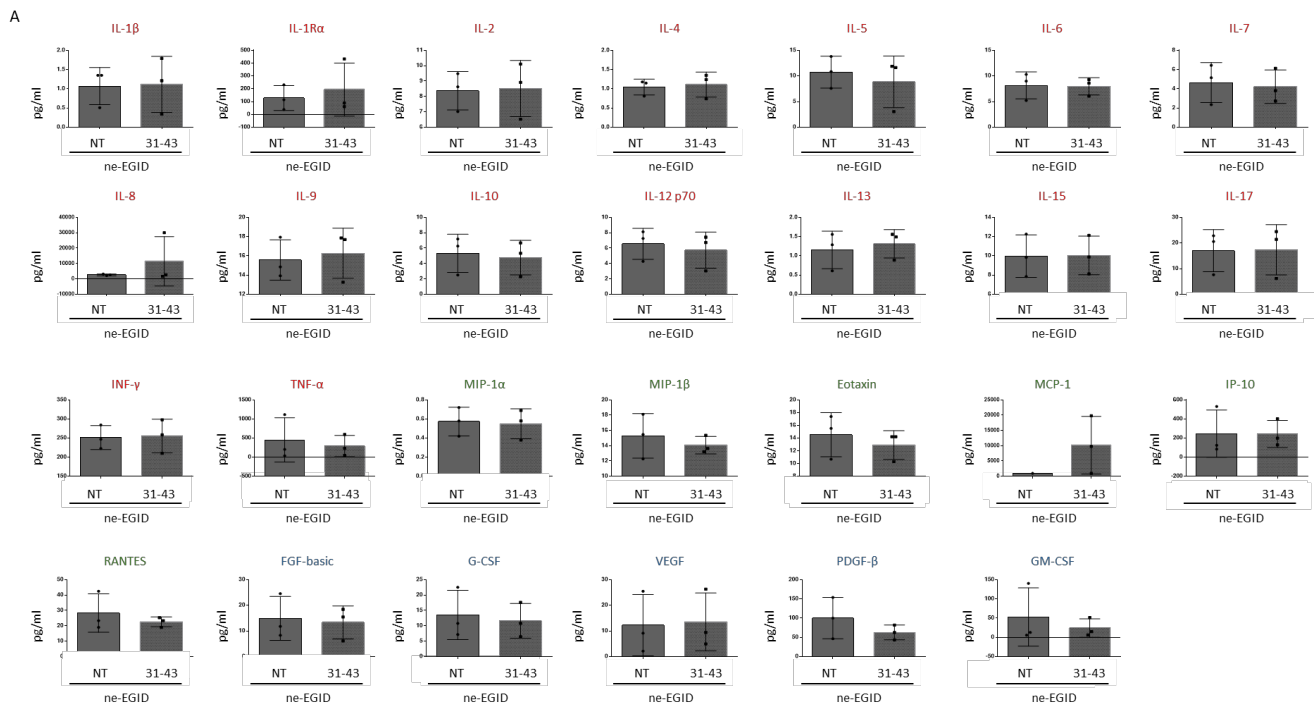


Figure 4. Culture medium of intestinal organoids with ne-EGID did not increase levels of pro-inflammatory cytokines, chemokines, and growth factors after treatment with gliadin peptide.

A. Bar chart of cytokines, chemokines, and growth factors concentration, expressed as pg/ml, in cell medium of ne-EGID intestinal organoids treated with and without gliadin peptide. Columns represent the mean, and bars represent the standard deviation.

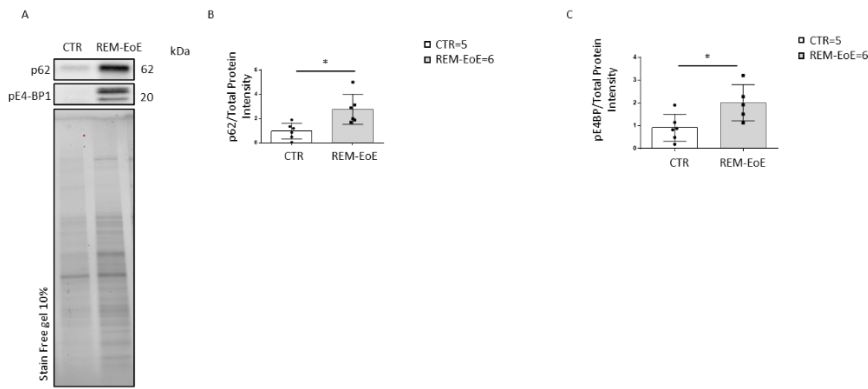


Figure 5. Autophagy and mTOR pathway were altered in intestinal organoids from REM-EoE respect to controls

A. Western blot analysis of total protein lysates of intestinal organoids from controls and REM-EoE intestinal organoids. Upper lines were blotted with an antibody against p62 and the middle lines with an antibody against pE4-BP1 against total protein intensity. Representative images were selected.

B. Densitometric analysis of the pE4-BP1/Stain free gel to respect total proteins intensity from CTR and REM-EoE intestinal organoids. The numbers of organoids analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's t-test = * $p < 0.05$.

C. Densitometric analysis of the p62/Stain free gel to respect total proteins intensity from CTR and REM-EoE intestinal organoids. The numbers of organoids analysed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's t-test = * $p < 0.05$.

6.Tables

Table 1. Cytokines, chemokines and growth factors descriptions.

Cytokines, Chemokines, Growing factors	Role in intestinal mucosa	Role in autophagy	Relate to NFKB pathway	References Role in intestinal mucosa	References Role in autophagy	References Related to NFKB pathway
IL-1β	IL-1 β is pro-inflammatory cytokine relevant in several inflammatory diseases. It is able to modulate the intestinal TJ barrier, induce an increase of intestinal permeability mediated, in part, by the activation of the NF- κ B pathway.	Autophagy limits IL-1 β activation or release; IL-1 β inhibits autophagy.	Yes, this cytokine is an effector.	41	42	43
IL-5	This cytokine have a role in the epithelium of ulcerative colitis (UC). In this condition, eosinophils are attracted to the intestinal tissue by chemotactic factors, such as IL-5.	IL-5 induces autophagy to promote the production of eosinophil cationic protein and eosinophil airway inflammation.	Yes as enhancer.	44	45	46
IL-7	Elevated levels of IL-7 promote spontaneous T cell activation and T cell-mediated	IL-7 can both promote or inhibit autophagy.	Yes, as enhancer.	47	48,49	35

	inflammation in the intestine and other organs.					
IL-9	IL-9 have a role in barrier function of the intestinal epithelium through regulation of Claudin-2, during intestinal inflammation. It is overexpressed in UC patients and increase in serum IL-9 in severe prognosis of IBD.	NO	Yes, as effector of Th9 cells.	50		51
IL-15	IL-15 is produced by intestinal epithelial cells, and it is required in development and maintenance of IELs. It is also critical for the survival and function of NK cells. it has been shown that IL-15 is the major mediator of the innate immune response and cooperates with active epidermal growth factor (EGF) to alter vesicular trafficking.	IL-15 stimulates autophagy pathway.	Yes, as enhancer.	52,53	54	55
IL-17	The expression of IL-17 is significantly	IL-17 can both induce	Yes, as enhancer.	56	57	58

	increased in the serum and inflamed mucosa in patients with IBD. IL-17 plays a role in chronic intestinal inflammation. Although it can drive inflammation and plays protective roles in limiting infection of the gut.	or inhibit autophagy.				
TNF-α (Tumor Necrosis Factor α)	It's a modulator of cell death or proliferation; it plays a critical role for the pathogenesis of IBD, infectious diseases, intestinal wound healing, and tumour formation. Tumor necrosis factor (TNF)- α , a key mediator of intestinal inflammation, causes an increase in intestinal epithelial tight junction.	TNF- α positively regulates autophagy.	Yes, as enhancer.	59,60	61,62	60
MIP-1A (macrophage inflammatory protein 1a)	CCL3 induced M1 macrophage polarization in intestinal mucosa, promoting inflammatory	NO	Yes, as effector.	63		35

	injury of intestinal tissue.					
MIP-1B (macrophage inflammatory protein 1b)	Systemic administration of MIP-1B in mice enhanced colitis with developing of large transmural ulcers.	NO	Yes, as effector.	64		35
Eotaxin	Eotaxin-1 is constitutively expressed in the lamina propria within the gastrointestinal tract. It is responsible of eosinophil recruitment in the colon and it is an important mediator of inflammation in IBD.	*	The promoter region of the eotaxin-1 gene contains binding sites for both NF- κ B and STAT6.	65		66
FGF-basic	FGF-basic treatment can significantly reduce the above intestinal mucosal damage and ameliorated intestinal mucosal permeability, in rats. FGF2 cooperates with IL-17 to protect intestinal epithelium during DSS-induced colitis.	FGF-2 suppresses autophagy.	Yes as enhancer.	67	68	69

G-CSF	G-CSF could have pro or anti-inflammatory effects by influencing interaction between T cell and dendritic cell. G-CSF is a modulator of intestinal immune system, and it plays a role in assisting in mucosal healing. Increased G-CSF antibodies levels are associated with: alterations of epithelial barrier function, bowel permeability, epithelial cell survival and proliferation.	G-CSF activates autophagy.	Yes, as effector.	70	71	72
VEGF	It is essential for both physiological and pathological angiogenesis. VEGF is involved in early development of intestinal epithelium, responsible for maintaining homeostasis after mucosal damage.	VEGF induces autophagy.	Yes, as effector.	73	74	75,76

Table 1: List of the cytokines, chemokines and growth factors increased in culture media from REM-EoE organoids respect to CTR. Their role in the intestinal epithelium and/or in the regulation of the autophagy and/or their relation to the NF- κ B pathway was highlighted.

Table 2

Patients	Range Age	Sex	Biopsy (Marsh Classification*)	Average number of Eosinophils per HPF in the oesophagus
CTR (N=4)	8-16	M=3 F=2	T0	Absent
ne-EGID (N=3)	7-15	M=1 F=2	T0	Absent
REM-EoE (N=6)	2-16	M=3 F=4	T0	Less than 15

Table 2. Patient's characteristic

Description of patients enrolled for the development of intestinal organoids. For each group, the range age, the number of male and female, the Marsh classification, and the number of eosinophils in the oesophagus were identified.

Table 3

Patients with remission EoE	Drug	Diet Therapy	Reactivated Patients
#1	Lansoprazolo (PPI)	Cow's milk, since 4 months of age	Persistently in remission
#2	Flurispiral (corticosteroids)	Milk, oranges, Legumes.	After 18 months
#3	no	Milk, Soya, Eggs, Legume (except beans)	Persistently in remission
#4	Flurispiral (corticosteroids)	Milk, Soya, Eggs, Nuts, Legumes (except beans)	Persistently in remission
#5	No	No (high IgE levels for egg and B-lactoglobulin)	Mild/moderate count of eosinophils
#6	Limpidex (PPI)	Milk and peanut	After 14 months
#7	Viscous budesonide (corticosteroids)	Free diet	Persistently in remission

Table 3. Patient's Drug and Diet Therapy

Summary of drug, diet therapy and state of the disease, during their follow-up, of patients with remission stage of eosinophilic esophagitis at the time in which we isolated intestinal organoids.

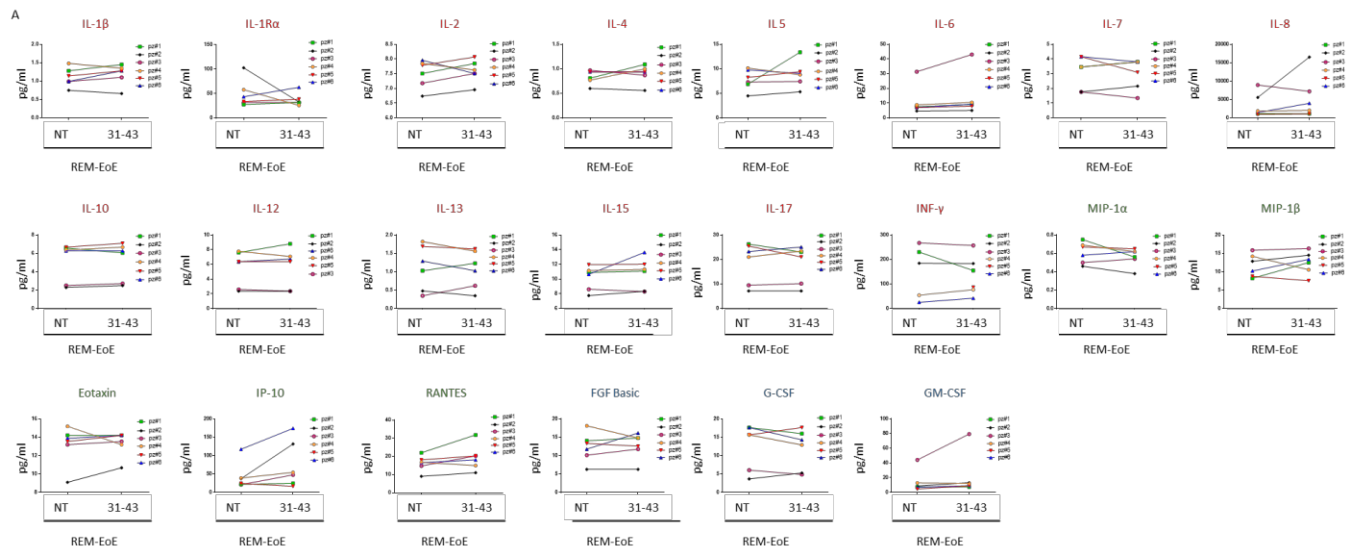
Table 4

CTR	CD3	TCR$\gamma\delta$⁺	CD25⁺	Intestinal deposits of anti-TG2
CTR#1	25,2	2,7	1	negative (1)
CTR#2	7,8	0,4	22	very weak (2)
CTR#3	15	1,4	8	negative (1)
CTR#4	16,75	1,2	10	negative (1)
REM-EoE	CD3	TCR$\gamma\delta$⁺	CD25⁺	Intestinal deposits of anti-TG2
REM-EoE#1	5	0,2	6	negative (1)
REM-EoE #4	12,2	1,75	7	very weak (2)
REM-EoE #7	8	1	Not Evaluated	Not Evaluated
REM-EoE #6	21,5	3,6	9	very weak (2)

Table 3. Immunohistochemistry and intestinal deposits of anti-TG2 in intestinal biopsies from CTR and REM-EoE patient's

Levels of CD3, TCR $\gamma\delta$ ⁺, CD25⁺ and Intestinal deposits of anti-TG2 were evaluated on freezing biopsies.

Supplementary Figure



Supplementary Figure 1 Gliadin peptides induce an altered concentration of cytokines, chemokines, and growth factors in culture medium of REM-EoE intestinal organoids

(A) Superimposed symbols with connecting lines of 22 out of 27 cytokines, chemokines, and growth factors, expressed as pg/ml, in cell medium of all REM-EoE intestinal organoids treated with and without gliadin peptide.

Conclusion

Intestinal inflammation is a hallmark in several diseases, including CeD. Inflammatory markers are present at the level of the intestinal epithelium even before the intestinal alterations of the diseases. Intestinal organoids from CeD patients reproduce the increase of the inflammatory markers found in CeD biopsies. They represent a good model not only to study CeD and other inflammatory disease (Chapter 1), but also to test postbiotics or other bacterial product to understand their role in preventing inflammation in CeD. In this thesis (Chapter 2) and in two manuscripts previously published (Appendix 4 and 5) we have tested 3 postbiotics derived from *Lactobacillus plantarum* CECT 749, *Lactobacillus paracasei* CBA L74 and *Lactobacillus rhamnosus* GG (LGG) showing that all of them were able to prevent gliadin peptides induced inflammation in CaCo-2 cells and reduce constitutive inflammation in CeD organoids. These preclinical studies provide a good basis for initiating clinical trials in celiac patients to prevent the proinflammatory effects of gliadin peptides. It would be interesting to test the ability of postbiotics to prevent the disease in potential patients who have anti-transglutaminase antibodies but have not yet developed the intestinal lesions typical of celiac disease. In Chapter 3 we have confirmed (Appendix 1) that intestinal biopsies and intestinal organoids from CeD patients were inflamed and presented a delay of the autophagy/activation of the mTOR pathway (Chapter 3). In GCD- and GFD-CeD organoids the delay of both the autophagy/activation of the mTOR pathway and inflammation were strictly connected in an interplay that ultimately may render the epithelial cells of CeD subjects more prone to pro-inflammatory stimuli from the environment, including gliadin peptides that can insist on the same pathways already altered in CeD intestinal epithelium. All these data opening new hypothesis of intervention in CeD prevention and therapy. In Chapter 4 we showed that in intestinal organoids from all REM-EoE patients were inflamed, were able to produce inflammatory cytokines and at least half of them responded to gliadin peptide P31-43 by increasing inflammatory markers. We have also described in these organoids that there was a delay of autophagy flux and activation of the mTOR pathway. Showing that both in REM-EoE and CeD organoids there are surprisingly similarities, although the two diseases are different as localization, histology markers and clinical features. Many questions remain unanswered in REM-EoE duodenal alterations, what is the role of the inflammation and/or autophagy delay and/or mTOR pathway activation in duodenum, a region far away from the oesophagus, of REM-EoE patients? Or when happens the inflammation and/or autophagy delay and/or mTOR pathway before or after the oesophagus alteration? The possibility to forecast the wheat/gliadin sensitivity and perhaps of other allergens in these patients in a personalised way is tempting considering that duodenal organoids are easy to develop and manage. Moreover, they could allow to avoid, the need of several EGD in these patients.



Article

Inflammation Is Present, Persistent and More Sensitive to Proinflammatory Triggers in Celiac Disease Enterocytes

Monia Porpora ¹, Mariangela Conte ¹, Giuliana Lania ¹, Claudia Bellomo ¹ , Luciano Rapacciuolo ¹, Fernando Gabriel Chirido ² , Renata Auricchio ¹, Riccardo Troncone ¹, Salvatore Auricchio ¹, Maria Vittoria Barone ^{1,*} and Merlin Nanayakkara ¹

¹ ELFID (European Laboratory for the Investigation of Food Induced Diseases), Department of Translational Medical Science, Section of Paediatrics, University Federico II, Via S. Pansini 5, 80131 Naples, Italy; monia.porpora@hotmail.it (M.P.); maryconte_92@hotmail.it (M.C.); giuliana.lania@gmail.com (G.L.); bellomo.claudia96@gmail.com (C.B.); lrapacci@unina.it (L.R.); r.auricchio@unina.it (R.A.); troncone@unina.it (R.T.); salauric@unina.it (S.A.); merlinnanayakkara@gmail.com (M.N.)

² Departamento de Ciencias Biologicas, Facultad de Ciencias Exactas, Instituto de Estudios Inmunologicos y Fisiopatologicos—IIFP (UNLP-CONICET), Bv. 120 1489, La Plata 1900, Argentina; fchirido@biol.unlp.edu.ar

* Correspondence: mv.barone@unina.it



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Abstract: Celiac disease (CD) is a chronic inflammatory disease caused by a genetic predisposition to an abnormal T cell-mediated immune response to the gluten in the diet. Different environmental proinflammatory factors can influence and amplify the T cell-mediated response to gluten. The aim of this manuscript was to study the role of enterocytes in CD intestinal inflammation and their response to different proinflammatory factors, such as gliadin and viruses. Intestinal biopsies from CD patients on a gluten-containing (GCD-CD) or a gluten-free diet (GFD-CD) as well as biopsies from potential CD patients (Pot-CD) before the onset of intestinal lesions and controls (CTR) were used to investigate IL-1 β and IL-6 mRNA levels in situ. Organoids from CD patients were used to test the levels of NF- κ B, ERK, IL-6, and IL-1 β by Western blot (WB), ELISA, and quantitative PCR. The Toll-like receptor ligand loxoribine (Lox) and gliadin peptide P31-43 were used as proinflammatory stimuli. In CD biopsies inflammation markers IL-1 β and IL-6 were increased in the enterocytes, and also in Pot-CD before the onset of the intestinal lesion and in GFD-CD. The inflammatory markers pNF- κ B, pERK, IL-1 β , and IL-6 were increased and persistent in CD organoids; these organoids were more sensitive to P31-43 and Lox stimuli compared with CTR organoids. Taken together, these observations point to constitutive inflammation in CD enterocytes, which are more sensitive to inflammatory stimuli such as food components and viruses.

Keywords: small intestine; potential celiac disease; NF- κ B; ERK

1. Introduction

Celiac disease (CD) is an immune-mediated enteropathy triggered in genetically susceptible individuals by a group of wheat proteins and related prolamins from cereals [1]. The HLA-restricted gliadin-specific intestinal T cell response plays a central role in the pathogenesis of CD [1]. Moreover, CD is associated with various extra-intestinal diseases including several skin manifestations [2].

CD is known to be characterized by a combination of gluten-induced symptoms, the generation of CD-associated autoantibodies, and enteropathy [3], but it remains unclear why T cells are activated by gliadin. Studies conducted in mice have demonstrated that mucosal inflammation due to reovirus infection may disrupt oral tolerance to gliadin by suppressing regulatory T cell conversion and promoting Th1 immunity [4]. These results indicate that in an inflamed environment enriched in cytokines, T cells tend to acquire a proinflammatory phenotype. The factors that create a proinflammatory environment in the CD intestine could have multiple origins. Recent studies have described the influence of

several different factors in CD, such as cellular vulnerability, the proinflammatory effects of gluten [5,6] and other wheat proteins [7], Western diet [8] and other environmental triggers, such as viruses [4,9], which may prepare and/or amplify the TC-mediated response to gluten [10].

Cellular vulnerability in CD has been suggested by recent studies that have shown that despite clinical and histological remission, celiac disease patients fed a gluten-free diet (GFD) have altered protein composition of gut tissue with signs of ongoing inflammation. In fact, changes indicative of epithelial inflammation, minor crypt hyperplasia, and low-grade inflammation in the serum have been demonstrated in GFD-CD intestinal biopsies before gluten challenge in vivo [11]. Moreover, alterations in differentiation/proliferation pathways have been found in GFD-CD patients before in vivo challenge with gluten [12]. Interestingly, after in vivo challenge with gluten, an increase in the same proteins that were altered at baseline in the GFD-CD was found [11,12]. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), a major mediator of the inflammatory response [13], has been found to be increased in GCD-CD and GFD-CD biopsies from several different genetic [14] and expression studies [15–17]. Fernandez-Jimenez et al. [15] showed that the expression of NF- κ B genes measured by RT-PCR (real-time polymerase chain reaction) was altered in GFD-CD and GCD-CD compared to control biopsies and in cultured biopsies from CD (GCD and GFD) patients challenged with gliadin. These results showed that genes that were constitutively upregulated in GFD patients belonged to the NF- κ B signaling system. Interestingly, according to GFD biopsies, gluten challenge also increased NF- κ B pathway activation in vitro. Similar increases in the activation of the NF- κ B pathway were observed in GFD-CD biopsies and fibroblasts, both derived from the intestines of CD patients and from skin explants (distant from the intestine). Only biopsies and fibroblasts from CD patients were sensitive to low doses of gliadin peptide P31-43, which did not affect controls [18]. Taken together, these data indicate that in CD, a vulnerability (probably constitutive) is present that renders cells more sensitive to proinflammatory stimuli.

The intestinal epithelium has assumed an emerging role in CD pathogenesis; in fact, morphological [5,18] and functional alterations [18,19] have been described in epithelial cells in CD patients. Moreover, the inflammasome pathway was increased in intestinal epithelial cells isolated from GFD-CD biopsies after gluten challenge [20]. Moreover, IL-1 β and IL-6 are part of the NF- κ B pathway [21,22] and are important mediators of the inflammation at the level of the intestine [23].

Gluten and, in particular, one of its undigested peptides (P31-43) can induce inflammation in cultured cells, in CD biopsies (GCD and GFD), and in mice [6]. P31-43 is not presented to T cells and can induce several different effects on cells, in CD biopsies and mice, including proliferation, activation of innate immune markers, and inflammation [6,24]. Moreover, it can act synergistically with viral ligands (Poli I:C and loxoribine) to induce innate immunity activation both in vitro and in vivo [25].

Therefore, the emerging role of epithelial inflammation and the proinflammatory effects of viruses and gliadin in CD prompted us to study inflammatory markers in situ in the enterocytes of biopsies derived from CD patients at different stages of the disease (GCD-, GFD-, and Pot-CD). Moreover, we examined whether intestinal organoids derived from CD biopsies are a good model for studying inflammation and sensitivity to proinflammatory agents, such as P31-43 and the viral ligand loxoribine, in CD epithelium.

2. Results

2.1. In CD Biopsies, the Inflammatory Markers IL-1 β and IL-6 Are Increased in Enterocytes

IL-1 β and IL-6 are part of the NF- κ B pathway [21,22], they are important mediators of the inflammation at the level of the intestine [23] and have been related to inflammatory bowel diseases [26,27]. Expression of IL-1 β and IL-6 mRNA in situ was analyzed in intestinal biopsies from CD patients at different stages of the disease as well as those from controls.

IL-1 β mRNA was absent in control biopsies at both the level of the intestinal epithelial cells of the crypts (Figure 1A,E) and of the villi (Supplemental Figure S1A). In contrast, IL-1 β mRNA was increased in all CD patients independent of the stage of the disease and almost exclusively at the level of the crypts (Figure 1B–D; Supplemental Figure S1B–E). In particular, IL-1 β mRNA was highly expressed (59 ± 36 red dots/crypt, p value < 0.05) in CD patients fed a gluten-containing diet (GCD-CD) (Figure 1B,E) in the acute phase of the disease and in potential patients (Pot-CD) (43.6 ± 11 red dots/crypt, p value < 0.01) (Figure 1C,E) before the onset of lesions of the small intestine, who still consumed gluten. Patients in the remission phase of the disease (GFD-CD) on a gluten-free diet also presented an increased expression of IL-1 β in the intestinal epithelium, although at lower levels, than patients in the GCD-CD and Pot-CD groups (17.6 ± 2.5 red dots/crypt, p value < 0.001) (Figure 1D,E).

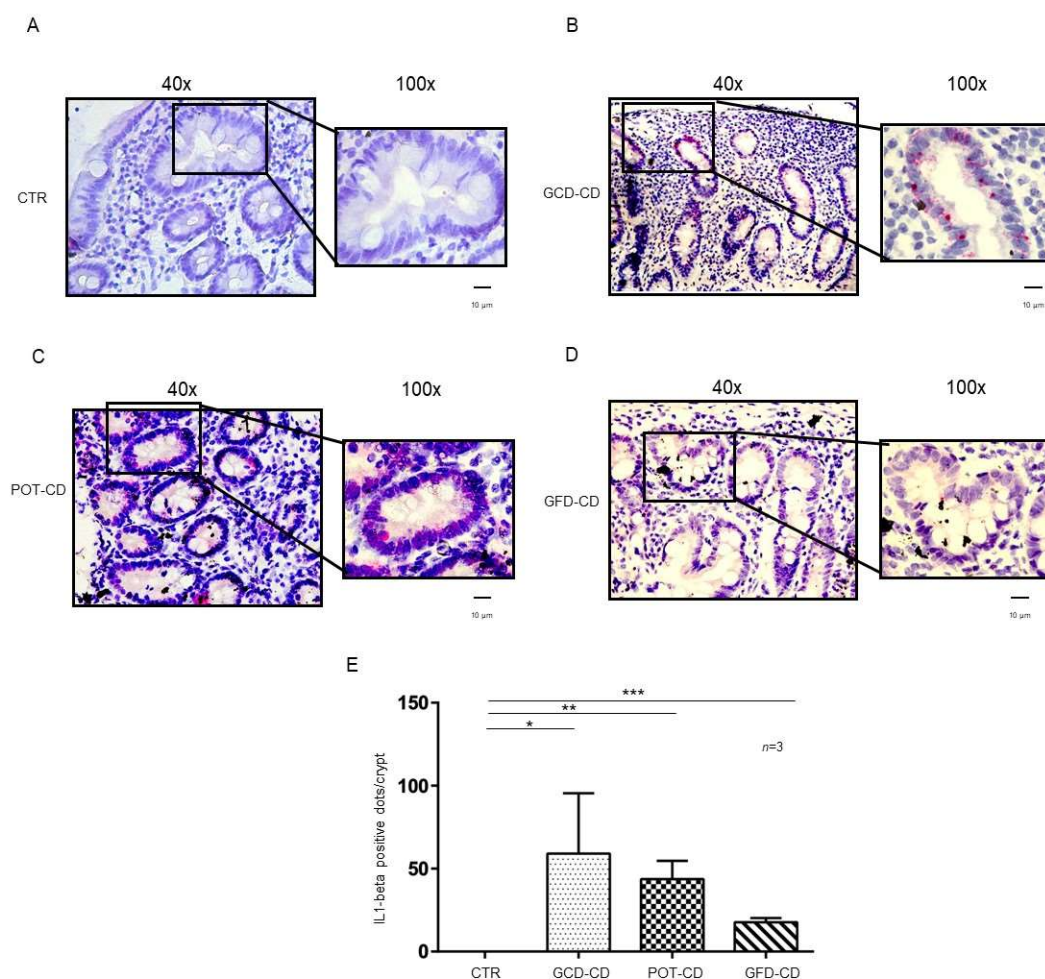


Figure 1. IL-1 β is increased in the epithelium of the crypts in CD biopsies: In situ mRNA analysis of IL-1 β in biopsies from controls (CTR) (A), gluten-containing diet celiac disease patients (GCD-CD) (B), Potential CD patients (Pot-CD) (C), and gluten-free diet celiac patients (GFD-CD) (D). Blue indicates hematoxylin–eosin staining of the nuclei, and red indicates IL-1 β mRNA. Black squares show different enlargements of crypts. Lines indicate 10 micrometers at 100 $\times$ objective. (E). IL-1 β -positive red dot counts in the crypts of intestinal biopsies from CD patients and controls. At least 10 crypts/subject were counted on different slides. The numbers of subjects analyzed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's t test: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

IL-6 mRNA was present in control biopsies both at the level of the crypts (Figure 2A,E) and of the villi (Supplemental Figure S2A), as expected [28]. On the other hand, IL-6, similar to IL-1 β (Figure 2), was overexpressed with respect to the control in the GCD-CD, GFD-CD, and Pot-CD groups (Figure 2B–D).

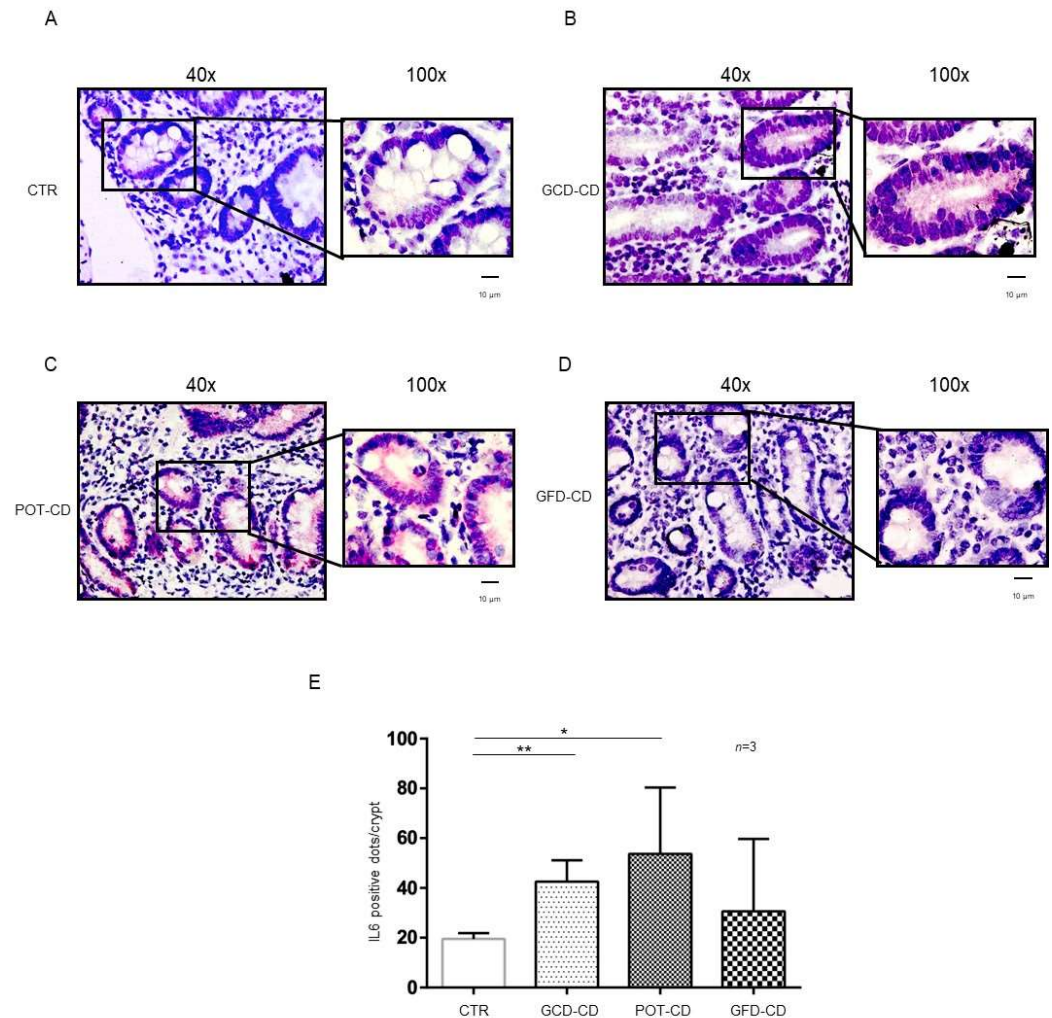


Figure 2. IL-6 levels were increased in the epithelium of the crypts in CD biopsies: In situ mRNA analysis of IL-6 in biopsies from controls (CTR) (A), gluten-containing diet celiac disease patients (GCD-CD) (B), Potential CD patients (Pot-CD) (C), and gluten-free diet celiac patients (GFD-CD) (D). Blue indicates hematoxylin–eosin staining of the nuclei, and red indicates IL-6 mRNA. Black squares show different enlargements of crypts. Lines indicate 10 micrometers at 100 $\times$ objective. (E). IL-6-positive red dot counts in the crypts of intestinal biopsies from CD patients and controls. At least 10 crypts/subject were counted on different slides. The numbers of subjects analyzed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t* test: * = *p* < 0.05; ** = *p* < 0.01.

In particular, IL-6 mRNA was highly expressed (42.5 ± 8.6 red dots/crypt) in CD patients fed a gluten-containing diet (GCD-CD) (Figure 2B,E; *p* value < 0.01) in the acute phase of the disease and in potential patients (Pot-CD) (53.67 ± 26.7 red dots/crypt; *p* value < 0.05) (Figure 2C,E) before the onset of lesions of the small intestine, who still consumed gluten. Patients in the remission phase of the disease and fed a gluten-free diet (GFD-CD) (Figure 2D) also presented altered expression of IL-6 in the intestinal epithelium; although, the difference did not reach statistical significance (30.67 ± 29.01 red dots/crypt) (Figure 2D,E).

2.2. The Inflammatory Markers pNF- κ B, pERK, IL-1 β , and IL-6 Were Increased and Persistent in CD Organoids

Intestinal organoids were derived from GCD-CD and CTR intestinal biopsies and tested after 4 weeks of culture for the inflammatory markers pNF- κ B, pERK, IL-1 β , and IL-6. In particular, pNF- κ B (p value < 0.01) and pERK (p value < 0.05) were increased in CD organoids compared to CTR organoids, as evaluated by Western blot (WB) analysis (Figure 3A–D). Quantitative mRNA (Figure 3E,F) showed a marked increase in IL-1 β (p value < 0.05) and IL-6 (p value < 0.05) expression in CD organoids compared with that of CTR organoids. An ELISA of the supernatant of the organoid cultures (Figure 3G,H) showed a marked increase in IL-1 β (p value < 0.05) and IL-6 (p value < 0.01) levels in the CD supernatant compared with those of CTR supernatant.

To understand whether the inflammation in intestinal organoids was a residual effect of the inflamed intestinal environment or inherent to the intestinal CD epithelium, we cultivated organoids from CD and CTR patients for several weeks (from 4 to 12 weeks) and then tested them again for the inflammatory markers pNF- κ B and pERK by WB analysis (Figure 4A–C). Interestingly, the expression of the inflammatory markers pNF- κ B (p value < 0.01, < 0.001) and pERK (p value < 0.01) in CD organoids remained more elevated than in CTR organoids after several weeks in culture.

In intestinal organoids from inflammatory bowel disease (IBD) patients, markers of inflammation are elevated only for a few weeks (1–2 w) after culture and then decrease to the level of CTR organoids [29]. In Figure 4D–F, we compared the inflammatory markers pNF- κ B and pERK in intestinal organoids from CD and IBD patients after 4 weeks in culture. We confirmed that in IBD, both pNF- κ B and pERK were at the level of the controls. Only CD organoids showed higher levels of pNF- κ B (p value < 0.01) and pERK (p value < 0.01), than the CTR organoids after 4 weeks in culture. Taken together, this indicates that in CD, contrary to IBD, inflammation is persistent.

2.3. 3D and 2D Organoids from CD Patients Had Increased Inflammatory Markers Compared to Those from CTR Patients

Organoids are considered good models for studying inflammation and infection of the intestine [30].

To provide correct treatment for the apical side of the intestinal cells of organoids, it is necessary to open them up, shifting them to 2D because in 3D, the apical side of the cells is enclosed in the spherical organoids. Light microscopy analysis of organoids in 3D and 2D did not show any difference in their dimensions. Three-dimensional CD organoids were denser than CTR organoids, as already described [31] (Figure 5A). The expression of villin and cytokeratin, differentiation markers of epithelial cells, was similar in 3D and 2D organoids, as assessed by immunofluorescence and WB (Figure 5B–H). Phosphorylation levels of NF- κ B and ERK in 3D organoids were compared to those in 2D organoids, for both CD and CTR patients (Figure 5I–L). pNF- κ B was increased in both 2D (p value < 0.05) and 3D (p value < 0.01) CD organoids compared to CTR organoids. Additionally, pERK was increased in both 2D (p value < 0.05) and 3D (p value < 0.01) CD organoids compared to CTR organoids. Moreover, IL-1 β and IL-6 measured by RT PCR and ELISA were increased in CD organoids respect to controls as shown in Supplemental Figure S3. We confirmed that opening the organoids did not affect inflammatory marker expression (Figure 5I–L).

2.4. Organoids from CD Patients Were More Sensitive to P31-43

Basal differences between patients and controls organoids for the expression of inflammatory markers NF- κ B and ERK showed by WB in Figure 3A,C, Figure 4A,D and Figure 5I,L prompted us to study these markers after pro-inflammatory stimuli such as P31-43. CD cells responded to inflammatory stimuli such as the A-gliadin peptide P31-43 by increasing inflammatory markers expression [6]. To determine if there was a difference in sensitivity between CTR and CD organoids, we treated them with P31-43 at concentrations that did not affect CTR organoids (Figure 6).

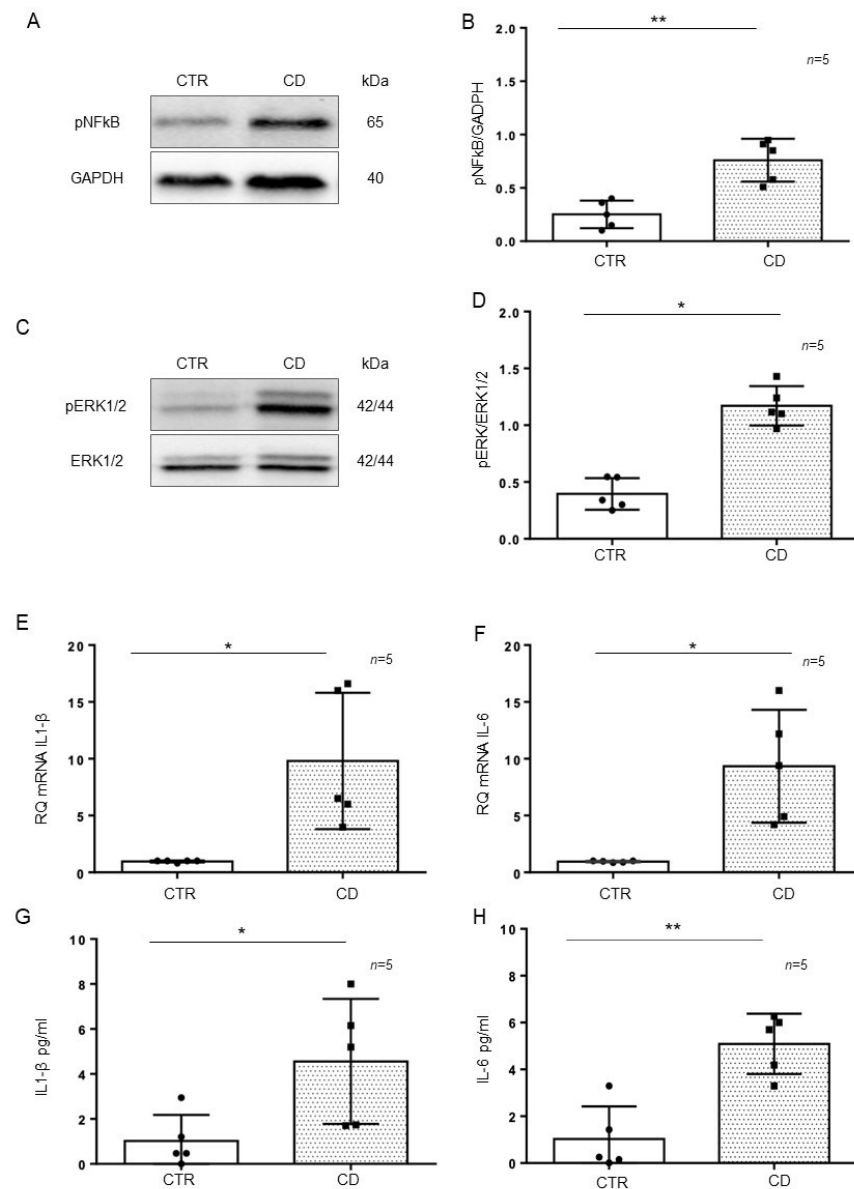
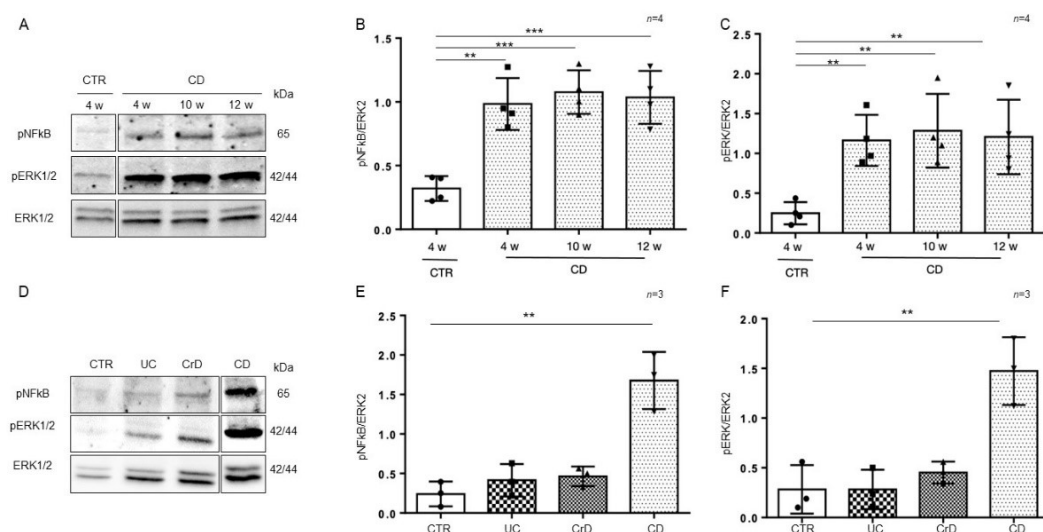


Figure 3. Inflammatory markers were increased in CD organoids: (A). Western blot analysis of total protein lysates of organoids from controls (CTR) and CD patients fed a gluten-containing diet (CD). Upper lines were blotted with an antibody against the phosphorylated form of NF-κB (pNF-κB). Bottom lines were blotted with anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody as a loading control. Representative images were selected. (B). Densitometric analysis of the pNF-κB/GAPDH bands from CTR and CD. The numbers of organoids analyzed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t* test: ** = $p < 0.01$. (C). Western blot analysis of total protein lysates of organoids from CTR and CD. Upper lines were blotted with an antibody against the phosphorylated form of ERK (pERK). Bottom lines were blotted with anti-ERK antibody as a loading control. Representative images were selected. (D). Densitometric analysis of the pERK/ERK bands from CTR and CD. The numbers of organoids analyzed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t* test: * = $p < 0.05$. (E,F). Quantitative PCR analysis of IL-1β and IL-6 mRNA levels in CD patients compared to CTR patients. The number of organoids analyzed is indicated. The columns represent the mean, and bars represent the standard deviation. Student's *t* test: * = $p < 0.05$. (G,H). ELISA showing IL-1β and IL-6 protein levels in the culture media of CD organoids compared to CTR organoids. The numbers of organoids are indicated. The columns represent the mean, and bars represent the standard deviation. Student's *t* test: * = $p < 0.05$; ** = $p < 0.01$.



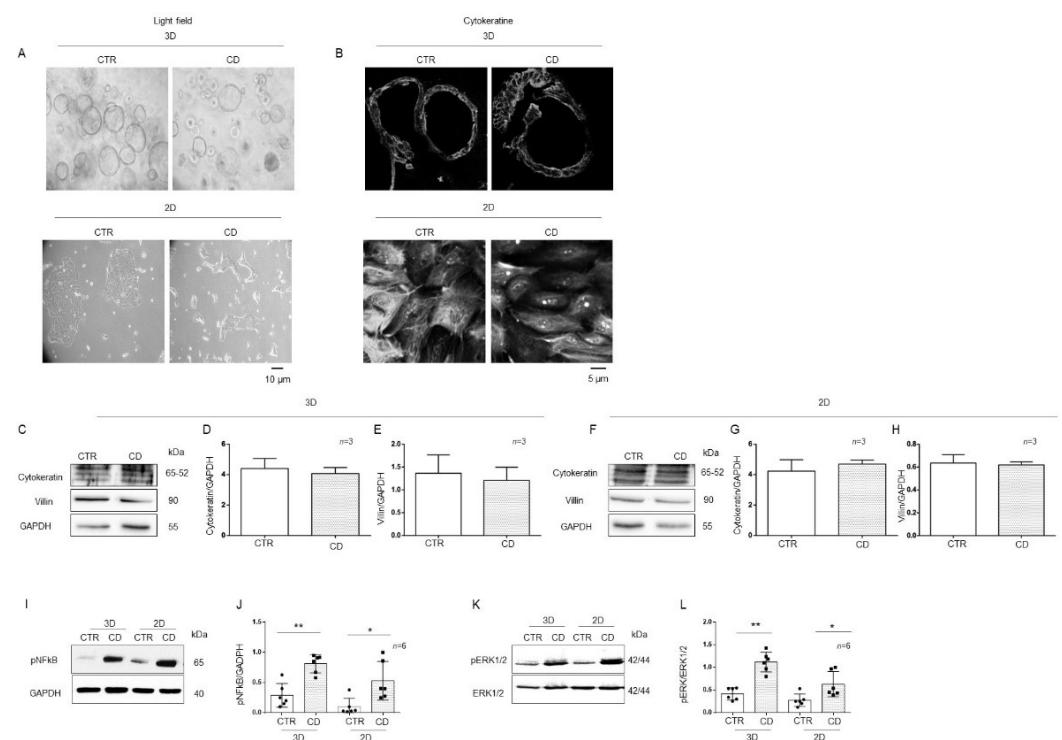


Figure 5. Markers of inflammation were increased in both 3D and 2D organoids from CD patients compared to CTR patients: **(A)**. Light field microscopy of 3D and 2D organoids from CD and CTR. 20X objective. The black line indicates 10 microns. **(B)** Immunofluorescence images of 3D and 2D organoids stained with anti-cytokeratin antibodies. 60× objective. The black line indicates 5 microns. **(C)**. Western blot analysis of total protein lysates from CTR and CD 3D organoids. Upper lines were blotted with an antibody against cytokeratin. Middle lines were blotted with an antibody against villin. Bottom lines were blotted with GAPDH antibody as a loading control. Representative images were selected. Densitometric analysis of the cytokeratin/GAPDH **(D)** and villin/GAPDH **(E)** bands from 3D organoids from CTR and CD. Columns represent the mean, and bars represent the standard deviations. The numbers of subjects analyzed are indicated. **(F)**. Western blot analysis of total protein lysates from CTR and CD 2D organoids. Upper lines were blotted with an antibody against cytokeratin. Middle lines were blotted with an antibody against villin. Bottom lines were blotted with GAPDH antibody as a loading control. Representative images were selected. Densitometric analysis of the cytokeratin/GAPDH **(G)** and villin/GAPDH **(H)** bands from 2D organoids from CTR and CD. Columns represent the mean, and bars represent the standard deviations. The numbers of subjects analyzed are indicated. **(I)**. Western blot analysis of total protein lysates of 3D and 2D organoids from CTR and CD patients. Upper lines were blotted with an antibody against the phosphorylated form of NF-κB (pNF-κB). Lower lines were blotted with anti-GAPDH antibody as a loading control. Representative images were selected. **(J)**. Densitometric analysis of the pNF-κB/GAPDH bands from 3D and 2D organoids from CTR and CD. The numbers of organoids analyzed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t* test: * = $p < 0.05$; ** = $p < 0.01$. **(K)**. Western blot analysis of total protein lysates of 3D and 2D organoids from CTR and CD. Upper lines were blotted with an antibody against the phosphorylated form of ERK (pERK). Bottom lines were blotted with anti-ERK antibody as a loading control. Representative images were selected. **(L)**. Densitometric analysis of the pERK/ERK bands from CTR and CD. The numbers of organoids analyzed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t* test: * = $p < 0.05$; ** = $p < 0.01$.

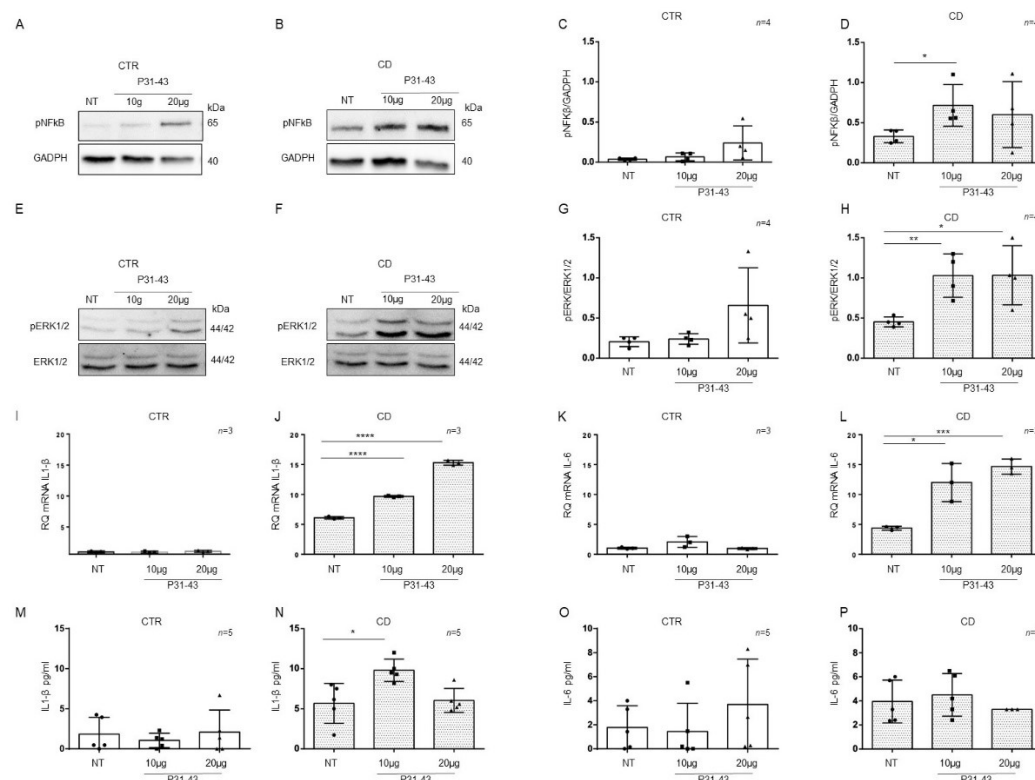


Figure 6. Organoids from CD patients, but not from CTR patients, are inflamed by P31-43: Western blot analysis of total protein lysates of organoids from CTR (A) and CD (B) before (NT) and after treatment with low concentrations of P31-43 10 μg/mL (10 μg) and 20 μg/mL (20 μg). Upper lines were blotted with an antibody against the phosphorylated form of NF-κB (pNF-κB). Bottom lines were blotted with anti-GAPDH antibodies as a loading control. Representative images were selected. (C,D). Densitometric analysis of the pNF-κB/GAPDH bands from CTR and CD before and after treatment with P31-43 as indicated. The numbers of organoids analyzed are indicated. Columns represent the mean, and bars represent the standard deviations. Student's t test: * = $p < 0.05$. (E,F). Western blot analysis of total protein lysates of organoids from CTR (E) and CD (F) patients before (NT) and after treatment with P31-43 as indicated. Upper lines were blotted with an antibody against the phosphorylated form of ERK (pERK). Bottom lines were blotted with anti-ERK antibody as a loading control. Representative images were selected. (G,H). Densitometric analysis of the pERK/ERK bands from CTR and CD before and after treatment with P31-43 as indicated. The numbers of subjects analyzed are indicated. Columns represent the mean, and bars represent the standard deviation. Student's t test: * = $p < 0.05$; ** = $p < 0.01$. (I,J). Quantitative PCR analysis of IL-1β mRNA levels in organoids from CD patients compared to CTR organoids. The numbers of subjects analyzed are indicated. Student's t test: **** = $p < 0.0001$. (K,L). Quantitative PCR analysis of IL-6 mRNA levels in organoids from CD patients compared to CTR organoids. The numbers of organoids analyzed are indicated. Student's t test: * = $p < 0.05$; *** = $p < 0.001$. (M,N). ELISA showing IL-1β protein levels in the culture media of CD organoids compared to CTR organoids before (NT) and after treatment with P31-43 as indicated. The numbers of organoids analyzed are indicated. The columns represent the mean, and bars represent the standard deviation. Student's t test: * = $p < 0.05$. (O,P). ELISA showing IL-6 protein levels in the culture media of CD and CTR organoids before (NT) and after treatment with P31-43 as indicated. The numbers of organoids analyzed are indicated. The columns represent the mean, and bars represent the standard deviation.

2.5. Organoids from CD Patients Were More Sensitive to Lox

CD cells and biopsies responded to the inflammatory stimuli of Toll-like receptor 7 viral ligand, Lox, by increasing the levels of inflammatory markers [9,25]. Considering the basal differences between patients and controls organoids for the expression of inflammatory

markers NF- κ B and ERK showed by WB in Figures 3A,C, 4A,D and 5I,L, we tested the sensitivity to another pro-inflammatory stimuli such as Lox.

To understand if there was a difference in sensitivity between CTR and CD organoids to viral ligand stimuli, we treated them with Lox at concentrations that did not affect CTR organoids (Figure 7).

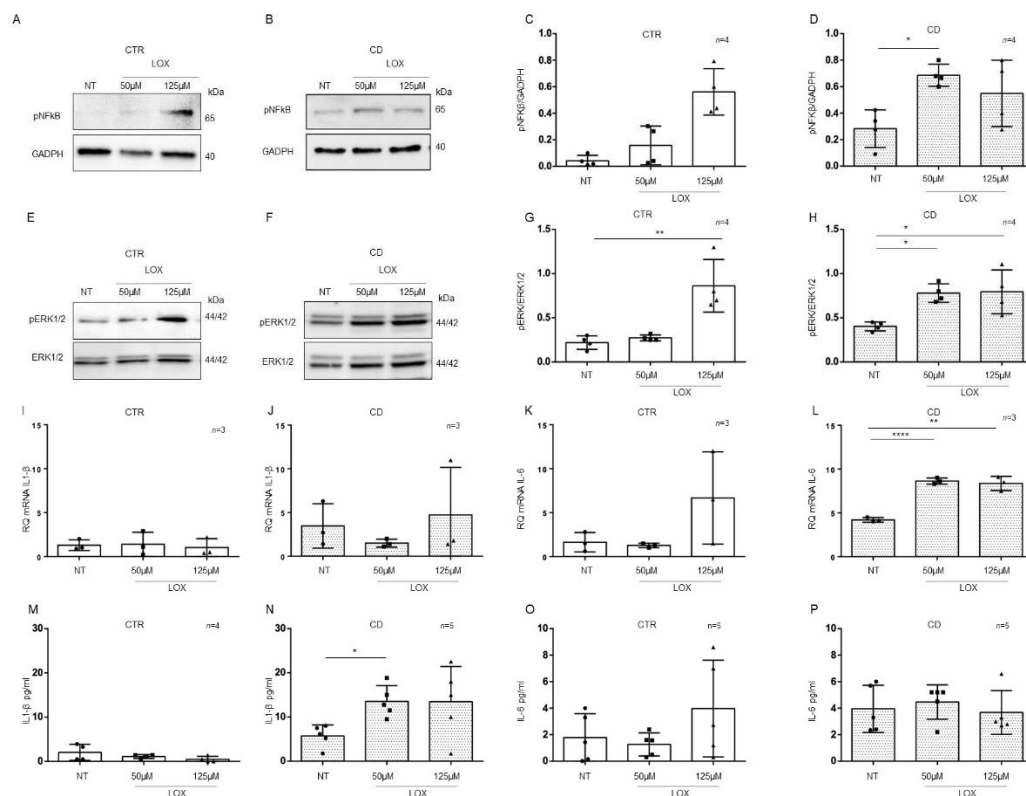


Figure 7. Organoids from CD patients, but not from CTR patients, are inflamed by Lox: Western blot analysis of total protein lysates of organoids from CTR (A) and CD (B) patients before (NT) and after treatment with low concentrations of Lox (50 μ M and 125 μ M). Upper lines were blotted with an antibody against the phosphorylated form of NF- κ B (pNF- κ B). Bottom lines were blotted with anti-GAPDH antibody as a loading control. Representative images were selected. (C,D). Densitometric analysis of the pNF- κ B/GAPDH bands from CTR and CD before and after treatment with Lox as indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t* test: * = *p* < 0.05. (E,F). Western blot analysis of total protein lysates of organoids from CTR (E) and CD (F) patients before (NT) and after treatment with Lox as indicated. Upper lines were blotted with an antibody against the phosphorylated form of ERK (pERK). Bottom lines were blotted with anti-ERK antibody as a loading control. Representative images were selected. (G,H). Densitometric analysis of the pERK/ERK bands from CTR and CD before and after treatment with Lox as indicated. Columns represent the mean, and bars represent the standard deviation. Student's *t* test: * = *p* < 0.05; ** = *p* < 0.01. (I,J). Quantitative PCR analysis of IL-1 β mRNA levels in organoids from CD patients compared to those from CTR patients. The numbers of organoids analyzed is indicated. Columns represent the mean, and bars represent the standard deviation. (K,L). Quantitative PCR analysis of IL-6 mRNA levels in organoids from CD patients compared to those from CTR patients. The numbers of organoids analyzed are indicated. Student's *t* test. ** = *p* < 0.01; **** = *p* < 0.0001. (M,N). ELISA showing IL-1 β protein levels in the culture media of CD organoids compared to CTR organoids before (NT) and after treatment with Lox as indicated. The numbers of subjects analyzed are indicated. The columns represent the media, and bars represent the standard deviation. Student's *t*-test: * = *p* < 0.05. (O,P). ELISA showing IL-6 protein levels in the culture media of CD and CTR organoids before (NT) and after treatment with Lox as indicated. The numbers of organoids analyzed are indicated. The columns represent the media, and bars represent the standard deviation.

In control organoids, 50 μ M and 125 μ M Lox (Figure 7) did not increase the expression of the inflammatory markers pNF- κ B, pERK, IL-1 β , and IL-6, with the exception of pERK, which was significantly increased only by 125 μ M Lox (p value < 0.01). As before, we analyzed pNF- κ B and pERK by WB and IL-1 β and IL-6 by quantitative PCR and ELISA (Figure 7).

On the other hand, in CD organoids, treatment with Lox increased the expression of the inflammatory markers pNF- κ B and pERK at both 50 μ M (pNF- κ B, p value < 0.05) and 125 μ M (pERK, p value < 0.05) concentrations, according to WB analysis (Figure 7A–H).

Stimulation with 50 μ M (p value < 0.0001) and 125 μ M (p value < 0.01) Lox increased the levels of IL-6 mRNA (Figure 7K,L) but not IL-1 β mRNA.

ELISAs (Figure 7M–P) on CD organoid culture media revealed that IL-1 β was increased after 50 μ M Lox (p value < 0.05) treatment, whereas IL-6 was not increased. These results indicate that CD enterocytes were more sensitive than control enterocytes to inflammatory stimuli of different origins.

3. Discussion

The mucosa of the small intestine is the primary target of CD. Gluten peptides interact with the epithelium, cross the epithelial barrier, and induce an adaptive immune response against gluten in individuals with MHC class II DQ2/8 haplotypes. Only a few individuals with these haplotypes will develop CD after exposure to gluten, indicating that other factors influence the initiation and maintenance of the disease. One of these factors could be the altered functionality of the innate epithelial response [20].

In this manuscript, we described epithelial inflammation in CD biopsies using IL-1 β and IL-6 as markers in patients at the acute phase of the disease (GCD-CD), after remission of the intestinal lesion (GFD-CD), and in patients with anti-TTG antibodies, who were genetically predisposed to CD (Pot-CD) yet had normal intestinal morphology for 2–3 years (on average) prior to the onset of the intestinal lesion. We derived organoids from intestinal biopsies of GCD-CD and CTR patients and tested them for markers of inflammation, such as pNF- κ B, pERK, IL-1 β , and IL-6, and found that these markers were altered in CD organoids. In CD organoids the inflammation was persistent. Moreover, CD organoids were more sensitive than controls to inflammatory stimuli such as gliadin peptide P31-43 and the TLR7 receptor ligand Lox.

Intestinal inflammation in CD has been described by many different studies, in patients at both the GCD and GFD stages and before and after gluten challenge [9,11,12,14,17]. It is now clear that in CD, several different factors, such as cellular vulnerability, the proinflammatory effects of gluten and other wheat proteins, Western diet, and other environmental triggers, such as viruses, converge to prepare, and/or amplify the TC-mediated response to gluten [6,9].

Moreover, changes at the mRNA and protein levels of the inflammasome pathway were found in intestinal epithelial cells purified from CD intestinal biopsies and analyzed for gene expression. These results indicate that intestinal epithelial cells play a key role in small intestinal inflammation in CD [20].

The IL-1 β and IL-6 cytokines are important mediators of the inflammatory response and are involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis [28,32].

In this manuscript, we found that IL-1 β and IL-6 levels increased in CD enterocytes, not only in the acute phase of the disease but also in the remission phase and in potential patients before the onset of intestinal disease. Interestingly, in our experiments, both IL-1 β and IL-6 levels were altered mainly in the epithelium in all stages of the disease. The presence of inflammation in the Pot-CD group is particularly interesting, as it indicates that inflammation of the epithelium precedes mucosal remodeling, and points to the intestinal epithelium as a key component of the inflammatory response in CD.

The possibility of growing small intestinal organoids has given many researchers a new tool to study the role of the intestinal epithelium in several different diseases [30].

Organoids are a miniaturized and simplified version of an organ produced in vitro in three dimensions with realistic microanatomy. Intestinal organoids are derived from crypt stem cells. Several different groups have used organoids to study CD, revealing the presence of increased staminality, permeability, inflammasome activity, and innate immunity genes with respect to healthy individuals [20,33]. Extracellular matrix (ECM) genes were decreased in CD organoids compared to control individuals [31]. Taken together, these observations indicate that CD intestinal epithelial cells are constitutively different from those in healthy individuals.

In intestinal organoids from IBD, inflammation disappears after 1 week in culture and can be regained only upon INF- α treatment [29]. Therefore, in IBD, inflammation in intestinal organoids is regarded as a residual effect of the tissue of origin. We derived organoids from the intestinal epithelium of CD patients, IBD patients, and healthy individuals. We confirmed the data on IBD organoids available in the literature. In our experiments, IBD (both Crohn's and ulcerative colitis) organoids were no longer inflamed after 4 weeks in culture, and both pNF- κ B and pERK levels were not different from the control organoids.

In contrast, in CD organoids, we found increased markers of inflammation, such as pNF- κ B, pERK, IL-1 β , and IL-6, at the protein and mRNA levels. In contrast to that in IBD, inflammation in CD organoids was persistent, as the levels of pNF- κ B and pERK did not decrease after more than ten weeks in culture. This suggests that the inflammation in CD organoids is not a residual effect of the tissue of origin but is constitutive.

Possible constitutive alterations in CD, which appear to be independent of the stage of the disease and the gluten content in the diet, have been recently described in vivo in the literature [12,34,35]. Interestingly, several cytokines related to the inflammatory pathway were increased in at-risk CD infants before the onset of the disease and the introduction of gluten to the diet [35].

Moreover, in CD biopsies and fibroblasts, increased sensitivity to inflammatory triggers such as gliadin peptide P31-43, IL-15, and Toll-7-specific viral ligand Lox has been described [18,25,36,37].

For this reason, we treated CD and CTR organoids with two different environmental inflammatory stimuli, gliadin peptide P31-43 and Lox. P31-43 and Lox, ineffective in CTR organoids, were able to induce the activation of NF- κ B and ERK and increase IL-1 β and IL-6 levels in CD organoids. In CD biopsies and in intestinal organoids, increased sensitivity to inflammatory stimuli from bacteria has been described [19], indicating that intestinal organoids from CD patients are more sensitive to proinflammatory stimuli.

In conclusion, the factors that create a proinflammatory environment in the CD intestine can be exogenous, such as food and viruses, but can also be endogenous. In fact, low-grade inflammation of the CD epithelium, probably constitutive, is present even before intestinal damage. Intestinal organoids reproduced this constitutive inflammation and thus represent a good model for studying epithelial inflammation in CD. Moreover, the intestinal epithelium in CD is more sensitive to proinflammatory stimuli, including gliadin and viruses. Taken together, these observations point to constitutive alterations, probably genetic or epigenetic, which render the CD epithelium more sensitive to inflammatory stimuli such as food components, virus, and microbiota.

4. Materials and Methods

4.1. Organoids

One to two duodenal biopsies per individual from CD patients and from controls were taken with standard endoscopic EGDS during routine gastroduodenoscopy (Table 1) and placed in ice-cold: 10 mL isolation buffer (5.6 mmol/L Na₂HPO₄ (Sigma S7907; Sigma-Aldrich, Milan, Italy), 8.0 mmol/L KH₂PO₄ (Sigma P5655; Sigma-Aldrich, Milan, Italy), 96.2 mmol/L NaCl (Sigma S5886; Sigma-Aldrich, Milan, Italy), 1.6 mmol/L KCl (Sigma P5405; Sigma-Aldrich, Milan, Italy), 43.4 mmol/L sucrose (Fisher BP220-1; Thermo Fisher Scientific, Waltham, MA, USA), and 54.9 mmol/L D-sorbitol (Fisher BP439-500; Thermo Fisher Scientific Milan, Italy)) in deionized water. Crypt units were isolated according

to the protocol of Yuli Wang et al. [38] with minor variations. Briefly, after 60 min, the biopsy samples were further enzymatically digested with collagenase (2 mg/mL, C0130 Sigma-Aldrich, Milan, Italy) in washing buffer (WB) containing penicillin/streptomycin (100 units mL⁻¹, cat.15140122), L-glutamine (2 mM, cat. 25030081), and FBS (10%, *vol/vol*, cat.10270-106) in DMEM/F12 Nutrient Mix (cat.11330-032, Gibco, Milan, Italy) with HEPES (cat.15630-049 Gibco, Milan, Italy) on ice for 30 min. The digest was filtered through a 70 µm strainer (Falcon, NY, USA) and the strainer was rinsed with an additional 10 mL of WB. Crypts were collected by centrifugation at 500× *g* for 5 min. The supernatant was discarded, the crypts were carefully resuspended in 40 µL of ice-cold Matrigel matrix (Corning cat.35623, Milan, Italy) to enable three-dimensional growth in 48-well plates; the plates were incubated in a cell culture incubator at 37 °C and 5% carbon dioxide for 10 min to allow the Matrigel to solidify. Afterwards, 300 µL cell culture medium enriched with supplements (CM-S) was added to each well and was replaced every second day. The organoids were used for assays or cryopreserved at −150 °C. To cryopreserve organoids, they were washed with ice-cold PBS EDTA to remove Matrigel and collected by centrifugation at 500× *g* for 5 min. Organoid pellets were suspended in 1 mL WB, 10% fetal bovine serum (FBS, Gibco, Milan, Italy), and 10% dimethyl sulfoxide, slowly frozen to −80 °C in a cryo freezing container (Nalgene, Sigma, Milan, Italy), and then transferred to −150 °C for long-term storage. For further research, the cryopreserved organoids were quickly thawed at 37 °C, transferred to 10 mL WB, centrifuged at 2000× *g* for 5 min, plated with Matrigel, and cultured in CM-S medium. For 2D organoids, organoids were openly seeded in six wells pretreated with Matrigel diluted 1:40 in phosphate-buffered saline (PBS)

Table 1. Patient characteristics.

Patients	Range Age (Years)	Sex	Biopsy (Marsh Classification *)	Serum AntiTG2 (U/mL)	Anti-Endomysial Antibody (EMA)
Controls (N = 8)	10–18	M = 4, F = 4	8 = T0	0–1.5	Negative
GCD-CD (N = 8)	3–15	M = 3, F = 5	10 = T3 ^c 2 = T3 ^{c/b}	>50	Positive
GFD-CD (N = 5)	5–18	F = 5	3 = T0 2 = T1	0–1.5	Negative
POT-CD (N = 4)	7–12	M = 2, F = 2	3 = M0 1 = T1	9.3–57	3 = positive 1 = ND

* T0: Normal; T1: infiltrative lesion; T3: Flat destructive lesion (^b: moderate, ^c: total).

4.2. Culture Medium to Maintain Organoids (CM-S)

Mouse L-cells that expressed Wnt3a, R-spondin, and Noggin were commercially purchased (ATCC CRL-3276, Genova, Italy), and a conditioned medium (L-WRN) was prepared according to the instructions and protocol of the manufacturer. Culture medium with supplements (CM-S) was prepared using 50% conditioned L-WRN medium and 50% fresh primary culture media: Advanced DMEM/F-12 (cat.12634-010, Invitrogen Milan, Italy) 1 mM N-Acetyl-L-cysteine (cat. A7250, Sigma, Milan, Italy), 1 × B-27[®] supplements (cat.12587-010 Gibco, Milan, Italy), 50 ng/mL epidermal growth factor, (cat. PMG8041 Gibco Milan, Italy) 10 mM nicotinamide (cat. N0636, Sigma, Milan, Italy), 10 nM Leu15-gastrin I (cat. G9145 Sigma, Milan, Italy), 500 nM A8301 (inhibitor for ALK4/5/7, cat. 70024-90-7 Sigma, Milan, Italy), 10 µM SB202190 (p38 MAP kinase inhibitor, cat. S7076 Sigma, Milan, Italy), and 10 µM Y-27632 (p160 ROCK inhibitor; cat.1254 Tocris, Milan, Italy) in accordance with the protocols of Sato et al. [39], VanDussen et al. [40] and Yuli Wang et al. [38]. The organoids were cultured with 300 µL culture medium, which was changed every second or third day. After seven days, when the organoids had formed large circular structures, they were isolated from the Matrigel matrix and split. For each passage, the

spheroid/Matrigel was scratched into 500 μ L of PBS containing 0.5 mM EDTA (PBS-EDTA). The spheroids were pelleted by centrifugation at $500\times g$ for 5 min, and the supernatant was discarded. The spheroids were dissociated by incubation in 200 μ L of 0.25% trypsin in PBS-EDTA for 60–90 s. An additional 5 mL of WB was then added to inactivate the trypsin. The spheroids were pelleted by centrifugation at $500\times g$ for 5 min. The supernatant was carefully removed, and the pellet was resuspended in Matrigel.

4.3. Fixing of Organoids, OCT Embedding, and Cryosectioning

After removing the organoid culture media, the human spheroids were washed from each well of a 6-well plate with 5 mL of 1X PBS at room temperature. The structures were fixed with 5 mL of 2% paraformaldehyde (PFA) and 0.1% glutaraldehyde (GA) in 1X PBS for 30 min at room temperature. After washing extensively with 5 mL of 1X PBS to remove the fixing solution, the organoid domes were carefully removed with a scoop or spatula and placed in a 50 mL conical tube containing 20% sucrose in 1X PBS. The tube was left at 4 °C overnight or for three days, until the domes fell to the bottom of the tube. The domes were removed from the sucrose solution and placed in a mold containing optimal cutting temperature (OCT cat. 05-9801 Bio Optica, Milan, Italy) compound. Several domes were placed on each mold; they were snap frozen and stored at -80°C . Using a cryotome, we cut the organoid block into cryosections approximately 10 μ m thick.

4.4. Immunostaining

After washing the slides with 1X PBS to remove OCT for 3D organoids, the 2D organoids were openly seeding in six wells pretreated with Matrigel diluted 1:40 in PBS; the tissues were permeated with 0.15% Triton/1X PBS for 15 min at room temperature. The cells were washed 3 times with 1X PBS for 10 min each time. The slides were blocked with 3% BSA/1X PBS (blocking solution) or 10% FBS/1X PBS for 1 h at room temperature. The primary antibody anti-rabbit anti-cytokeratin (cat. Z0622, Dako Carpinteria, CA, USA) was diluted in blocking solution overnight at 4 °C in a humidified chamber. Then the cells were washed 3 times with 1X PBS for 10 min each time. Alexa Fluor 546 donkey anti-rabbit secondary antibody (A10040 Invitrogen, Milan, Italy) was added to the organoids. The cells were then washed 3 times with 1X PBS for 10 min each time, mounted with Mowiol (cat. 475904-M Sigma-Aldrich, Milan, Italy) and observed under a confocal microscope (Zeiss LSM 510, Jena, Germany) [18].

4.5. Western Blot

The human spheroids were seeded in six multiwells (Corning, Milan, Italy) coated with Matrigel diluted 1:40 in phosphate buffered saline (PBS) for 3 days. After they were stimulated with P31-43 (Caslo, Kongens Lyngby, Denmark) or LOX (cat. 121288-39-9, Invivogen Toulouse, France) for various times at 37 °C, the organoids were homogenized in tissue homogenization buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 5 mM MgCl_2 , 1% Triton, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM VO_4 , aprotinin, and LAP; all purchased from Sigma, Milan, Italy, except for LAP, which was purchased from Roche, Milan, Italy). The cell lysates were analyzed using SDS-PAGE with a standard running buffer (25 mM Trizma, 192 mM glycine, and 0.1% SDS) and were transferred onto nitrocellulose membranes using Transbolt Turbo (cat.1704158, BioRad, Milan, Italy). The membranes were blocked with 5% nonfat dry milk and probed with rabbit anti-ERK1/2 (cat.31374, Elabscience, Milan, Italy) and rabbit anti-pY-ERK1/2, (cat.20869, Elabscience, Milan, Italy) mouse anti-GAPDH, (cat. G8795, Sigma-Aldrich, Milan, Italy), and rabbit anti-pY-NF- κ B, (cat.3033P, Cell Signalling, Euroclone, Milan, Italy). The bands were visualized using ECL (cat. RPN2209, GE Healthcare, Amersham, Buckinghamshire, UK) with exposure times of 2–10 min. The band intensity was evaluated by integrating all the pixels of the band after subtraction of the background to calculate the average of the pixels surrounding the band [18].

4.6. ELISA

The levels of IL-1 β and IL-6 were measured using commercial test kits (IL-1 β cat.850.006.192, IL-6 cat.950.030.192, Diaclone, Besancon Cedex, France) on cell culture supernatants.

4.7. RNAscope to Detect IL-1 β and IL-6 mRNA

Expression of IL-1 β and IL-6 mRNA in situ was analyzed in intestinal biopsies from CD patients at different stages of the disease as well as those from controls. (Table 1) IL-1 β and IL-6 mRNA levels were detected by RNAscope™ 2.5 HD Assay—RED (cat.321720, IL-1 β 310361, IL-6 cat.310371, ACD—Biotechnie, Milan, Italy). Sample preparation, probe hybridization, and signal detection were carried out according to the kit instructions. Positive signals were indicated by dot-shaped red granules in the crypts. Positive and negative probes were used as positive and negative controls. The red-stained cells were counted. Positive staining in more than 10% of the cells was considered positive, while less than 10% or colorless staining was defined as negative.

Briefly, biopsies included in paraffin were cut at 5 μ m, slides were deparaffinized with xylene twice for 5 min and 100% alcohol twice for 1 min. Deparaffinized slides were incubated with hydrogen peroxide for 10 min at room temperature, submerged in the target retrieval reagent for 30 min at 99°, transferred to 100% alcohol for 3 min, and dried at room temperature overnight. Each slide was incubated with protease plus for 30 min at 40° and then washed 3 times with distilled water. Slides were incubated with the appropriate probe for 2 h at 40°, washed with wash buffer and incubated with hybridized Amp (from Amp 1 to Amp 6) according to the protocol. To detect the signal, slides were incubated with fast red solution, submerged in staining dishes containing 50% hematoxylin and mounted with EcoMount.

4.8. PCR

Total RNA was extracted from organoids using a RNeasy Mini Kit (cat. 74104 Qiagen, Milan, Italy). The mRNA concentration was measured using a Nanodrop spectrophotometer, and the RNA quality was analyzed using agarose gel electrophoresis in Tris/Borate/ethylenediaminetetraacetic acid (EDTA) buffer (TBE, Sigma, Milan, Italy). RNA (1 μ g) was reverse transcribed into cDNA using a QuantiTect Reverse Transcription Kit (cat. 205311 Qiagen, Milan, Italy) according to the manufacturer's protocol. The experiments were performed with approximately 40 ng of cDNA templates, according to the manufacturer's protocol (cat. 4331182 TaqMan Gene Expression Assay, Applied Biosystems, Monza, Italy), using a 7900 HT Fast Real-Time PCR system. The gene expression assay used to detect the IL-1 β gene was Hs01555410_m1 (Applied Biosystems, Thermo Fisher Scientific Inc., Monza, Italy), and the probe was located on Chr.2: 112829758–112836842 on Build GRCh38; for the IL-6 gene Hs00174131_m1 (Applied Biosystems, Thermo Fisher Scientific Inc., Monza, Italy) was used, and the probe was located on Chr.7: 22725889–22732002 on Build GRCh38. The expression of each gene was normalized to the expression of an endogenous housekeeping gene (HPRT1). Relative quantification was performed using the $\Delta\Delta$ Ct method. SDS software (ABI, version 1.4 or 2.4) was used to analyze the raw data.

4.9. Statical Analysis

GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA) was used for statistical analyses and to construct graphical representations. Statistical analyses of the differences included Student's *t* tests. A *p* value < 0.05 was considered statistically significant. Two-tailed comparisons were used for all statistical analyses. The sample size was chosen after considering the variance of the control samples, and the number of samples needed to assess the extent of the expected effect was estimated to be three or four. Therefore, the chosen sample size was three, four, or (more often) five.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms23041973/s1>.

Author Contributions: M.P. performed the mRNA in situ experiments and contributed data analysis; M.C., G.L., and C.B. performed W.B. experiments and RT-PCR and L.R. prepared the immunostaining slides and cared for the biobank; R.A. and R.T. led the clinical recruitment of patients; F.G.C. revised the manuscript; S.A. contributed to study conception and writing the manuscript; M.V.B. led the study conception, design, performance, and wrote the text. M.N. led the study conception and design and performed experiments. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the University of Naples Federico II, Protocol number: 115/09/ESPROT).

Informed Consent Statement: Written informed consent was obtained from all patients or from the next of kin, caretakers, or guardians on behalf of the minor/child participants involved in our study.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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Article

PTPRK, an EGFR Phosphatase, Is Decreased in CeD Biopsies and Intestinal Organoids

Merlin Nanayakkara ¹, Claudia Bellomo ¹, Francesca Furone ¹, Mariantonia Maglio ², Antonella Marano ², Giuliana Lania ², Monia Porpora ², Martina Nicoletti ¹, Salvatore Auricchio ² and Maria Vittoria Barone ^{1,2,*}

¹ Department of Translational Medical Science, Section of Pediatrics, University Federico II, Via S. Pansini 5, 80131 Naples, Italy

² ELFID (European Laboratory for the Investigation of Food Induced Diseases), University Federico II, Via S. Pansini 5, 80131 Naples, Italy

* Correspondence: mv.barone@unina.it; Tel.: +39-0817464568; Fax: +39-0817463116

Abstract: Background & Aims: Celiac disease (CeD) is an immune-mediated enteropathy triggered in genetically susceptible (HLA-DQ2/8) individuals by a group of wheat proteins and related prolamins from cereals. The celiac intestine is characterized by an inversion of the differentiation/proliferation program of the enterocytes, with an increase in the proliferative compartment and crypt hyperplasia, which are the mechanisms that regulate the increased proliferation in CeD that are not completely understood. The aim of this study is to understand the role of Protein Tyrosine Phosphatase Receptor Type K (PTPRK), a nodal phosphatase that regulates EGFR activation in the proliferation of the enterocytes from CeD biopsies and organoids. Methods: The levels of PTPRK were evaluated by RT-PCR, western blot (WB) and immunofluorescence techniques in intestinal biopsies and organoids from CeD patients and controls. Additionally, pEGFR and pERK were evaluated by WB and proliferation by BrdU incorporation. PTPRK si-RNA was silenced in CTR organoids and was overexpressed in CeD organoids. Results: PTPRK was reduced in Gluten Containing Diet–Celiac Disease (GCD–CeD) and Potential-Celiac Disease (Pot–CeD) biopsies ($p < 0.01$ – $p < 0.05$) whereas pEGFR ($p < 0.01$ $p < 0.01$), pERK ($p < 0.01$ $p < 0.01$) and proliferation were increased. ($p < 0.05$ $p < 0.05$) respect to the controls. The CeD organoids reproduced these same alterations. Silencing of PTPRK in CTR organoids increased pEGFR, pERK and proliferation. The overexpression of PTPRK in CeD organoids reduced pEGFR, pERK and proliferation. Conclusions: modulation of PTPRK levels can reduce or increase pEGFR, pERK and proliferation in CeD or CTR organoids, respectively. The CeD organoids can be a good model to study the mechanisms of the disease.

Keywords: PTPRK; EGFR; celiac disease; intestinal organoids



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1. Introduction

Celiac disease (CeD) is an immune-mediated enteropathy triggered in genetically susceptible (HLA-DQ2/8) individuals by a group of wheat proteins and related prolamins from cereals [1]. In particular, these proteins damage the intestinal mucosa through the inflammation generated by the immune response that is both innate and adaptive against gliadin, causing crypt hyperplasia through uncontrolled proliferation of enterocytes [2–5].

The celiac intestine is characterized by reduced differentiation of the enterocytes, resulting in a reversal of the differentiation/proliferation program of the intestinal tissue. This may lead to complete villous atrophy and increased proliferation with cryptic hyperplasia [6,7].

In two patient populations, Gluten Containing Diet–Celiac Disease (GCD–CeD) and Gluten Free Diet–Celiac Disease (GFD–CeD), at the level of the duodenal biopsies, increased activity both of the EGFR/EGF (Epidermal Growth Factor Receptor/Epidermal Growth Factor) system and of the ERK (Extracellular Signal-Regulated Kinases) molecule has

been demonstrated. Moreover, activation of EGFR/ERK pathway has been linked to the enhancement of enterocytes proliferation [5,7,8]. It is interesting to note that both gluten and gliadin peptide P31-43 can increase proliferation in GFD–CeD patients biopsies in vitro and after gluten challenge in vivo and that they are able to affect several other biological pathways in vitro in cells [8–13] and also in animal models [14,15]. HLA-DQ 2/8 isotypes have been strongly associated with CeD, this genetical predisposition is necessary but not sufficient to the insurgence of the disease. Repeated GWAS (Genome Wide Association Study) analysis led to the identification of a total of 39 non-HLA sensitivity loci that explain only 10–14% of the CeD heritability, these in combination with the HLA locus could explain 50% of the CeD heritability [16,17]. In order to prioritise and annotate functional SNPs (Single Nucleotide Polymorphisms), pathways and genes affected in CeD, Kumar et al. have applied integrative approaches [17]. Results from 1469 blood samples were combined with co-expression analyses to give priority to causative genes, from different blood immune cell populations. Data from pathways and tissue-specific expression analyses on these genes identified a role in CeD pathogenesis for Lipoma-Preferred Partner (LPP) [18–20], C1ORF106 (C1 Orfan 106), ARGHAP31 [21] and Protein Tyrosine Phosphatase Receptor Type K (PTPRK) genes, which play a role in actin-cytoskeleton rearrangement, cell-cell adhesion and in the Epidermal Growth factor (EGF)/EGF Receptor (EGFR) pathway activation [17,22]. PTPRK, an EGFR phosphatase, is reduced in CeD biopsies respect to controls [17,23].

Proliferation, metabolism, differentiation, motility, cytoskeletal organization, cell-cell interactions, development and other cellular processes are under control of reversible protein phosphorylation regulating signal transduction pathways [24]. In particular, both Protein-Tyrosine Kinases (PTKs) and Protein-Tyrosine-Phosphatases (PTPs), two classes of counteracting enzymes, control the level of protein tyrosine phosphorylation. New evidence indicates that, depending on the specific pathway, protein tyrosine dephosphorylation may be of equal or greater importance to the regulation of cell function than protein tyrosine phosphorylation [25]. The EGFR is a component of Receptor Protein-Tyrosine Kinase (RPTK) superfamily. When a ligand binds to the N-terminal extracellular domain of EGFR, it stabilises homodimerization and heterodimerisation with other members of EGFR family and promotes trans tyrosine phosphorylation of the intracellular C-terminal domain. It has been demonstrated that the aberrant regulation of EGFR promotes multiple tumorigenic processes by stimulating angiogenesis, proliferation and metastases. EGFR and/or its ligands have a significant role in many different types of solid tumours and are most common in human epithelial cancers. The main mechanism by which EGFR modulates several transduction pathways is the phosphorylation of its tyrosine and this particular step must be strictly regulated. PTP-catalysed dephosphorylation of EGFR is one of these mechanisms for regulation. PTPRK has been demonstrated to dephosphorylate EGFR; in CHO (Chinese Hamster Ovary) cells that lack PTPRK, there is increased proliferation [26]. Aim of this study is to analyse and understand the role of PTPRK, an EGFR phosphatase, in the CeD intestinal epithelium from biopsies and organoids.

2. Materials and Methods

2.1. Biopsies

For organ culture studies, duodenal biopsies were obtained from patients at GCD–CeD (20 patients, mean age of 10 years), controls affected by gastroesophageal reflux (20 subjects, mean age of 13 years) and Pot–CeD patients (8 subjects, mean age of 11 years). The age range for all of them was 2–16 years. The group of patients with GCD–CeD (Marsh T3c) and the Pot–CeD group had positive serology (anti-tTg antibodies >50 U/mL and in a range between 9 and 60 U/mL and positive EMA, respectively) (Table 1). Eurospital kit EU-tTG(cat. 9105, Trieste, Italy). was used to evaluate anti-tTg antibody titre. Specimens were harvested, snap-frozen in liquid nitrogen, embedded in OCT (cat. 05-9801, Bio Optica, Milan, Italy) and stored at -80°C .

Table 1. Patients' characteristics.

Patients	Range Age (Years)	Sex	Biopsy (Marsh Classification *)	Serum AntiTG2 (U/mL)	Anti-Endomysial Antibody (EMA)
Controls (N = 20)	9–17	M = 8 F = 12	20 = T0	0–1.7	Negative
GCD–CeD (N = 20)	4–16	M = 10 F = 10	6 = T3c 14 = T3 c/b	>50	Positive
POT–CeD (N = 8)	8–13	M = 5 F = 3	6 = T0 2 = T1	9–60	Positive

* T0: Normal; T1: infiltrative lesion; T3: Flat destructive lesion (b: moderate, c: total).

2.2. Organoids

Two or more duodenal biopsies from GCD–CeD patients and controls were taken during routine gastro-duodenoscopy (Table 2) and placed in 10 mL of isolation buffer with the addition of 2 Mm EDTA (cat.15575020, Thermo Fisher, Milan, Italy) and 0.5 Mm DDT (D0632, Sigma-Aldrich, Milan, Italy), as described previously [27]. After 1 h of agitation at 4 °C, the biopsy samples were washed with the isolation buffer, filtered through a 70 µm strainer (Falcon, NY, USA) and centrifugated at 500× g for 5 min, then the crypts were resuspended in 40 µL of ice-cold Matrigel matrix (cat.35623, Corning, Milan, Italy) to permit three-dimensional growth in 48 well plates. Afterwards, 300 µL cell culture medium enriched with supplements (CM-S) was added to each well [27]. To pass from a 3D to a 2D structure, organoids were openly seeded on 35 mm multiwellspre-treated with Matrigel (1:40 in PBS). We generally processed the 2D organoids within 24–72 h after seeding.

Table 2. Organoids characteristics.

Patients	Range Age (Years)	Sex	Biopsy (Marsh Classification *)	Serum AntiTG2 (U/mL)	Anti-Endomysia Antibody (EMA)
Controls (N = 5)	4–15	M = 3 F = 2	5 = T0	0–1.9	Negative
GCD–CeD (N = 5)	8–17	M = 2 F = 3	3 = T3c 2 = T3 c/b	>50	Positive

* T0: Normal; T3: Flat destructive lesion (b: moderate, c: total).

2.3. Immunofluorescence Staining of Biopsies

PTPRK protein levels and localisation were assessed by immunofluorescence. An amount of 5 µm cryostat sections from each biopsy were treated with acetone (10 min), Pot–CeD patients, controls and blocked with 1% bovine serum albumin (BSA) in PBS for 15 min at room temperature (RT) and stained with rabbit anti-PTPRK (cat. ab222249, Abcam, Microtech, Naples, Italy) antibody for 1h at RT. Then, the sections were probed with Alexa 488 anti-rabbit (1:50; cat.11001, Milan, Italy) for 45 min at room temperature and counterstained with Hoechst (cat. 23491454, Sigma-Aldrich, Milan, Italy), mounted with Mowiol (cat. 9002895, Sigma-Aldrich, Milan, Italy) and observed under a Zeiss LSM510 or Zeiss LSM700 confocal microscopes (laser scanning microscope) (Germany). Images were obtained with a 63× objective unless differently stated [10].

2.4. BrdU Cell Proliferation Assay

PTPRK was silenced, or not, in CTR organoids and was overexpressed, or not, in GCD CeD organoids seeded on 2D on 15 µ-Slid Angiogenesis (cat.81506, ibidi Milan, Italy) coated with Matrigel diluted 1:40 in PBS for 2 d.

BrdU (cat. B5002, Milan, Italy), a marker of S phase, was added in all samples for 18 h before fixation and immunofluorescence staining. BrdU was detected with a monoclonal antibody (RPN20AB, GE Healthcare, Buckinghamshire, UK) and with an anti-mouse-Alexa-633 (1:10, cat. A21063, Milan, Italy) conjugated as secondary antibody. Nuclei

were highlighted by Hoechst staining (Sigma-Aldrich, Milan, Italy). Cell proliferation was assessed by BrdU incorporation assay as described elsewhere [10]. LSM 510 Zeiss microscope was used to acquire and analyse the images.

2.5. Western Blot

Biopsy fragments from duodenum obtained from GCD–CeD and organoids, were processed [10,28] After removing matrigel by Cell Recovery Solution (cat. 354270, Corning Milan Italy), organoids were homogenized in 50 µL tissue homogenization buffer [28]. The cell lysates (10 µg/mL) were analysed by SDS-PAGE and transferred onto nitrocellulose membranes by Trans Blot Turbo (cat.1704158, BioRad, Milan, Italy). The membranes were blocked with 5% non-fat dry milk and probed with anti-PTPRk (cat. ab222249, Abcam, Microtech, Naples, Italy) and rabbit anti-pY-ERK1/2, (cat.20869, Elabscience, Microtech, Naples, Italy), anti-P-EGFR (Y1068) (cat.3777, Cell Signalling, Euroclone Milan, Italy) and anti-EGFR (cat.2232, Cell Signalling, Euroclone Milan, Italy), mouse anti-GAPDH, (cat. G8795, Sigma-Aldrich, Milan, Italy) and with rabbit anti-ERK1/2 (cat.31374, Elabscience, Microtech, Naples, Italy) were used as loading controls for biopsies and organoids, respectively. Then, two 10 min exposure using ECL (cat. RPN2209, GE Healthcare, Amersham, Buckinghamshire, UK) allowed to visualise the bands of interest. The bands intensity was evaluated by integrating all the pixels of the band after subtraction of the background to calculate the average of the pixels surrounding the band.

2.6. Crypt Epithelial Cell Proliferation Test

A total of 4µm cryostat sections duodenal biopsies from 4 GCD–CeD, 4 Pot–CeD and 4 CTR patients were used for evaluating crypt epithelial cell proliferation by immunohistochemistry using Ki67 antigen detection (Table 3). The sections, after a pre-incubation of 10 min with rabbit normal serum (1:100; X0902, Dako, Milan, Italy), were probed with the primary mouse monoclonal antibody Ki67 (1:200; M7240, Dako, Milan, Italy) for 1h and the secondary antibody with rabbit anti-mouse (1:25; Z0259, Dako, Milan, Italy) for 30 min followed by a 30min step with alkaline phosphatase and monoclonal mouse anti-alkaline phosphatase immuno-complexes (mouse APAAP 1:40; K0670, Dako, Milan, Italy). Lastly, the sections were incubated with New Fuchsin for few minutes. A light microscope Axioskop2 plus (Zeiss, Germany) was used to observe sections. Each sample shown more than 500 epithelial cells and the number of Ki67-positive cells was expressed as a percentage of the total number of positive crypt epithelial cells.

Table 3. Patients' characteristics.

Patients	Range Age (Years)	Sex	Biopsy (Marsh Classification *)	Ki67 %
Controls (N = 4)	8–16	M = 2 F = 2	T0	22.5–33
GCD–CeD (N = 4)	3–15	M = 3 F = 1	T3c	52.7–62.2
POT–CeD (N = 4)	2–14	M = 1 F = 3	T0/T1	37–53

* T0: Normal; T1: infiltrative lesion; T3: Flat destructive lesion (c: total).

2.7. mRNA Analysis

Total mRNA was extracted from biopsies of patients with GCD–CeD, Pot–CeD and controls using TRIZOL reagent (cat.10296028, Ambion®-Life Technologies Milan, Italy). The mRNA concentration was measured using a Nanodrop® spectrophotometer (Thermo Fisher, Milan, Italy), the RNA quality was analysed using agarose gel electrophoresis in Tris/Borate/EDTA buffer (TBE, Sigma, Milan, Italy). The RNA (1 µg) was reverse transcribed into cDNAs. The gene expression assay used for PTPRK gene was Hs00267788_m1 (Cat#4331182, Thermo Fisher Scientific). Real-time PCR were performed with approximately

40 ng of cDNA templates, according to the manufacturer's protocol (TaqMan[®] Gene Expression Assay (cat.4369016, Thermo Fisher, Milan, Italy) [8].

2.8. PTPRK Silencing

PTPRK mRNA (Hs PTPRK 1) was used for silencing experiments. To evaluate transfection efficiency was employed non-specific siRNA (Cat. 1022564) (MAPK1), scrambled mRNA and scrambled mRNA sequences (Cat.1027284) (All Stars Negative). HiPerFect Transfection Reagent (cat.301705) was used for transfections. All these products were purchased from QIAGEN, Milan, Italy. The CTR organoids were seeded on 35 mm multiwells (Corning, Milan, Italy) for 24 h. In sum, the cells grown in cell culture medium supplemented with CM-S were added with a transfection mix composed by 6 µg of siRNA, diluted in 100 micro-litres of culture medium without serum to give a final siRNA concentration of 50 nM and 20 µL of HiPerFect Transfection reagent. The transfection mix was vortexed and added drop wise onto the cells that were incubated for 72 h. The cells were then processed for Western blot (WB) analysis and proliferation assay [8,10].

2.9. PTPRK Overexpression

Following the manufacturer's instructions PTPRK transfection was conducted with TT210003 MegaTran2.0 Transfection Reagent (OriGene Clone, Milan, Italy). In sum, the human spheroids from GCD–CeD were incubated in cell culture medium supplemented with CM-S for 24 h. The transfection mix was done by mixing MegaTran 2.0 and DNA in a 3:1 ratio in 100 micro-litres of serum free DMEM. The day after, the transfection mix was incubated for 10–15 min at room temperature to allow transfection complexes to form and were then added to the cells that were incubated for 24 h. Cells were then processed for WB and proliferation assay.

2.10. Statistical Analysis

For statistical analyses and graphical representations was used GraphPad Prism 6.1nk software (GraphPad Software, San Diego, CA, USA). The statistical analysis of the differences was performed using Student's *t*-test and Mann–Whitney test A *p* value < 0.05 was considered statistically significant.

3. Results

3.1. In Enterocytes from GCD–CeD and Pot–CeD Biopsies, PTPRK Is Decreased, pEGFR, pERK and Proliferation Are Increased

PTPRK protein is decreased in the intestine of acute CeD patients at GCD with villus atrophy as shown by WB analysis (Figure 1A,B). In fact, PTPRK protein was decreased in the protein's lysate from intestinal biopsies of GCD–CeD patients compared to controls. This observation was confirmed by immunofluorescence staining of the PTPRK proteins (Figure 1C,D), which showed a significant decrease of the fluorescence intensity of the PTPRK staining of intestinal biopsies from GCD–CeD both in the crypts and in the villi respect to controls. Moreover, the immunofluorescence staining revealed that PTPRK localization is mainly at the level of the intestinal epithelium of crypts and villi both in controls and in CeD biopsies (Figure 1C,D). Interestingly a similar decrease of the PTPRK protein and a similar localization in the epithelial cells was found in the biopsies from Pot–CeD with normal villi architecture at GCD (Figure 1A–D).

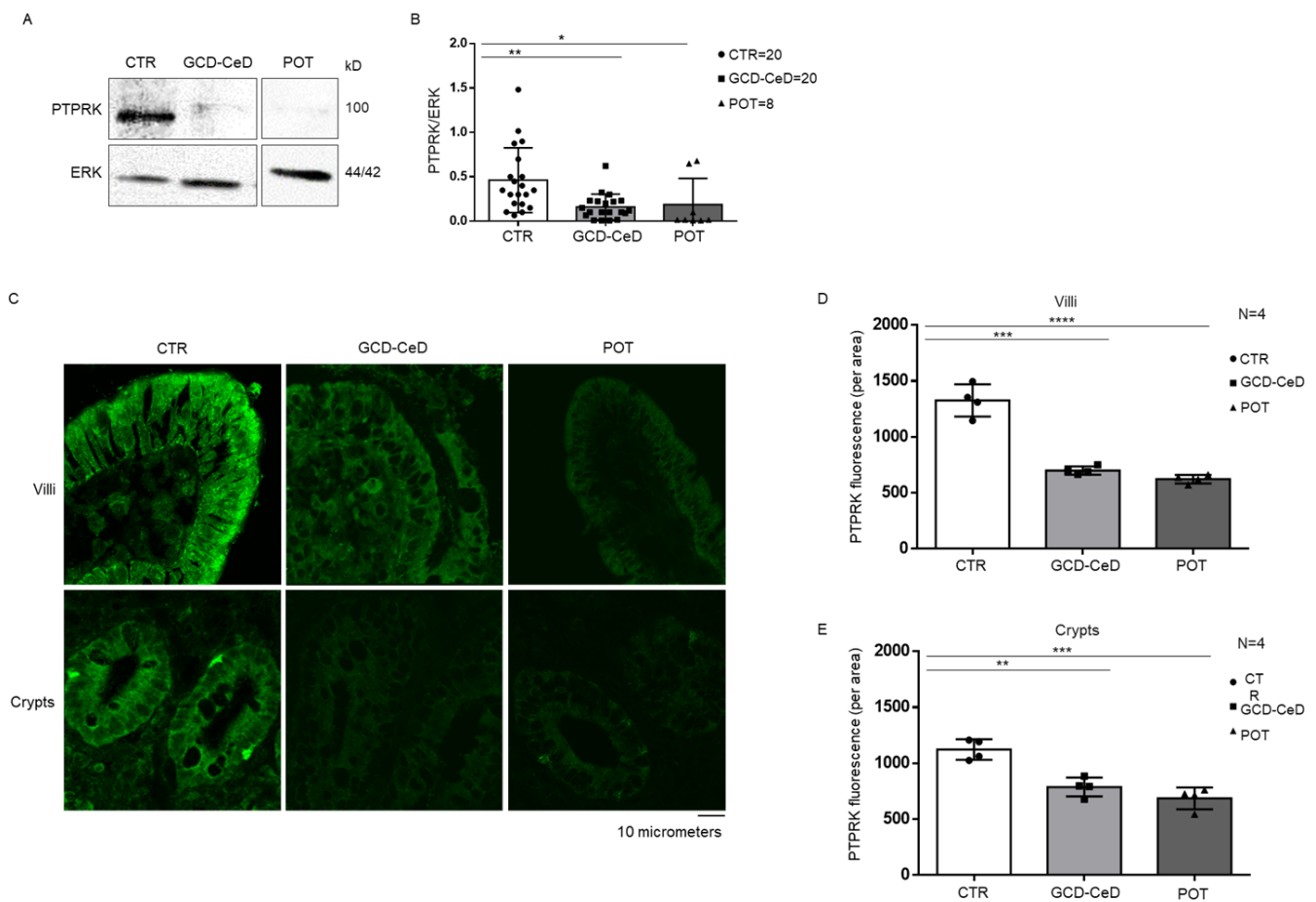


Figure 1. PTPRK protein is decreased in intestinal enterocytes from CeD biopsies (A). Western blot analysis of total protein lysates of intestinal biopsies from controls (CTR), CeD patients in the acute phase of the disease (GCD–CeD) and CeD potential patients (Pot–CeD). Upper lines were blotted with an antibody against PTPRK. Anti-ERK (extracellular-signal-regulated kinase) antibody was used as loading control (bottom lines). Representative images are shown. **(B).** Densitometric analysis of the PTPRK/ERK bands from CTR, GCD–CeD and Pot–CeD. Number of patients analysed are indicated (N = 5). Columns represent the mean and bars the standard deviation. Student's t test: * = $p < 0.05$; ** = $p < 0.01$. **(C).** Immunofluorescence images of intestinal biopsies crypts and villi from CTR, GCD–CeD and Pot–CeD stained with anti-PTPRK antibodies. **(D,E).** Statistical analysis of PTPRK immunofluorescent staining in villi and crypts from CTR, GCD–CeD and Pot–CeD. Columns represent the mean and bars the standard deviation. Student's t test: * $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$. N represent number of organoids evaluated.

The decrease of PTPRK is generally linked to the increase in EGFR (pEGFR) and ERK (pERK) phosphorylation together with the increase in proliferation. For this reason, we have evaluated GCD–CeD and Pot–CeD biopsies for pEGFR levels respect to CTR biopsies. In Figure 2A,B, it is shown by WB analysis that pEGFR is increased in GCD–CeD and Pot–CeD respect to CTR biopsies. As expected, pERK was also increased in GCD–CeD and Pot–CeD respect to CTR biopsies (Figure 2C,D). Moreover, in these same biopsies the proliferation marker Ki67 was increased in the intestinal crypts respect to controls as shown by immunohistochemistry experiments (Figure 2E,F and Table 4). The combination of these experiments show that, in GCD–CeD and Pot–CeD with low levels of PTPRK, the phosphorylated form of EGFR and ERK were both increased together with proliferation.

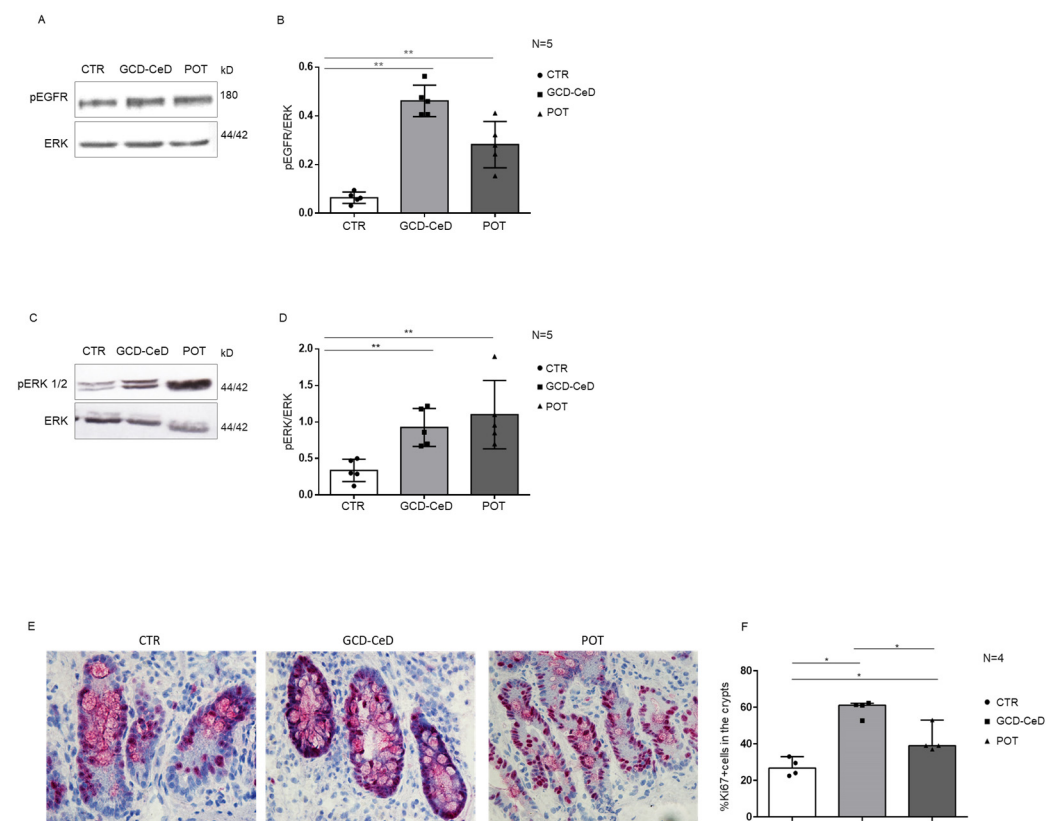


Figure 2. pEGFR, pERK proteins and proliferation are increased in CeD biopsies with low levels of PTPRK. (A). Western blot analysis of total protein lysates of intestinal biopsies from CTR, GCD–CeD and Pot–CeD. Upper lines were blotted with an antibody against pEGFR. Bottom lines were blotted with anti-ERK antibodies as loading control. Representative images were selected. (B). Densitometric analysis of the pEGFR/ERK bands from CTR, GCD–CeD and Pot–CeD. The numbers of patients analysed are indicated. Columns represent the mean and bars the standard deviation. Student’s *t* test: ** = $p < 0.01$. (C). Western blot analysis of total protein lysates of intestinal biopsies from CTR, GCD–CeD and Pot–CeD. Upper lines were blotted with an antibody against the phosphorylated form of ERK (pERK). Bottom lines were blotted with anti-ERK antibodies as loading control. Representative images were selected. (D). Densitometric analysis of the pERK/ERK bands from CTR, GCD–CeD and Pot–CeD. The numbers of patients analysed are indicated. Columns represent the mean and bars the standard deviation. Student’s *t* test: ** = $p < 0.01$. (E). Immunohistochemistry analysis of crypts from biopsies of CTR, GCD–CeD and Pot–CeD patients stained with antibodies against Ki67. Red dense spots indicate positive nuclei. Total nuclei are in blue. (F). Statistical analysis of the percentage of Ki67 positive nuclei in the crypts respect to total nuclei. Columns represent the median and bars the range. Mann–Whitney test. * = $p < 0.05$.

Table 4. Quantitative values of an increase or a decrease in CeD patients’ biopsies.

CeD–Patients	PTPRK	pEGFR	pERK	Ki67 Positive Nuclei
12980 T3c	−2	+7.8	+2	+2.2
12985 T3c	−4.6	+9.3	+3.6	+2.3
12686 T3c	−2.3	+6.3	+3.5	+1.9
12994 T3c	−2	+7.1	+2.6	+2.2

CeD patients’ biopsies with low levels of PTPRK present high levels of EGFR and ERK phosphorylation together with increased Ki65 positive cells. The numbers represent quantitative values of an increase or a decrease of at least 2 times respect to the mean of controls’ biopsies for the same parameter.

3.2. Intestinal Organoids from CeD Patients Reproduce the Increase in pEGFR, pERK and Proliferation Found in GCD–CeD and Pot–CeD Biopsies

Intestinal organoids derived from CeD patients at GCD were analysed for PTPRK mRNA levels by quantitative PCR analysis. In Figure 3, a Real Time PCR analysis of intestinal organoids derived from CeD patients have less PTPRK mRNA respect to intestinal organoids derived from controls. Additionally, PTPRK protein levels were also reduced in these organoids as shown in Figure 3B,C. Moreover, in these same organoids pEGFR (Figure 3B,D) and pERK (Figure 3B,D) were increased as shown by WB analysis.

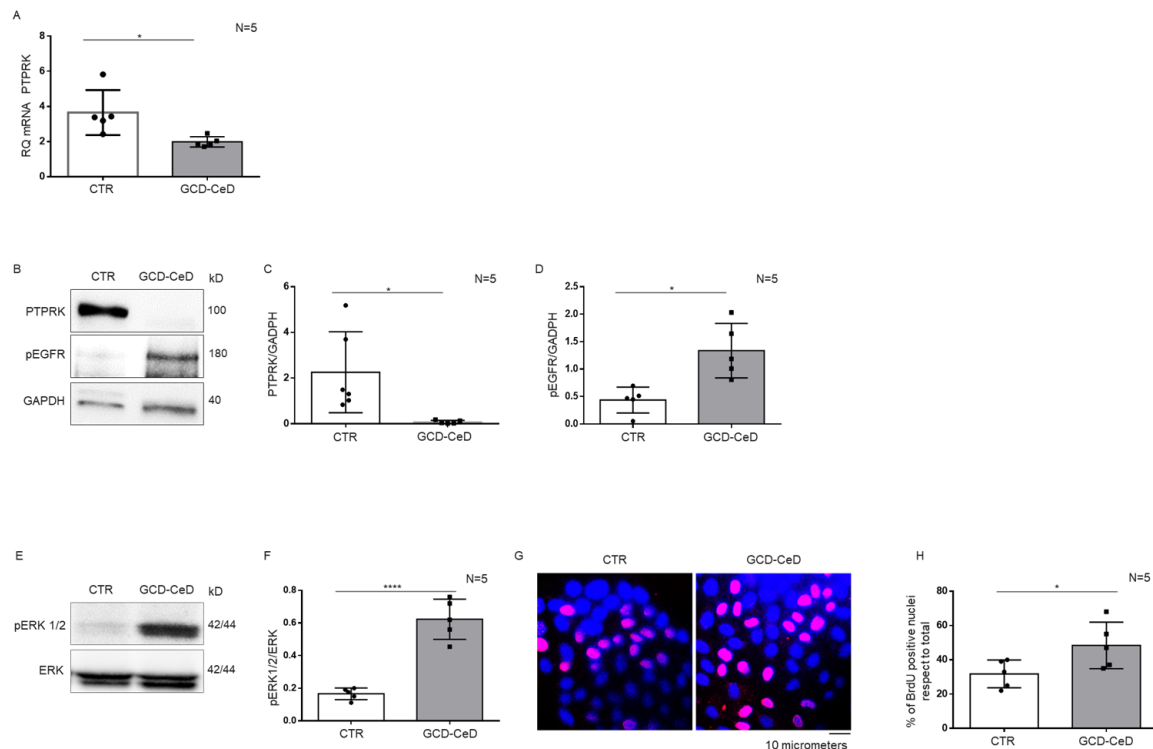


Figure 3. CeD organoids have low levels of PTPRK mRNA, and protein, pEGFR, pERK and proliferation are increased. (A). PTPRK mRNA levels in CTR and GCD–CeD organoids analysed by Real Time PCR. Columns represent the mean and bars the standard deviation. Student’s *t* test: * = $p < 0.05$. (B). Western blot analysis of total protein lysates of intestinal organoids from CTR and GCD–CeD. The membrane was blotted with antibodies against PTPRK, pEGFR and GAPDH as loading control. (C,D). Densitometric analysis of the PTPRK/GAPDH and pEGFR/GAPDH bands. Columns represent the mean and bars the standard deviation. Student’s *t* test: * = $p < 0.05$. (E). Upper lines were blotted with an antibody against pERK. Bottom lines were blotted with anti-ERK antibodies as loading control. (F). Densitometric analysis of the pERK/ERK bands. Columns represent the mean and bars the standard deviation. Student’s *t* test: **** = $p < 0.0001$. (G). Immunofluorescence images of anti-BrdU staining on intestinal organoids from CTR and GCD–CeD patients. Red nuclei are positive for BrdU staining. (H). Statistical analysis of the percentage of BrdU positive nuclei in intestinal organoids from CTR and GCD–CeD. Columns represent the mean and bars the standard deviation. Student’s *t* test: * = $p < 0.05$.

Furthermore, BrdU incorporation, a marker of S phase entry of the cell cycle, was increased in CeD organoids nuclei respect to controls (Figure 3G,H) showing that CeD organoids enterocytes proliferate more respect to controls. Taken all together these data indicate that intestinal organoids from CeD patients reproduce the same increase in pEGFR, pERK and proliferation present in intestinal biopsies from GCD–CeD and Pot–CeD intestinal biopsies.

3.3. Silencing of PTPRK Protein in CTR Organoids Induced Increase in pEGFR, pERK and Proliferation

To demonstrate the correlation between the low levels of PTPRK and the increase in pERGF, pERK and proliferation found in CeD organoids, we decided to reduce the PTPRK levels in CTRs organoids by silencing PTPRK (Figure 4A,B). In CTR organoids with PTPRK silenced both pEGFR and pERK increased respect to the not silenced controls (Figure 4A,C–E). Moreover, in CTR organoids with siPTPRK, proliferation measured by BrdU incorporation was increased.

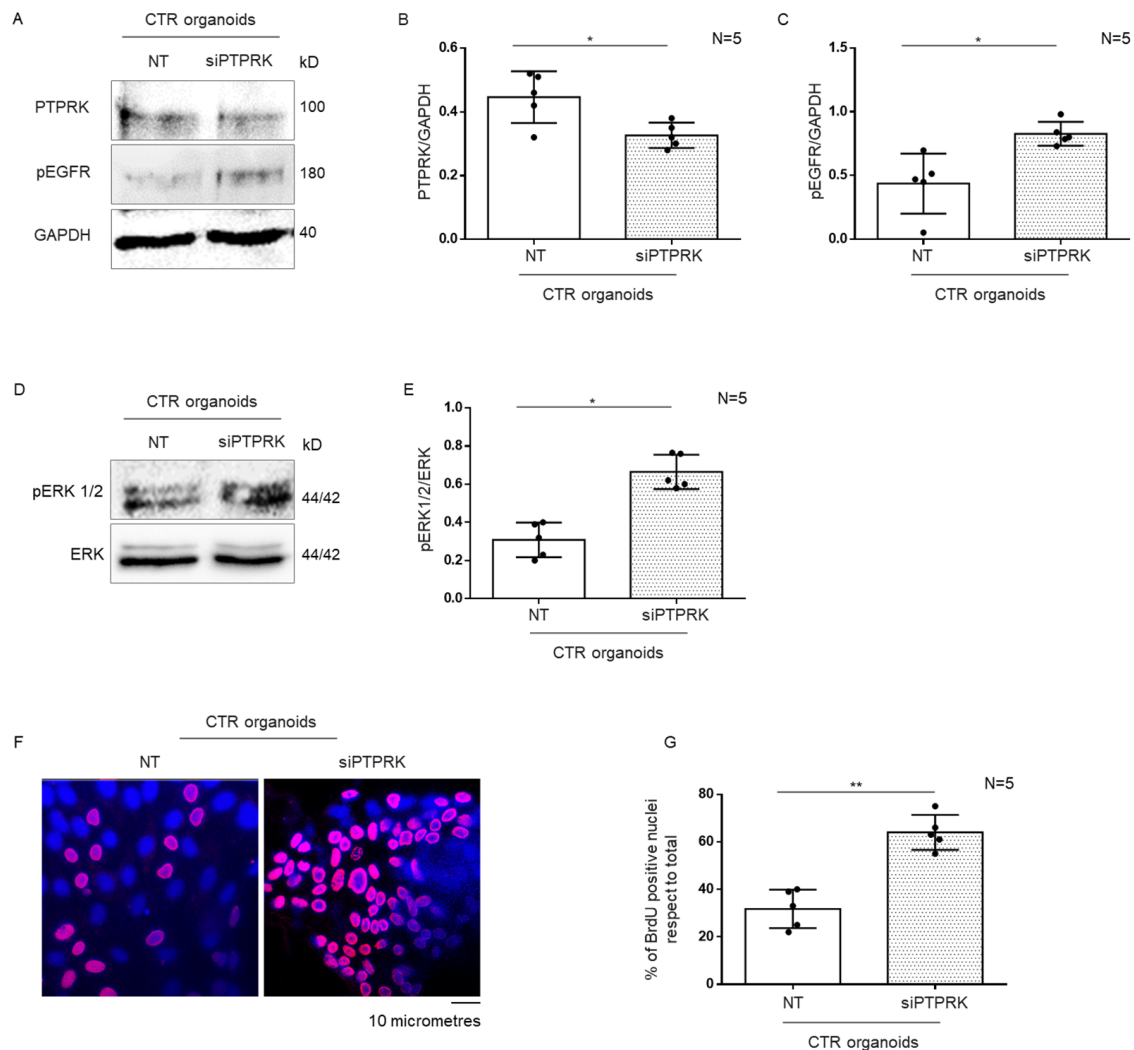


Figure 4. Silencing PTPRK in CTR organoids induced increase in pEGFR, pERK and proliferation. (A). Western blot analysis of total protein lysates of CTR organoids transfected and not with silencing PTPRK (siPTPRK). The membrane was blotted with antibodies against PTPRK, pEGFR and GAPDH as loading control. (B,C). Densitometric analysis of the PTPRK/GAPDH and pERK/GAPDH bands. Columns represent the mean and bars the standard deviation. Student's *t* test: * = *p* < 0.05. (D). Upper lines were blotted with an antibody against pERK. Bottom lines were blotted with anti-ERK antibodies as loading control. (E). Densitometric analysis of the pERK/ERK bands. Columns represent the mean and bars the standard deviation. Student's *t* test: * = *p* < 0.05. (F). Immunofluorescence images of anti-BrdU staining on intestinal organoids from CTR. Red nuclei are positive for BrdU staining. (G). Statistical analysis of the percentage of BrdU positive nuclei in intestinal organoids from CTR. Columns represent the mean and bars the standard deviation. Student's *t* test: ** = *p* < 0.01.

Showing that the reduction in the levels of the PTPRK protein by silencing mRNA was able to modulate the levels of pEGFR, pERK and the proliferation in CTRs reproducing the same alterations found in CeD organoids.

3.4. Overexpression of PTPRK Protein in CeD Organoids Reduced pEGFR, pERK and Proliferation

Afterward, we have overexpressed PTPRK in CeD organoids to confirm that the levels of pEGFR, pERK and proliferation were dependent on PTPRK levels. In CeD organoids with PTPRK overexpression, both pEGFR and pERK decreased respect to the not transfected CeD organoids (Figure 5A,C–E). BrdU incorporation also decreased in CeD organoids overexpressing PTPRK. This shows that the increase in the levels of the PTPRK protein by overexpression was able to modulate the levels of pERGF, pERK and the proliferation in CeD organoids.

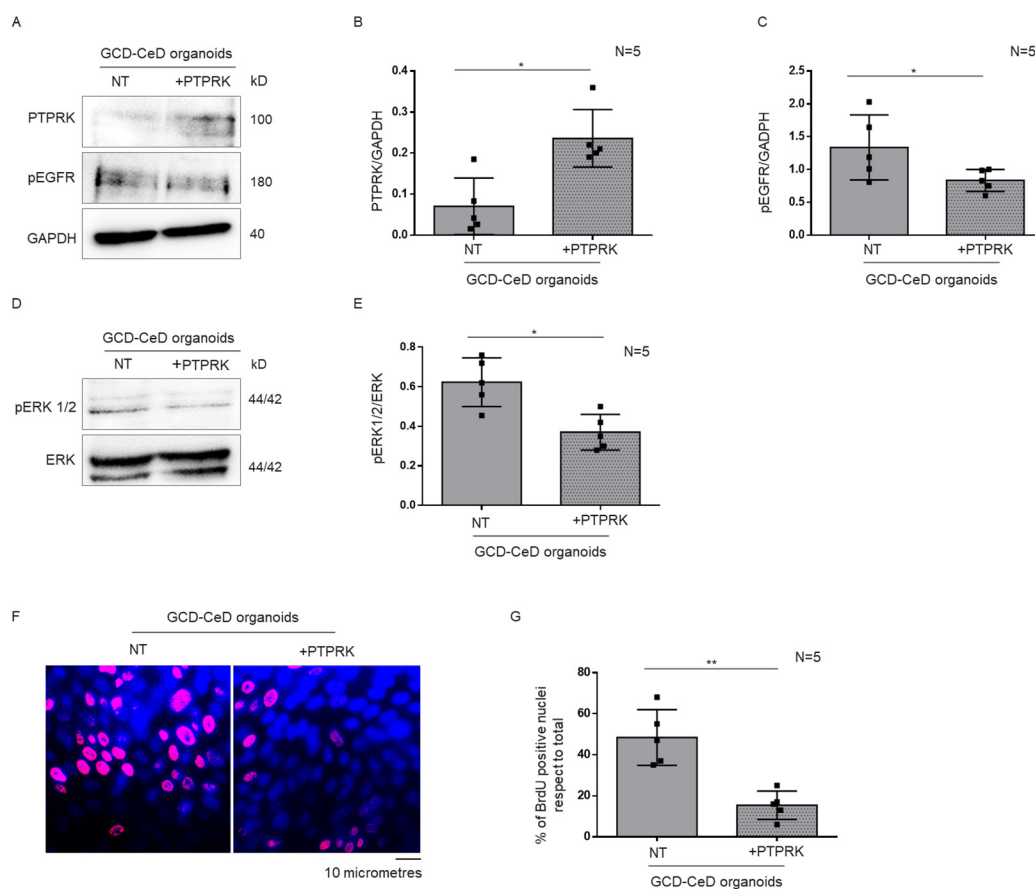


Figure 5. Overexpression of PTPRK in CeD organoids induced a decrease in pEGFR, pERK and proliferation. (A). Western blot analysis of total protein lysates of CeD organoids transfected and not with PTPRK. The membrane was blotted with antibodies against PTPRK, pEGFR and GAPDH as loading control. (B,C). Densitometric analysis of the PTPRK/GAPDH and pEGFR/GAPDH bands. Columns represent the mean and bars the standard deviation. Student's *t* test: * = $p < 0.05$. (D). Upper lines were blotted with an antibody against pERK. Bottom lines were blotted with anti-ERK antibodies as loading control. (E). Densitometric analysis of the pERK/ERK bands. Columns represent the mean and bars the standard deviation. Student's *t* test: * = $p < 0.05$. (F). Immunofluorescence images of intestinal organoids from GCD–CeD patients stained with anti-BrdU antibodies. Red nuclei are positive for BrdU staining. (G). Statistical analysis of the percentage of BrdU positive nuclei in intestinal organoids from CTR and GCD–CeD. Columns represent the mean and bars the standard deviation. Student's *t* test: ** = $p < 0.01$.

4. Discussion

In this manuscript we have analysed the role of the PTPRK phosphatase on the increase in pEGFR, pERK and proliferation in CeD biopsies and intestinal organoids. In CeD biopsies, both GCD–CeD and Pot–CeD patients the levels of PTPRK that decreased in respect to controls, indicating that the reduction in the PTPRK protein was independent from the intestinal atrophy. Moreover, in the biopsies with low levels of PTPRK there was an increase in pEGFR, pERK and crypts epithelial cells proliferation, indicating that also in intestinal epithelium, PTPRK can have a role in the regulation of the signalling from EGFR to proliferation. Subsequently, we showed that intestinal organoids derived from CeD biopsies had low levels of PTPRK, together with increased pEGFR, pERK and crypts enterocytes proliferation. Therefore, intestinal organoids from CeD patients reproduce the alterations of the EGFR/ERK/proliferation pathway found in CeD biopsies.

To show the causal link between the PTPRK levels and the state of activation of EGFR and ERK together with the increased number of proliferating enterocytes, we have silenced PTPRK in CTR organoids to create the same phenotype present in CeD organoids. In CTR organoids with reduced levels of PTPRK there was an increase in pEGFR and pERK together with increased proliferation. Indicating that the levels of PTPRK protein can regulate the EGFR pathway that leads to proliferation. On the other side, the overexpression of the PTPRK protein in CeD organoids was able to reduce both EGFR/ERK phosphorylation state and the cell proliferation.

In CeD, the role of the EGFR/ERK/proliferation pathway has been established [5,9]. In fact, in CeD biopsies and in fibroblasts, pEGFR and pERK are increased together with increased proliferation, that is EGFR and ERK dependent [7,8,11]. In CeD biopsies and fibroblasts EGFR is delayed in the early endocytic vesicles and stays longer activated respect to CTRs [8]. Moreover, gliadin and gliadin peptide P31–43, further delay the endocytic pathway and activate the same EGFR/ERK/proliferation pathway both in CeD biopsies and in control's biopsies [7,11]. Interestingly, EGFR is also involved in the innate immune response mediated by IL15R-alpha upon gliadin treatment in CeD intestinal biopsies [28].

PTPRK is a nodal phosphate that regulates the signalling from tyrosine kinase receptors, including EGFR [26]. EGFR (ErbB1) belongs to the receptor protein-tyrosine kinase (RPTK) superfamily. Aberrant regulation of EGFR has been shown to promote multiple tumorigenic processes by stimulating proliferation, angiogenesis, and metastasis [29,30]. PTPRK has been found co-expressed with THEMIS in the celiac intestinal mucosa [23].

Moreover, other phosphatases of the PTP family are involved in the pathogenesis of inflammatory bowel diseases (IBD), where an association with the lack of PTP proteins has been linked to inflammation [30].

Intestinal organoids from CeD patients not only reproduced the alterations found in CeD biopsies, but also allowed challenge the intestinal epithelium by silencing and overexpressing PTPRK. In this way, by silencing PTPRK in CTR organoids, we were able to reproduce some of the mechanisms of the disease such as the increase in pEGFR/pERK and proliferation found in CeD organoids. On the other side overexpressing PTPRK in CeD organoids reduced to normal the levels of pEGFR, pERK and proliferation. In sum, we were able to recreate the CeD phenotype in CTRs organoids and to “cure” the CeD organoids modulating the PTPRK protein levels using as read outs pEGFR/pERK levels and proliferation. Additionally, the link between PTPRK and TGF- β is known as is the one between Notch and TGF- β [31]. It is possible to hypothesize that they can have a role in the mechanisms of the villus atrophy, in which way it is too soon to be established.

In conclusion, organoids turned out to be a good model to study some pathogenetic mechanisms of the disease, as they reproduced the alterations found in CeD biopsies. They can be a very simple model allowing to study the interaction of the intestinal epithelium and food or other environmental agents. This model can be extended to other diseases in which the intestinal epithelium plays a nodal role in the pathogenetic mechanism such as IBD and diabetes. On the other side CeD is a complex disease, in which several different factors and mechanisms can contribute to the pathogenetic events. Nevertheless, it is possible

to increase the complexity of the intestinal epithelial organoids to finally reproduce the complete intestinal model and study the contribution of each intestinal cellular compounds to the mechanisms of the disease.

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Role of Protein Tyrosine Phosphatase in Inflammatory Bowel Disease, Celiac Disease and Diabetes: focus on the intestinal mucosa

Claudia Bellomo^{1&}, Francesca Furone^{1&}, Roberta Rotondo^{2‡}, Ilaria Ciscognetti^{1‡}, Martina Carpinelli¹, Francesco Silvestro¹, Violeta Catalina Greu¹, Francesca Natasha Hewa Munasinghe¹, Majed Mordaa¹, Martina Nicoletti¹, Leandra Sepe³, Maria Vittoria Barone^{1,4 *} and Merlin Nanayakkara⁵

¹ Department of Translational Medical Science, Section of Pediatrics, University Federico II, Via S. Pansini 5, 80131 Naples, Italy.

² Department of Medicine and Surgery, University of Parma, Parma, Italy.

³ Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, 80131 Naples, Italy.

⁴ ELFID (European Laboratory for the Investigation of Food-Induced Diseases), University Federico II, Via S. Pansini 5, 80131 Naples, Italy.

⁵ Federico II University Hospital, 80131 Naples, Italy

&, ‡ These authors contributed equally to the manuscript

Abstract: Protein tyrosine phosphatases (PTPs) are a family of enzymes essential for numerous cellular processes, such as cell growth, inflammation, differentiation, immune-mediated responses, and oncogenic transformation. The aim of this review is to review the literature concerning the role of several PTPs —PTPN22, PTPN2, PTPN6, PTPN11, DUSP2, DUSP6 and PTPRK— at the level of the intestinal mucosa in inflammatory bowel disease (IBD), celiac disease (CeD), and type 1 diabetes (T1D) in both *in vitro* and *in vivo* models. The results revealed shared features, at the level of the intestinal mucosa, between these diseases characterised by alterations of different biological processes, such as proliferation, autoimmunity, cell death, autophagy and inflammation.

Keywords: Protein tyrosine phosphatases; Celiac disease; Diabetes; Inflammatory bowel diseases; intestinal mucosa.

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1. Introduction

Protein tyrosine phosphatases (PTPs) are protein enzymes that remove phosphate groups from tyrosine residues on other proteins. Tyrosine kinases are, on the other hand, enzymes that add phosphates to tyrosine residues. Owing to the activity carried out by protein kinases and phosphatases, the regulation of phospho-tyrosine signalling is possible, so cells are ready to respond to external signals, inducing changes in the intracellular environment. When receptors on the cell surface bind to ligands, this results in the transduction of external signals within the cell. Therefore, RTKs (receptor tyrosine kinases) can dimerise upon ligand binding, leading to trans autophosphorylation of the receptor. This event leads to the recruitment of proteins that bind phospho-tyrosine residues to the receptor, which propagates and amplifies a variety of signal cascades (1).

PTP proteins play a nodal role in the regulation of signals mediated by phosphatases. Therefore, PTPs are signalling molecules that regulate a variety of cellular processes, such as cell growth, differentiation, the cell cycle, and oncogenic transformation (2).

PTP1B was the first phosphatase identified. This protein has a sequence analogous to a segment of the CD45 (Cluster of Differentiation) receptor protein (3). Owing to this structural analogy, it is possible to identify the existence of a conserved catalytic domain that has become the main feature of the PTP gene family. Further, there is a connection between PTPs and the immune system, as the latter relies heavily on protein phosphorylation to recognise and transmit intra- and extracellular signals (4).

Members of the PTP family are classified according to their structural and biochemical properties. Most PTP protein phosphatases have a conserved catalytic domain that has a cysteine amino acid residue that performs a nucleophilic attachment on the substrate residues. There are three classes of PTP proteins. Class I includes both classical PTP phosphatases that add phosphorylated tyrosine residues and dual-specific phosphatases (DUSPs) that target phosphorylated tyrosine, serine, and threonine residues (Fig. 1).

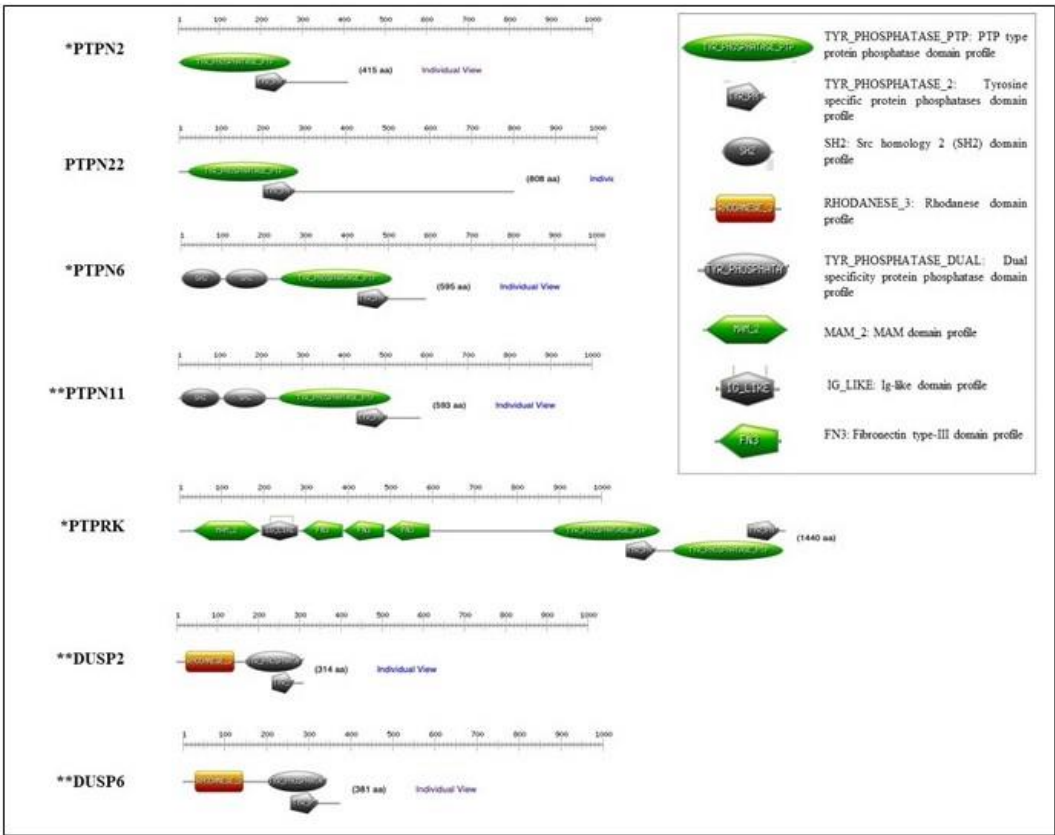


Figure 1. Graphical representation of the PTP domain profile.

PTPs are also classified for their main functions in the immune response (PTPN22, PTPN6, DUSP2, DUSP6, and PTPN11) and signalling pathways (PTPN2, PTPN6, PTPN11, PTPσ, DUSP6, DUSP2, and PTPRK) (1).

Most studies in the literature have described both the molecular mechanisms and therapeutic target of PTPs in cancer (5, 6). While, the role of PTPs in inflammatory diseases such as inflammatory bowel disease (IBD), celiac disease (CeD), and Type 1 Diabetes (T1D) has been established (4, 7-9).

IBD, CeD, and T1D share genetic alterations of genes involved mainly in the pathways of intestinal barrier dysfunction, immune/inflammatory pathways and autophagy (10, 11). A notable aspect of these three diseases is the presence of increased intestinal permeability that precedes the onset of inflammation (12).

IBD and CeD share a specific target organ, the intestine. Both are complex diseases in which genetics and the environment contribute to the dysregulation of innate and adaptive immune responses, leading to chronic inflammation and disease. T1D has as organ target not only pancreatic β -cells but also the intestinal mucosa. In fact, clinical and experimental research has revealed that diabetes mellitus is characterized by intestinal hypomotility, gut microbial dysbiosis, increased gut permeability, and dysfunction of intestinal stem cells, which may be linked to inflammation of the intestinal mucosa (13-15).

All previously described conditions are characterised by the presence of genetic and environmental factors and inflammation. Among the environmental factors that trigger inflammation, nutrients play a significant role in inducing low-grade inflammation, which is considered a "common background" for various diseases, including diabetes, IBD, and CeD (16). A new concept emerging in recent literature suggests that genetic predisposition can increase the susceptibility of cells to inflammatory triggers, leading to cellular vulnerability and autoimmunity (16, 17). How this cellular vulnerability is characterized is not yet clear but signalling molecules, including PTPs, are emerging as possible culprits.

The aim of this review is to review the recent literature on the role of PTP proteins at the level of the intestine in inflammatory diseases, such as CeD, IBD, and T1D.

2. Materials and Methods

We selected the most recent literature on IBD, T1D, and CeD, which are correlated with/associated with PTP proteins. Citing 80/83 from the last 20 years, 50/83 from the last 10 years and 28/83 from the last 5 years and only are between 1993 and 1998 as they are the original references for the PTP described.

3. PTP and Inflammatory Bowel Disease

In this section, we describe the role of the PTPs involved in the development of IBDs (Crohn's disease (CD) and ulcerative colitis (UC)) at the level of the intestine: PTPN22, PTPN2, PTPN6, PTPN11, PTP σ DUSP2, and DUSP6 (Fig. 2) (Table1).

PTPN22 (Protein tyrosine phosphatases non-receptor 22) is expressed mainly in haematopoietic cells and is associated with multiple inflammatory disorders (1, 4, 18). Gene association study demonstrated that SNPs in PTPN22 (loss-of-function variant: rs2476601 and rs1310182) were associated respectively with CD and UC (19-21). In mouse models of experimental arthritis and DSS (dextran sulphate sodium)-induced colitis, PTPN22 deficiency resulted in increased intestinal susceptibility to inflammatory agents and pronounced disease progression, indicating that PTPN22 is required to protect from systemic and gastrointestinal inflammation. Katakura et al. (22) and Yarilina et al. (23) reported that in mice PTPN22 promotes intestinal inflammation suppression in models of colitis and can ameliorate intestinal mucosa injury (24). In mice models PTPN22 represses acute inflammatory immune responses in the gut in the presence of IL-10 (interleukin-10) but promotes acute gut inflammation in the absence of IL-10. During intestinal chronic inflammation, however, PTPN22 promotes gut inflammation irrespective of the presence of IL-10 (25). In addition, PTPN22 at the level of the intestine is involved in autophagy and inflammatory regulation through the dephosphorylation of NOD-like receptor protein 3 (NLRP3) to allow efficient inflammasome activation. Loss of functional autophagy counteracts the reduction in NLRP3 activation observed in PTPN22-deficient cells, leading to increased IL-1 β secretion (26, 27). Defects in autophagy result in impaired goblet cell function, and variants in genes involved in autophagy are associated with an increased risk of developing IBD (28) (Fig. 2 and Tab.1).

The **PTPN2** protein, also called TC-PTP (T cell protein tyrosine phosphatase), is a splicing variant of the PTPN2 gene with the highest expression levels in lymphoid tissue. Previous studies in mice have provided insight into the crucial role of PTPN2 in intestinal

homeostasis, including the regulation of proinflammatory cytokine secretion, autophagy, intestinal epithelial barrier function, and gut microbiota homeostasis (29, 30). The anti-inflammatory role of PTPN2 is highlighted by the fact that PTPN2-deficient mice die a few weeks after birth because of systemic inflammation and severe colitis (31). PTPN2 crucially contributes to normal macrophage–IEC (intestinal epithelial cells) interactions that govern macrophage differentiation and epithelial barrier function and represents a target for therapeutic intervention in IBD. PTPN2 has also been shown to play an important role in Paneth cell regulation. PTPN2 knockout (KO) mice presented a reduced number of Paneth cells, increased Paneth cell endoplasmic reticulum stress, and dysbiosis (29, 32). Clinically relevant loss-of-function mutations in the PTPN2 gene contribute to the etiopathogenesis of chronic inflammatory intestinal disease, at least in part through dysregulation of epithelial barrier properties. Consistent with these findings, PTPN2 levels were increased in the intestinal epithelium in active CD patients. (12, 33, 34) In addition, PTPN2 is involved in autophagy regulation and inflammasome activation (31). PTPN2 was shown to regulate inflammasome activation and IL-1B secretion by limiting the phosphorylation of the adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC), which is required for inflammasome assembly. In mice, the loss of PTPN2 in myeloid cells results in increased susceptibility to DSS-induced colitis due to increased inflammasome assembly and elevated IL1B production at the level of the intestine (35) (Fig. 2 and Tab.1).

PTPN11 was upregulated in both UC patients and colitis model mice (36). It is Src homology 2 (SH2) domain-containing tyrosine phosphatase-2 (SHP2, encoded by the gene PTPN11) (37). As a ubiquitously expressed cytoplasmic protein tyrosine phosphatase that functions as a positive regulator of the Ras–mitogen-activated protein kinase (MAPK) signalling pathway downstream of various growth factors and cytokines, it is thought to contribute to the regulation of cell proliferation and differentiation. On the other hand, Yamashita et al demonstrated that SHP2 lacking mice specifically in IECs developed severe colitis, alterations of intestinal mucosa with numbers of absorptive enterocytes and goblet cells markedly reduced in the small intestine and colon respect to controls. SHP2 is necessary for the homeostasis of IECs, particularly absorptive enterocytes and goblet cells, as well as for protection against colitis, as evaluated by a previous study (38) in the small intestine and the development of intestinal organoids from isolated crypts in mice. Xiao et al. demonstrated that Shp2 expressed in the monocyte/macrophage lineage serves as a proinflammatory factor in the intestine (39). In contrast, mice with SHP2 deficiency in intestinal epithelial cells exhibit abnormal development of the intestinal epithelium and disrupted host–microbiota equilibrium (40, 41). However, the precise role of SHP2 in the regulation of gastrointestinal stem cell and progenitor functions, especially in vivo, has remained unclear (Fig. 2 and Tab.1).

PTPσ, encoded by PTPRS (Protein Tyrosine Phosphatase Receptor S), consists of a cell adhesion molecule-like ectodomain containing three immunoglobulin (Ig)-like repeats and three to eight fibronectin type III repeats, a transmembrane domain, and a cytosolic region with two PTPase domains, of which the first (D1) is catalytically active. PTPσ expression is developmentally regulated and found primarily in the nervous system and specific epithelia. PTPσ $-/-$ mice presented high neonatal mortality. Analysis of the intestinal tissue of surviving mice revealed the presence of mucosal inflammation, intestinal crypt branching, and villus blunting associated with human inflammatory bowel disease (IBD). Additionally, PTPσ targets apical junction complex proteins in the intestine and regulates epithelial permeability (42, 43) (Fig. 2 and Tab.1).

PTPN6, previously known as SH-PTP1, is an Src homology region 2 domains–containing tyrosine phosphatase that is predominantly expressed in hematopoietic and epithelial cells. Shp-1 was associated with increased proliferation and altered goblet and Paneth cell differentiation. Previous studies demonstrate that the levels of PTPN6 were reduced in active UC and CD respect to controls (44). In another study, two PTPN6 SNPs (rs7310161 and rs759052) were genotyped in a population of 107 IBD patients from

Southern Tunisia and a weak association with UC was identified (45). For this reason, a link between PTPN6 and IBD still needs to be defined (Fig. 2 and Tab.1).

DUSP2 (also known as Procaspase-Activating Compound 1– PAC-1) was first identified as a mitogen-inducible gene in human T cells. DUSP2 is a nuclear protein phosphatase that is regulated by the [dephosphorylation](#) of the extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 MAPKs. DUSP2 is a positive mediator of inflammation (46). DUSP2^{−/−} mice exhibit more severe disease, increased mucosal hyperaemia, increased colonic [ulceration](#), and high levels of proinflammatory cytokines, including IL-6, IL-17, Tumor necrosis factor α (TNF α), and IL-1 β . In a study of UC patients, the levels of DUSP2 mRNA expression were reduced in their blood because of the methylation of CpG islands (47) (Fig. 2 and Tab.1).

DUSP6 is a cytoplasmic phosphatase specific to the ERK protein kinase (48). The signal transduction pathway regulated by ERK is responsible for the regulation of multiple cellular responses. In DUSP6 KO mice have shown that ERK induces CD4⁺ T cell polarization and affects the differentiation of T-helper 1 (TH1), TH2, and TH17 cells. DUSP6-deficient mice presented not only increased CD4⁺ T cell activation and cell proliferation induced by ERK but also an increased rate of cell death. This depletion also causes severe inflammation in mouse models of IBD, as confirmed by histological examination. It shows prominent intestinal epithelial hyperplasia, depletion of caliciform cells and infiltration of mononuclear cells. Furthermore, colon explants secrete increased levels of TNF- α and Interferon- γ (IFN- γ), whereas IL-17 α levels are reduced. In these mice, ERK inhibition significantly reduces colitis. Additionally, Beaudry K. et al., by *ex vivo* DUSP6 KO organoid cultures, demonstrated that loss of DUSP6 caused an increase in crypt depth and epithelial cell proliferation (49) (Fig. 2 and Tab.1).

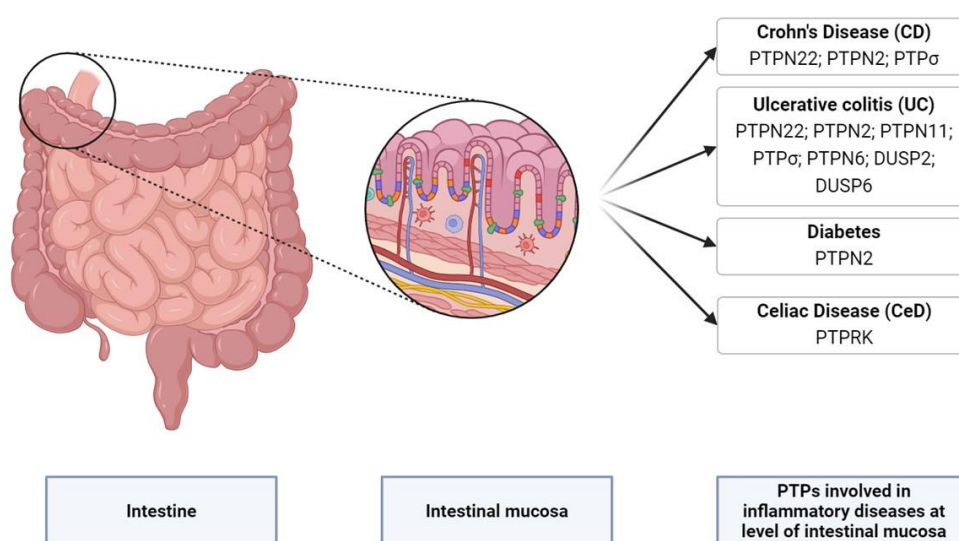


Figure 2. Schematic representation of PTPs (PTPN22; PTPN2; PTP σ ; PTPN11; PTPN6; DUSP2; DUSP6; PTPRK) involved in Crohn's disease, ulcerative colitis, diabetes and celiac disease at level of intestinal mucosa.

4. PTP and Diabetes

T1D is an autoimmune disease characterized by the destruction of insulin-producing β -cells in the pancreas. The onset of T1D is thought to be due to a complex interplay of genetic and environmental factors. The primary genetic risk factors include specific human leukocyte antigen (HLA) alleles, particularly HLA-DQ and HLA-DR, which are essential for immune regulation. Environmental triggers, such as viral infections and certain

dietary components, can act as catalysts in genetically predisposed individuals. Autoreactive T cells, activated by these environmental factors, infiltrate the pancreatic islets, triggering an immune response against β -cells. This response leads to the release of proinflammatory cytokines and chemokines, resulting in the destruction of β -cells (50–52). Several studies have focused on the autoimmune processes involved in pathogenesis, which are described in depth in the literature (7, 8). On the other hand, recent studies indicate that, in addition to this mechanism, altered intestinal permeability, impaired barrier function, and the intestinal microbiota can significantly influence the development of diabetes (13, 14).

Changes in the intestinal mucosa, such as reduced cytokine production (IL-17A, IL-22, and IL-23A) and shifts in microbiota composition (e.g., a decrease in SFB), have been observed in various mouse models of T1D. These changes do not seem to depend solely on hyperglycaemia but are associated with intestinal inflammation and compromised epithelial cell integrity (13, 14).

Studies suggest that treating intestinal inflammation could restore the microbiota balance and improve the condition of intestinal epithelial cells, potentially positively influencing T1D. In particular, the use of anti-TNF α therapies, which are common in inflammatory bowel diseases such as Crohn's disease, has been shown to reduce intestinal inflammation and improve diabetic pathology (14).

Diabetes disrupts intestinal homeostasis through a mechanism mediated by insulin-like growth factor binding protein 3 (IGFBP3). Chronic hyperglycaemia and inflammation associated with diabetes alter the liver's release of factors, including reducing insulin-like growth factor 1 (IGF-I) and increasing IGFBP3. This excessive IGFBP3 targets crypts in the small and large intestines, leading to the apoptosis of intestinal stem cells (ISCs). The remaining ISCs fail to differentiate into other cell types, such as enterocytes, enteroendocrine cells, and goblet cells, resulting in impaired intestinal crypt turnover and mucosal atrophy. This condition causes malabsorption, worsening hyperglycaemia and inflammation and establishing a feedback loop that further disrupts intestinal homeostasis (13, 14).

PTPs are crucial regulators of cellular signalling pathways and have been implicated also in the pathogenesis of T1D. PTPs serve as pivotal negative regulators of multiple signalling pathways, influencing both immune responses and glucose metabolism (8, 53–59). Understanding the specific roles and mechanisms of different PTPs offers valuable insights into their potential as targets for therapeutic strategies aimed at modulating autoimmune responses and managing T1D.

The loss of PTPN2 function seems to be associated with increased intestinal permeability and increased expression of proinflammatory mediators, indicating that PTPN2 plays a protective role in maintaining intestinal barrier integrity and regulating the inflammatory response. A polymorphism in PTPN2 rs2542151 GT or GG genotype is associated with an earlier onset of type 1 diabetes (60). In addition, the PTPN2 rs2542151 has been linked to a higher prevalence of type 2 diabetes mellitus (T2DM) and greater severity of non-alcoholic fatty liver disease (NAFLD) (59). PTPN2 is known for its role as a negative regulator of IFN- γ -mediated proinflammatory signalling. IFN- γ increases intestinal permeability and promotes inflammation. This is a process that PTPN2 appears to counteract by limiting the expression of proteins such as claudin-2, which is involved in the formation of pores in the epithelial barrier. These changes may facilitate the access of intestinal antigens to the immune system, contributing to chronic inflammation and insulin resistance (59). A recent study revealed that reduced expression of the T1D candidate gene PTPN2 exacerbates damage caused by IFN- α and TNF- α . PTPN2 seems to provide protection against both factors, revealing a shared downstream signalling pathway between them. Inhibition

of PTPN2 also impairs PDX1 (pancreatic and duodenal homeobox 1) expression and insulin production after TNF- α exposure (61).

PTPN2 is a crucial regulator of the harmful effects of TNF- α in human β -cells, suggesting that patients with PTPN2 risk polymorphisms may benefit from therapies targeting TNF- α . PTPN2 may also influence T1D development through its role in regulating intestinal permeability. Further research is needed to explore the specific molecular mechanisms underlying T1D progression, which could provide new perspectives for therapeutic intervention and disease management (61) (Fig. 2 and Tab.1).

4. PTP and Celiac Disease

CeD is a multifactorial disease in which environmental triggers combined with genetic predispositions determine its onset. Repeated GWASs (genome wide association studies) were able to identify 39 non-HLA (Human Leukocyte Antigens) susceptibility loci associated with CeD, and together, these loci can explain approximately 10–14% of the CeD heritability (62). Combined with the HLA locus, only 50% of the CeD heritability is explained. Among these 39 non-HLA loci (Rho involved in CeD), four were not directly involved in immunological processes. C1ORF106 (C1 Orfan 106), ARHGAP31 (GTPase activating protein 31), LPP (lipoma-preferred partner) and PTPRK (protein tyrosine phosphatase receptor type K) play roles in actin rearrangement in the cytoskeleton, in the cell-cell adhesion process and in the maintenance of intestinal epithelial barrier function (62). PTPRK also regulates EGFR (epidermal growth factor receptor) signalling through dephosphorylation of catalytic sites and inactivation of the receptor.

The PTPRK gene is located on the long arm of chromosome 6 (63). PTPRK mRNA is widely expressed; except in immune cells, skeletal muscles, and testes, and it is upregulated by the transforming growth factor β (TGF- β) signal transduction pathway.

PTPRK is a receptor-type PTP that consists of an extracellular region, a single transmembrane region and two tandem catalytic domains (64). Many functions are performed by the PTPRK gene (65–69) including regulation of EGFR activation (65, 66, 70). Altered regulation of EGFR has been shown to promote uncontrolled cell proliferation, angiogenesis and metastasis development. Proliferation is also a marker of CeD pathogenesis, mainly through the signalling of EGFR (71). In biopsies and fibroblasts from celiac patients, there is a delay in EGFR trafficking in early endocytic vesicles such that the receptor remains activated longer than that in controls (72). Gliadin further delays the endocytic pathway and can prolong the activation of the EGFR/ERK (Extracellular signal-regulated kinase) signalling pathway and cell proliferation in biopsies from controls and celiac patients (73, 74). Elevated activation of the EGFR/MAPK pathway is also found in the enterocytes of celiac patients, resulting in alterations in the cytoskeleton. Furthermore, altered expression of genes involved in autophagy and the regulation of the EGFR pathway is also observed in CeD patients (72).

The effects of PTPRK in regulating the proliferation of enterocytes and keratinocytes have also been studied via biopsies and organoids (9, 75, 76). PTPRK phosphatase expression levels are reduced in biopsies from celiac patients with a gluten-containing diet (GCD) and from potential celiac patients (Pot-CeD), whereas EGFR phosphorylation, ERK and cell proliferation levels are increased compared with those in biopsies from controls (CTRs). These findings suggest that the reduction in PTPRK protein expression levels is independent of intestinal villous atrophy (9).

Another study found a region on chromosome 6 through GWAS that includes two genes, the THEMIS gene, which codes for a molecule involved in the selection of thymocytes, and the PTPRK gene. Both genes perform immuno-related functions and are involved in

the pathogenesis of CeD. Gene expression analysis of duodenal biopsies from celiac patients revealed higher mRNA levels of the THEMIS gene than those in samples taken from the same patients after gluten-free diet (GFD) treatment or from CTRs. These findings point to the correlation between low PTPRK expression levels in the CeD and increased permeability of the intestinal barrier, and PTPRK inhibition may promote enterocyte hyperproliferation in intestinal crypts. Moreover, compared with CTR patients, CeD patients have similar mRNA levels, indicating that adhesion to a GFD is sufficient to restore the transcriptional activity of the THEMIS and PTPRK genes to normal levels (76).

Altered function of the intestinal epithelium plays an important role in the pathogenesis of inflammatory diseases (77). Intestinal organoids, derived from intestinal stem cells, recreate the crypt/villus axis in vitro, allowing the role of the intestinal epithelium in various pathologies to be studied (78-82). CeD intestinal organoids reproduce the low-grade inflammation of CeD intestinal biopsies (83). In CeD organoids, PTPRK low levels are associated with EGFR/ERK increased phosphorylation and proliferation. Nanayakkara et al. conducted functional experiments on intestinal organoids from CeD patients and controls to study the role of PTPRK in intestinal epithelial cells proliferation. In control organoids, the silencing PTPRK induces the increase of the phosphorylation of EGFR and ERK and proliferation. On the other hand, overexpressing PTPRK in CeD organoids was able to reduce the phosphorylation of EGFR and ERK and proliferation to normal levels. PTPRK expression in intestinal organoids from CeD patients and controls can modulate the phosphorylation of EGFR and ERK and proliferation (9) (Fig. 2-3 and Tab. 1).

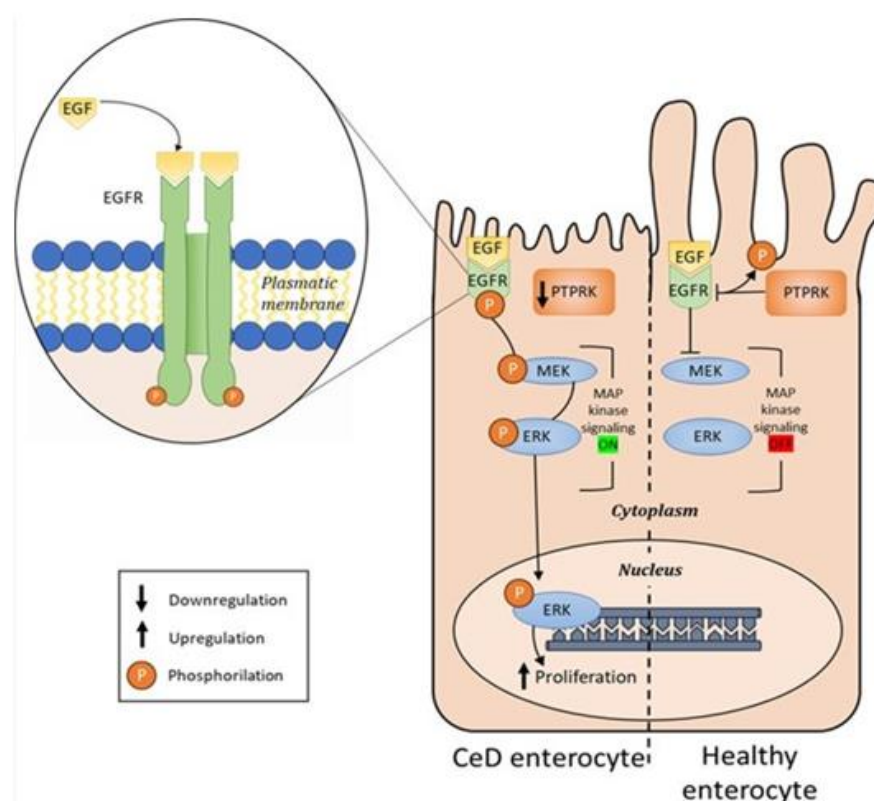


Figure 3 Schematic representation of CeD (left) and healthy (right) enterocytes with a description of the role of PTPRK in the regulation of the EGF pathway. In CeD enterocytes, with respect to healthy enterocytes, the downregulation of PTPRK leads to the upregulation of EGFR, and MAP-kinase signalling is always switched ON, increasing proliferation.

PTP protein	Disease	Alteration in the disease	Models of study	Function at the level of the intestine	Reference
PTPN22	CD-UC	Loss-of-function	Mice (22, 23,25); Cell culture (26)	1) PTPN22 has a dual context-dependent role: -in acute gut inflammation PTPN22 together with IL-10 is repressor of inflammatory response; PTPN22 minus IL-10 is promoter. -during chronic inflammation PTPN22 promotes gut inflammation irrespective of the presence of IL-10 2) protect from systemic and gastrointestinal inflammation 3) autophagy regulation 4) inflammasoma regulation	(1,4,18-28)
PTPN2	CD-UC	Loss-of-function	Mice (29,31,32) Intestinal epithelial cell (IEC) lines (T84;HT29cl.19a) (33,34) Intestinal biopsy (33)	INTESTINAL HOMEOSTASIS -regulation of proinflammatory cytokine secretion -autophagy -intestinal epithelial barrier function -Paneth Cells regulation -autophagy regulation -inflammasoma activation -gut microbiota homeostasis	(12,29-35)
PTPN2	Diabetes	Minor allele frequency	Patients	Involved in many leaky gut associated diseases	(57-59)
PTPN11	UC	Up-regulated and Down-regulated	Mice (38)(38)	Homeostasis of IECs of absorptive enterocytes and goblet cells; microbiota equilibrium; positive regulator of MAPK signaling pathway, regulation of cell proliferation and differentiation	(37-41)
PTPσ	UC	Down-regulated	Mice (42)	PTPσ is a regulator of mucosal inflammation, intestinal epithelial development and permeability	(42,43)
PTPN6	UC	Down-regulated	Patients	PTPN6 is associated with increased proliferation and altered goblet and Paneth cell differentiation	(44,45)
DUSP2	UC	Low levels (mRNA)	Mice (46)	DUSP2 is a regulator of mucosal inflammation and colonic ulceration	(46,47)
DUSP6	UC	Down-regulated	Mice; (47) HT29 and HCT116 colorectal carcinoma cells (47)	DUSP6 is a modulator of CD4+ T cell activation and epithelial cell proliferation	(48,49)
PTPRK	CeD	Down-regulated	Intestinal organoids (9)	PTPRK is a regulator of epithelial inflammation and enterocytes proliferation	(9,65,66,70,76)

Table 1. PTP proteins involved in inflammatory intestinal diseases and their function at the level of the intestine investigated in several study models.

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5. Conclusions

In conclusion, the involvement of PTP phosphatases in diseases such as IBD, T1D and CeD implicates the nodal role of the signalling mediated by phosphotyrosines in these diseases. PTPs are signalling molecules that operate in almost all cells of the body and are able to regulate a variety of cellular processes, such as cell growth, differentiation, the cell cycle and oncogenic transformation. The generation of different diseases is likely due to the fragility of specific cell tissues induced by a combination of exogenous and endogenous factors. Moreover, these data point to an emerging role of the intestinal epithelium not only in inflammatory intestinal chronic diseases, including IBD and CeD but also in T1D, where PTP alterations have been found both at the level of pancreas β -cells and at the intestinal epithelial cells. Taken together, these data link IBD, T1D and CeD, which were previously regarded as unrelated and mediated by independent pathogenetic events.

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Article

Gliadin Peptide P31-43 Induces mTOR/NFκβ Activation and Reduces Autophagy: The Role of *Lactobacillus paracasei* CBA L74 Postbiotic

Mariangela Conte ¹, Federica Nigro ², Monia Porpora ¹, Claudia Bellomo ¹ , Francesca Furone ¹, Andrea Luigi Budelli ³ , Roberto Nigro ³ , Maria Vittoria Barone ^{1,*} and Merlin Nanayakkara ¹

¹ ELFID (European Laboratory for the Investigation of Food Induced Diseases), Department of Translational Medical Science, Section of Paediatrics, University Federico II, Via S. Pansini 5, 80131 Naples, Italy; maryconte_92@hotmail.it (M.C.); monia.porpora@hotmail.it (M.P.); bellomo.claudia96@gmail.com (C.B.); francescafurone@gmail.com (F.F.); merlinnanayakkara@gmail.com (M.N.)

² I.T.P. Innovation and Technology Provider s.r.l., Via Bisignano a Chiaia 68, 80121 Naples, Italy; federica.nigro@itpna.it

³ DICMAPI, University of Naples Federico II, 80125 Naples, Italy; andrealuigi.budelli@unina.it (A.L.B.); rnigro@unina.it (R.N.)

* Correspondence: mv.barone@unina.it; Tel.: +39-0817464568

Abstract: Celiac disease (CD) is an autoimmune disease characterized by an altered immune response stimulated by gliadin peptides that are not digested and cause damage to the intestinal mucosa. The aim of this study was to investigate whether the postbiotic *Lactobacillus paracasei* (LP) could prevent the action of gliadin peptides on mTOR, autophagy, and the inflammatory response. Most of the experiments performed were conducted on intestinal epithelial cells Caco-2 treated with a peptic-tryptic digest of gliadin (PTG) and P31-43. Furthermore, we pretreated the Caco-2 with the postbiotic LP before treatment with the previously described stimuli. In both cases, we evaluated the levels of pmTOR, p70S6k, and p4EBP-1 for the mTOR pathway, pNFκβ, and pERK for inflammation and LC 3 and p62 for autophagy. For autophagy, we also used immunofluorescence analysis. Using intestinal organoids derivate from celiac (CD) patients, we analyzed the effect of gliadin after postbiotic pretreatment with LP on inflammation marker NFκβ. Through these experiments, we showed that gliadin peptides are able to induce the increase of the inflammatory response in a more complex model of intestinal epithelial cells. LP postbiotic was able to induce autophagy in Caco-2 cells and prevent gliadin effects. In conclusion, postbiotic pretreatment with LP could be considered for in vivo clinical trials.

Keywords: celiac disease (CD); gliadin peptide P31-43; mTOR/NFκβ activation; *Lactobacillus paracasei* CBA L74 postbiotic



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1. Introduction

The gut is the largest immune organ in the body [1]. Approximately 100 trillion bacteria are associated with our gastrointestinal tract. The microbiota is considered a “super-organism” and is an integral part of the gastrointestinal tract [2]

Numerous functions are attributed to the microbiota in the human gut; in fact, it competes (for space and nutrients) with potential pathogens, induces the secretion of antimicrobial peptides, and stimulates differentiation and proliferation through interaction with intestinal epithelial cells [3,4], which, in turn, regulate intestinal homeostasis [5–7]. In addition, they modulate the inflammatory response induced by nutrient excess as nutrients are modulators of various cellular functions and may be involved in tissue immune response and inflammation.

Celiac disease (CD) is a systemic immune-mediated disease caused by gluten and related prolamins contained in wheat, barley, and rye. It occurs in genetically predisposed

individuals and is characterized by a variable combination of gluten-dependent clinical manifestations, the presence of specific autoantibodies in serum, positivity for HLA DQ2, and/or DQ8 haplotypes and enteropathy. To date, the gluten-free diet represents the only therapeutic option for these patients, who must eliminate gluten-containing grains from their diet for life.

Microbial dysbiosis is widely reported in CD patients [8,9]. The activation of innate immunity by gliadin peptides is an important component of the early events of the disease. In particular, the “toxic” A-gliadin peptide P31-43 induces several pleiotropic effects, including epidermal growth factor receptor (EGFR)-dependent actin remodelling and proliferation in cultured cell lines and enterocytes from CD patients and inflammation [10,11].

The mechanistic target of the rapamycin (mTOR) network is an evolutionarily conserved signaling hub that senses and integrates environmental and intracellular nutrient and growth factor signals to coordinate basic cellular and organismal responses such as cell growth, proliferation, apoptosis, and inflammation [12]. Gliadin sustains the mTOR inflammatory response in celiac disease [13]. Moreover, gliadin-derived peptides are able to stimulate enterocytes [14,15] causing the upregulation of proinflammatory cytokine expression such as the activation of the nuclear transcription factor- κ B (NF κ B) pathway in the small intestinal mucosa of CD patients [16].

Autophagy is crucial for cytoplasmic recycling, fundamental homeostasis, and cell survival [17,18]. It is also an essential component of immune defense against bacterial pathogens such as *Mycobacterium tuberculosis*, *Salmonella enteric*, and *Escherichia coli* [19–21]. Thus, triggering autophagy is essential for cell survival during pathogen infection [22,23]. The induction of autophagy involves numerous proteins and multiple signaling pathways. Microtubule-associated light chain 3 protein 1 (LC3, a mammalian homolog of yeast Atg8), vital for autophagosome formation, is considered one of the markers of autophagy [24]. P62 (also known as SQSTM1), is a marker of autophagic flux, is selectively incorporated into autophagosomes, and is efficiently degraded by autophagy [25].

Recent interest was generated by the use of prebiotics, probiotics, and postbiotics to modify the intestinal microbiome. Major interest is focused mainly on the use of postbiotics, a substance released by or produced through the metabolic activity of the microorganism in the gut when feeding on fibrous compounds, which exerts a beneficial effect on the host, either directly or indirectly. The term postbiotic refers to compounds such as protein compounds, hydrogen peroxide (H₂O₂), bacteriocins, organic acid, EPS, and enzymes [26,27]. These compounds have positive antimicrobial, anticancer, immunomodulation, antioxidant, antihypertensive, cholesterol-lowering, and antiproliferative properties [28–32]. Presently, scientific literature confirms that postbiotic components can be used as promising tools for prevention and treatment strategies in gastrointestinal disorders with less undesirable side effects, particularly in infants and children [33].

In this paper, we analyzed the effect of the gliadin peptide P31-43 on the mTOR and autophagy pathways and the ability of the LP CBA L74 (LP) postbiotic to prevent these effects in Caco-2 cells, an intestinal epithelial cell line, and organoids from intestinal biopsies of CD patients

2. Results

2.1. Gliadin Peptides Were Able to Induce mTOR, p70S6 Kinase and 4EBP Phosphorylation

The mammalian/mechanistic target of rapamycin (mTOR) is a serine/threonine kinase that integrates a multitude of extracellular signals and intracellular cues to drive growth and proliferation.

We investigated the effects of the gliadin peptide P31-43 and PTG (peptic-tryptic digest of gliadin) on the mTOR and autophagy pathway in Caco-2 cells, an intestinal epithelial cell line that is responsive to gliadin. To determine whether the mTOR pathway is active in Caco-2 cells, we analyzed them before and after treatment with PTG and p31-43, the phosphorylated/active form of mTOR and downstream targets p70S6 Kinase 1 (S6K1) and eIF4E binding protein (4EBP), by Western blot.

p-mTOR, p-S6K1, and p-4EBP were increased with respect to untreated cells (NT) after treatment with both p31-43 and PTG. Pretreatment with LP postbiotic was able to prevent the activation of the mTOR pathway in Caco-2 cells (Figure 1).

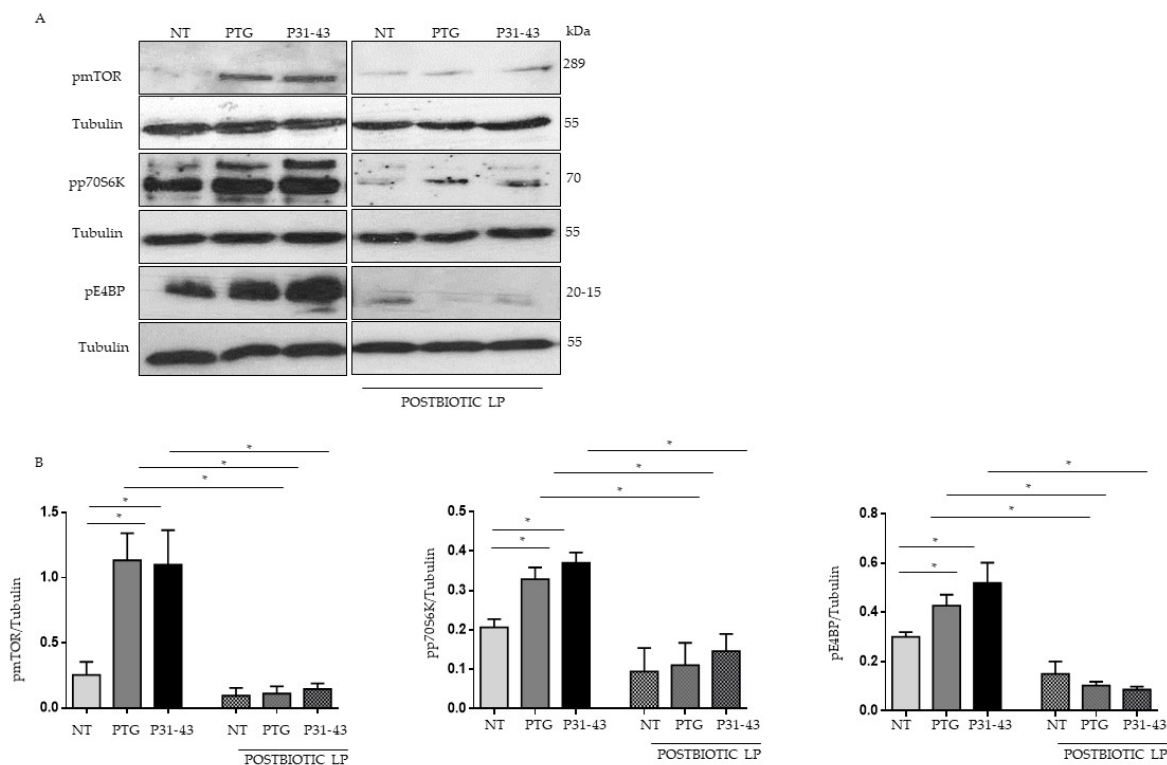


Figure 1. LP postbiotic prevents the gliadin-induced activation of the mTOR pathway in Caco-2 cells. (A) Western blot analysis of protein lysates from Caco-2 cells untreated (NT), treated with PTG and P31-43 for 30 min, and pretreated with LP postbiotic for 1 h were blotted with antibodies against pmTOR, pp70S6k, and p4EBP. Tubulin was used as a loading control. The immunoblotting analysis was representative of three independent experiments. (B) Densitometric analysis of bands from WB as in A. Columns represent the mean, bars the standard deviation of the relative intensity of pmTOR, pp70S6k, and p4EBP with respect to total tubulin protein. Student's *t*-test = * $p < 0.05$.

2.2. Gliadin Peptides Reduced the Levels of LC3 and Increased the Levels p62 Autophagy Markers

In the presence of nutrient restriction, mTOR is generally dephosphorylated, and autophagy is induced. The opposite is true in the presence of nutrients. For these reasons, we evaluated the autophagy pathway in Caco-2 cells after treatment with P31-43 and PTG.

Autophagy markers LC3II and p62 protein, a protein necessary for autophagy progression, were evaluated before and after treatment with PTG and P31-43 for 3 (Supplementary Materials Figure S2) and 24 h (Figure 2) by Western blotting in Caco-2 cells. As described by Manai et al. [34], we observed a reduction of LC3II levels and an increase of p62 that was statistically significant only after 24 h of treatment with PTG and P31-43. All this indicates that both the PTG and P31-43 can reduce autophagy and its progression. Interestingly, pretreatment with postbiotic LP prevents the effects of gliadin peptides on LC3 (Figure 2A–C) and p62 levels (Figure 2B–D). Moreover, these data were also confirmed by immunofluorescence (Figure 2E).

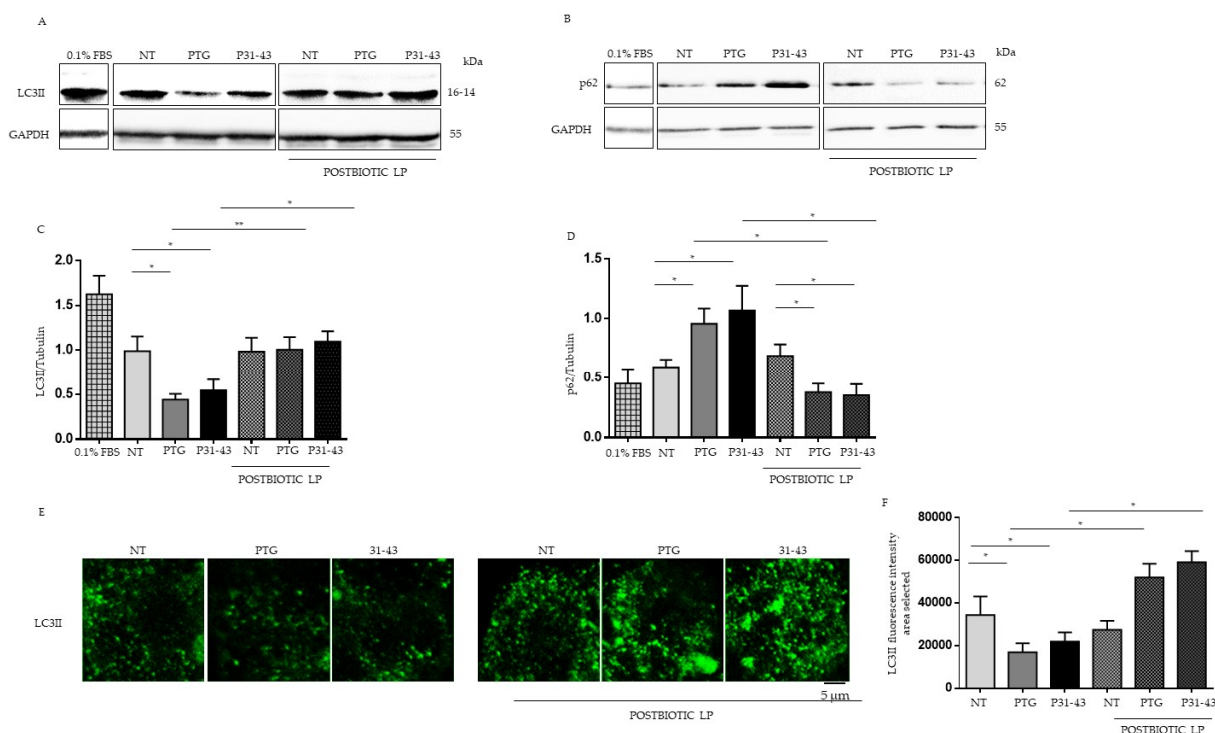


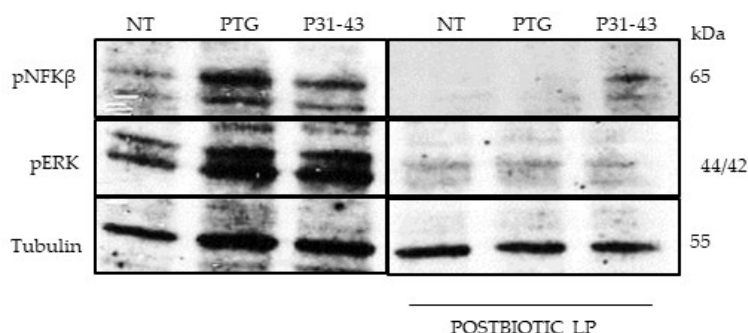
Figure 2. Pretreatment with LP postbiotic increased LC3II expression after treatment with PTG and P31-43. **(A,B)** Western blot analysis of protein lysates from Caco-2 cells untreated (NT), treated with PTG and P31-43 for 24 h, and pretreated with LP postbiotic were blotted with antibodies against LC3II and p62. GAPDH was used as a loading control. As a positive control of autophagy induction, the cells were starved with serum at 0.1% FBS. The immunoblotting analysis was representative of three independent experiments. **(C,D)** Densitometric analysis of bands from WB as in A. Columns represent the mean, bars the standard deviation of the relative intensity of LC3II and p62 with respect to total tubulin protein. Student's *t*-test = * $p < 0.05$, ** $p < 0.01$. **(E)** Immunofluorescence analysis of anti-LC3II from untreated Caco-2 cells and Caco-2 cells treated with PTG and P31-43 for 24 h and pretreated with LP. Images obtained using a 63× objective (two times digital zoom) were shown. The white bar represents 5 micrometers. **(F)** Statistical analysis of fluorescence intensity of LC3II in the area selected. Student's *t*-test = * $p < 0.05$.

In addition, we tested the effect of rapamycin, an inhibitor of mTORC1, on the mTOR/autophagy pathways both in the presence and absence of P31-43 in CaCo-2 cells by Western blotting. Interestingly, rapamycin was able to inhibit the mTOR pathway and increase autophagy (Supplementary Materials Figure S1).

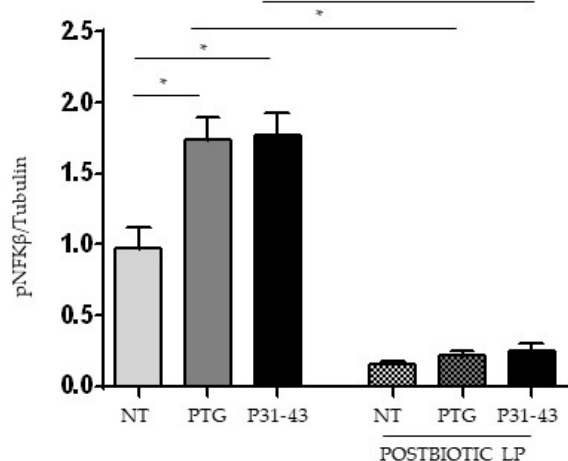
2.3. Pretreatment with Postbiotic from LP Decreased NFK β and ERK Phosphorylation Induced by Treatment with P31-43 and PTG

Both PTG and P31-43 are able to activate NFK β [15,35], and PTG is described to activate mTOR [13]. MAPKs are involved in I κ B-dependent NFK β activation [36]. We tested the effect of the LP postbiotic on PTG, and P31-43 inflammation markers increased. For this purpose, Caco-2 cells were pretreated with LP postbiotics for 1 h, and after being stimulated with PTG and P31-43, the activation of NFK β and ERK was evaluated by WB. Our data showed an increase in NFK β phosphorylation levels after stimulation with PTG and P31-43. The postbiotics of LP reduced this phosphorylation (Figure 3A,B). Additionally, ERK activation was increased after PTG and P31-43 treatment [15] and, again, pretreatment with postbiotics of LP prevented ERK activation (Figure 3A,C).

A



B



C

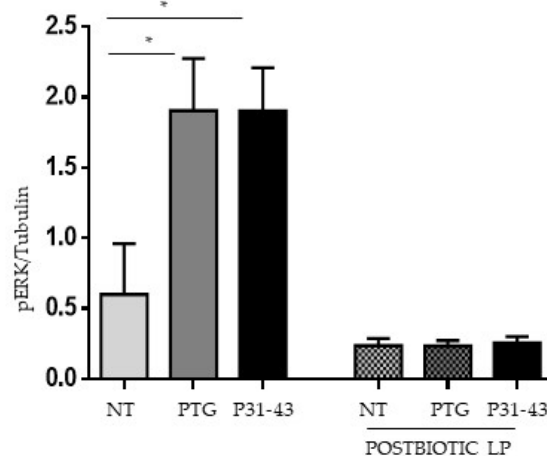


Figure 3. Pretreatment with LP decreased NFK β and ERK phosphorylation after treatment with P31-43 and PTG. (A) Western blot analysis of protein lysates from Caco-2 cells untreated (NT), treated with PTG and P31-43 for 30 min, and pretreated with LP postbiotic for 1 h were blotted with antibodies against pNFK β and pERK. Tubulin was used as a loading control. The immunoblotting analysis was representative of three independent experiments. (B,C) Densitometric analysis of bands from WB as in A. Columns represent the mean, bars the standard deviation of the relative intensity of pNFK β and pERK with respect to total tubulin protein. Student's *t*-test = * *p* < 0.05.

2.4. In CD Intestinal Organoids *Lactobacillus paracasei* Decreased NFK- β Phosphorylation and Prevented P31-43 Effects

To test the postbiotic LP in a more complex and physiologic cell model, we used intestinal organoids derived from CD patients in the active phase of the disease and control subjects. Organoids were derived from intestinal staminal cells and were cultivated for 4 weeks in 3D before opening them in 2D to allow treatment on the apical part of the epithelial cells.

Intestinal organoids from CD patients were inflamed [37] with respect to the controls, as shown in Figure 4A,B. In these conditions, we tested the effect of the LP postbiotic. Interestingly the LP postbiotic was able to reduce the constitutive inflammation present in CD organoids.

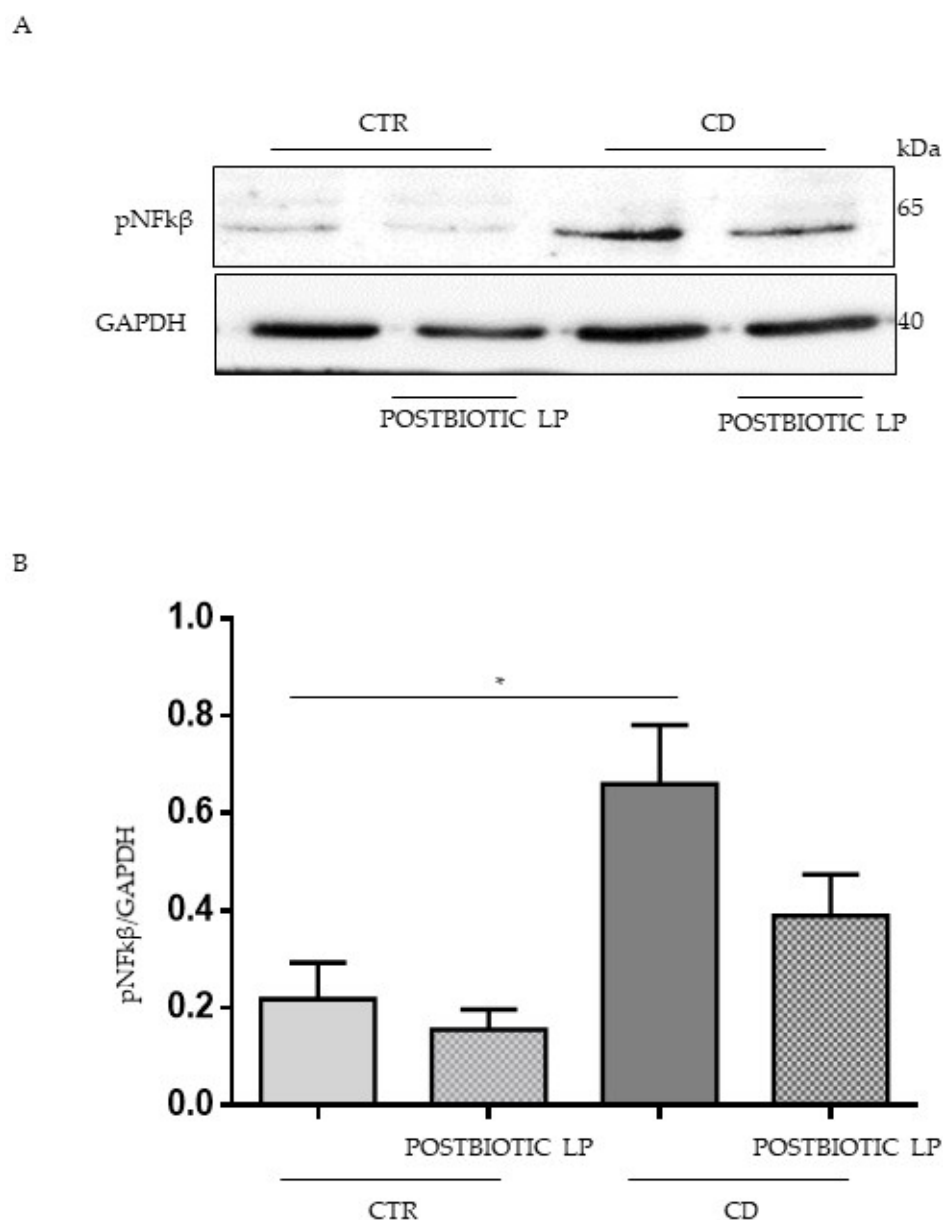


Figure 4. Inflammation in CD organoids can be prevented by LP postbiotic. **(A)** Western blot analysis of protein lysates from organoids from CD patients and controls, treated with LP postbiotic for 3 h were blotted with antibodies against NFκβ. GAPDH was used as a loading control. The number of subjects investigated was three. **(B)** Densitometric analysis of bands from WB. Columns represent the mean, bars the standard deviation of the relative intensity of NFκβ with respect to total tubulin protein. Student's *t*-test = * $p < 0.05$.

Next, we tested the effect of P31-43 on CD organoids using as a read out of the inflammation the phosphorylation of NFκβ. As shown in Figure 5A,B P31-43 caused a statistically significant increase in NFκβ phosphorylation; this effect was already described before [15]. LP postbiotic was able to prevent the increase of NFκβ phosphorylation induced by P31-43 on CD organoids.

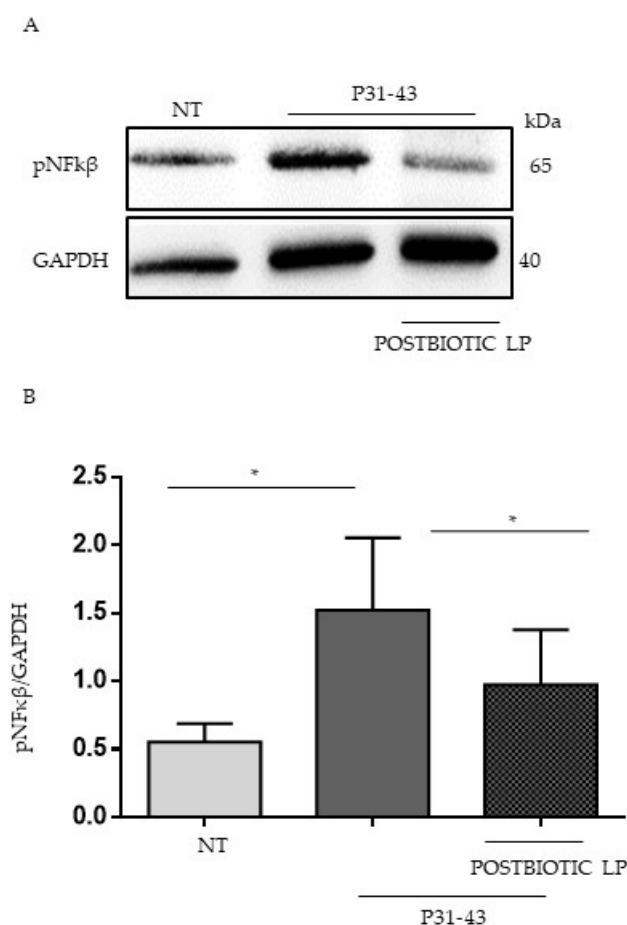


Figure 5. (A) Western blot analysis of protein lysates from organoids from CD patients, treated with P31-43 for 1 h and pretreated with LP postbiotic for 2 h, were blotted with antibodies against NFκβ. Tubulin was used as a loading control. The immunoblotting analysis was representative of three independent experiments. The number of subjects investigated is indicated. (B) Densitometric analysis of bands from WB. Columns represent the mean, bars the standard deviation of the relative intensity of NFκβ with respect to total tubulin protein. Student's *t*-test = * *p* < 0.05.

3. Discussion

In this study, we investigated the effects of gliadin on the mTOR/autophagy pathway and inflammation in epithelial cells and intestinal organoids from CD patients. Moreover, we evaluated the ability of the postbiotic *Lactobacillus paracasei* (LP) to prevent these effects.

mTOR is a tyrosine kinase that is able to regulate cell proliferation and inhibit the autophagy pathway. It is regulated by signals, such as growth factors and nutrients, that drive cell growth and proliferation [38]. The autophagy pathway is triggered when mTOR is dephosphorylated/inactivated. It is a pathway closely connected with cell regeneration and consists of an auto-cell digestion process generally activated to remove damaged macromolecules and organelles in order to maintain cellular homeostasis [39]. The activation of mTOR and the consequent deactivation of the autophagy pathway causes the induction of inflammatory markers such as NFκβ [40].

Caco-2 cells, intestinal epithelial cells derived from colon carcinoma and responsive to gliadin, were used to study the effects of gliadin on the mTOR and autophagy pathway. Both the P31-43 peptide and peptic-tryptic digest of gliadin (PTG) were able to activate mTOR and the downstream pathway by activation of the E4BP and 70S6k proteins. Since the activation of mTOR induces a reduction of autophagy [39], we investigated the activity of P31-43 and PTG on autophagy itself. We showed that in the presence of PTG and P31-43, there was a reduction of LC3-positive vesicles and the autophagic flux to the lysosomes

was decreased, as demonstrated by the increase in p62 only after 24 h of treatment [34]. Taken together, these data demonstrate that P31-43 and PTG induce a reduction in both autophagic vesicles and their flux to lysosomes. Interestingly, rapamycin was able to inhibit the mTOR pathway and increase autophagy. Furthermore, P31-43 and PTG were able to activate NF κ B in Caco-2 cells

Recent studies investigated the ability of probiotics to prevent the effects of gliadin *in vivo*. Their effects were studied both in subjects with celiac disease (CD) and in subjects with potential celiac disease [28]. Probiotics seem to have a good effect on CD symptoms when they are present, but they do not seem to have effects on the intestinal lesion.

Taken together, the data on the probiotic effects on CD indicates that the use of probiotics can act on gastrointestinal symptoms, giving an ameliorative effect, but have little effect on the natural history of the disease [28].

More recently, the interest of the scientific world is focused on postbiotics. A postbiotic is defined as the “preparation of inanimate microorganisms and/or their components: soluble factors secreted by live bacteria or released after their lysis, including various cell surface components, lactic acid, short-chain fatty acids (SCFA), and bioactive peptides that confer a health benefit on the host [41].

At present, no reports are available regarding the use of postbiotics in CD. For this reason, in this study, we investigated the ability of LP postbiotics to prevent the effects of gliadin and P31-43 peptide on mTOR/autophagy pathways and inflammation in Caco-2 cells. Moreover, we used intestinal organoids from CD patients to test the LP postbiotic effect on inflammation in a more complex and physiologic cell model.

We showed that after pretreatment with the postbiotic LP, both the peptide P31-43 and the PTG effects on mTOR activity and the downstream pathway were prevented. Moreover, pNF κ B was also reduced in the presence of LP postbiotic and PTG or P31-43.

We investigated the activity of P31-43 and PTG on Caco-2 cells pretreated with postbiotic LP on autophagy, demonstrating an increase in autophagy vesicles with the increase in the LC 3 marker and of the autophagic flux to the lysosomes with the reduction of p62.

Although postbiotic LP was able to inhibit the PTG and P31-43 stimulated effects, it is important to note that it also had an effect on the untreated sample. This indicates that the postbiotic LP effect could be independent of, and dominant over the PTG, P31-43 activities. On the other hand, the mechanisms of this activity are not clear, although a possible effect on the intracellular trafficking of the gliadin peptides and also other proinflammatory agents, such as bacteria, cannot be ruled out [9,42].

Recently, Freire R et al., using intestinal organoids developed from duodenal biopsies from both non-celiac (NC) and celiac (CD) patients, analyzed the role of microbiota-derived molecules in modulating the epithelium's response to gluten. They selected three bacterial bioproducts: butyrate, lactate, and PSA derived from *Bacteroides fragilis*. All bioproducts exerted a global protective effect by reducing the proinflammatory cytokine secretion triggered by PTG [43]. Using intestinal organoids derived from celiac (CD) patients, we analyzed the effect of P31-43 after pretreatment with LP postbiotic on inflammatory marker NF κ B, demonstrating a reduction. The use *in vitro* of patient-derived organoids to model CD pathogenesis could be a novel tool to further study CD treatment and prevention.

In conclusion, the LP postbiotic is able to prevent the effects of gliadin peptides on the pathway of mTOR, autophagy, and NF κ B *in vitro*.

These pre-clinical studies are a good basis for activating clinical trials in celiac patients to prevent the proinflammatory effects of gliadin peptides. In particular, it would be interesting to test the effect of the postbiotic for its ability to prevent disease in potential subjects who have anti-transglutaminase antibodies but have not yet developed the intestinal lesion typical of celiac disease. However, more studies are required to investigate the related safety parameters and biological activity of postbiotics *in vivo*.

Currently, the only available treatment for a patient with celiac disease is a strict gluten-free diet. Despite patients' best efforts, some subjects can result in continuous exposure due to the cross-contamination or traces of gluten in food. These risks could,

in some cases, compromise the health and quality of life of these subjects. It is, therefore, generally useful to study compounds that can prevent the inflammatory effects of gliadin with the hope of reducing the burden of living with celiac disease and improving long-term health outcomes.

4. Materials and Methods

4.1. Cell Cultures and Treatments

Human colon adenocarcinoma-derived cells (Caco-2) obtained from Interlab Cell Line Collection (Centro di Biotecnologie Avanzate, Genoa, Italy) were grown in DMEM supplemented with 10% fetal calf serum, 100 units penicillin-streptomycin/mL, and 1 mmol/L glutamine (all these products are Gibco Invitrogen, Milan, Italy). As a positive control of autophagy induction, the cells were starved with serum at 0.1% FBS. Cells were maintained in a humidified atmosphere (95%) of air and 5% CO₂ at 37 °C. LPS-free synthetic peptide P31-43 (Inbios, >95% purity, MALDI-toff analysis as expected) was obtained by Ultrasart-D20 (Sartorius AG, Goettingen, Germany) filtration. LPS levels were below detection (<0.20 EU/mg), assessed by a commercial QCL-1000 kit (Cambrex Corporation, NJ, USA). P31-43 sequence: LGQQQPFPPQQPY. Peptic-tryptic digests of A gliadin were obtained as described before [44]. The alcohol-soluble protein fraction was extracted from whole flour from wheat (*Triticum aestivum*, variety Sagittario) and then submitted to digestion as previously described [44]. Prolamin peptic-tryptic (PT) digests indicated as PTG were freeze-dried, lyophilized, and stored at −20 °C until used.

Gliadin peptides were used in the following concentrations: peptic-tryptic digests of gliadin (PTG) 500 µg/mL and P31-43 peptide were used at 100 µg/mL.

4.2. Organoids

One to two duodenal biopsies per individual were taken with standard endoscopic EGDS during routine gastroduodenoscopy (Table 1) and placed in an ice-cold 10 mL isolation buffer (5.6 mmol/L Na₂HPO₄ (Sigma S7907; Sigma-Aldrich, Milan, Italy), 8.0 mmol/L KH₂PO₄ (Sigma P5655; Sigma-Aldrich), 96.2 mmol/L NaCl (Sigma S5886; Sigma-Aldrich), 1.6 mmol/L KCl (Sigma P5405; Sigma-Aldrich), 43.4 mmol/L sucrose (Fisher BP220-1; Thermo Fisher Scientific, Waltham, MA, USA), and 54.9 mmol/L D-sorbitol (Fisher BP439-500; Thermo Fisher Scientific Milan, Italy) in deionized water. Crypt units were isolated according to the protocol of Yuli Wang et al. [45] with minor variations. Briefly, after 60 min, the biopsy samples were further enzymatically digested with collagenase (2 mg/mL, Sigma-Aldrich, Milan, Italy) in a washing buffer (WB) containing penicillin (100 units mL^{−1}), streptomycin (0.1 mg mL^{−1}), l-glutamine (2 mM), and FBS (10%, *v/v*) in DMEM/F12 with HEPES on ice for 30 min. The digest was filtered through a 70 µm strainer (Falcon, Milan, Italy), and the strainer was rinsed with an additional 10 mL of WB. Crypts were collected by centrifugation at 500× *g* for 5 min. The supernatant was discarded, the crypts were carefully resuspended in 40 µL of ice-cold Matrigel matrix (Corning 35623, Milan, Italy) to enable three-dimensional growth in 48-well plates; the plates were incubated in a cell culture incubator at 37 °C and 5% carbon dioxide for 10 min to allow the Matrigel to solidify. Afterwards, 300 µL cell culture medium enriched with supplements (CM-S) was added to each well and was replaced every second day. The organoids were used for assays or cryo-preserved at −150 °C. To cryopreserve organoids, they were washed with ice-cold PBS EDTA to remove Matrigel and collected by centrifugation at 500× *g* for 5 min. Organoid pellets were suspended in 1 mL WB, 10% faecal calf serum (FCS, Invitrogen, Milan, Italy), and 10% dimethyl sulfoxide, slowly frozen to −80 °C in a cryo freezing container (Nalgene, Milan, Italy), and then transferred to −150 °C for long-term storage. For further research, the cryopreserved organoids were quickly thawed at 37 °C, transferred to 10 mL WB, centrifuged at 2000× *g* for 5 min, plated with Matrigel, and cultured in CM-S medium. For 2D organoids, organoids were seeded in six wells pretreated with Matrigel diluted 1:40 in phosphate-buffered saline (PBS).

Table 1. Patient characteristics.

Patients	Range Age (Years)	Sex	Biopsy (Marsh Classification *)	Serum AntiTG2 (U/mL)	Anti-Endomysial Antibody (EMA)
Controls (N = 3)	10–18	M = 1, F = 2	3 = T0	0–1.5	Negative
GCD-CD (N = 3)	3–15	M = 1, F = 2	1 = T3 c 2 = T3 c/b	>50	Positive

* T0: Normal; T1: infiltrative lesion; T3: Flat destructive lesion (b: moderate, c: total).

4.3. Culture Medium to Maintain Organoids (CM-S)

Mouse L-cells that expressed Wnt3a, R-spondin, and Noggin were commercially purchased (ATCC CRL-3276, Genova, Italy), and a conditioned medium (L-WRN) was prepared according to the instructions and protocol of the manufacturer. Culture medium with supplements (CM-S) was prepared using 50% conditioned L-WRN medium and 50% fresh primary culture media: Advanced DMEM/F-12 (cat.12634-010, Invitrogen, Milan, Italy) 1 mM N-Acetyl-L-cysteine (cat. A7250, Sigma, Milan, Italy), 1 × B-27[®] supplements (cat.12587-010 Gibco, Milan, Italy), 50 ng/mL Epidermal Growth Factor (cat. PMG8041 Gibco Milan, Italy), 10 mM nicotinamide (cat. N0636, Sigma, Milan, Italy), 10 nM Leu15-gastrin I (cat. G9145 Sigma, Milan, Italy), 500 nM A8301 (inhibitor for ALK4/5/7, cat. 70024-90-7 Sigma, Milan, Italy), 10 μM SB202190 (p38 MAP kinase inhibitor, cat. S7076 Sigma, Milan, Italy), and 10 μM Y-27632 (p160 ROCK inhibitor; cat.1254 Tocris, Milan, Italy) in accordance with the protocols [45–47]. The organoids were cultured with a 300 μL culture medium, which was changed every second or third day.

4.4. Bacterial Growth Conditions

To prepare the postbiotic, we cultivated *L. paracasei*-CBA L74 in DMEM supplemented with 10% fetal calf serum and 1 mM glutamine until 10⁹ CFU/mL, as previously described [42,48]. The bacterial culture was then centrifuged at 3000 rpm for 10 min, and the supernatant was filtered through a 0 to 22 micron filter.

4.5. LC3 Immunofluorescence Staining

Cells, grown on sterile glass coverslips, were transferred into a 24-well plate and pretreated and not with the postbiotics for 2 h and then stimulated with PTG and P3-43 for 24 h. After fixation with 3% paraformaldehyde for 5 min at room temperature and permeabilization with 0.2% Triton (Biorad, Milan, Italy) for 5 min at room temperature, Caco-2 cells were stained for 1 h at room temperature with anti-LC3II (Cell signaling Milan Italy) antibody. Secondary antibodies Alexa-488 conjugated (Invitrogen) anti-rabbit for LC3II were added to the coverslips for 45 min at room temperature. The coverslips, after mounting on glass slides, were observed by confocal microscope (LSM 510 Zeiss, Milan, Italy), and images were analyzed with AIS Zeiss software to evaluate the intensity of the fluorescence (FI) of the microscopic field under consideration [49]. The magnification of the micrographs was the same for all the figures shown (63× objective).

4.6. Western Blot

After treatments, the Caco-2 cells were washed twice with cold PBS and resuspended in lysis buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 5 mM MgCl₂, 150 mM NaCl, 1% Triton, 1 mM PMSF, 1 mM VO₄, 100× Aprotinin, and 50× LAP, all of which were purchased from Sigma, Milan, Italy, except for LAP, which was obtained from Roche, Milan, Italy). The human spheroids were seeded in six multiwells (Corning, Milan, Italy) coated with Matrigel diluted 1:40 in phosphate-buffered saline (PBS) for 3 days. After they were stimulated with P31-43 or PTG and postbiotic were homogenized in tissue homogenization buffer 50 mM Tris-HCl (pH 8), 150 mM NaCl, 5 mM MgCl₂, 1% Triton, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM VO₄, aprotinin, and LAP. The cell lysates were analyzed using SDS-PAGE with a standard running buffer (25 mM Tris, 192 mM

glycine, and 0.1% SDS) and transferred onto nitrocellulose membranes (WhatmanGmbh, Dassel, Germany) using a transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, and 20% methanol, all of which were purchased from Sigma-Aldrich, Milan, Italy). The membranes were blocked with 5% nonfat dry milk and probed with rabbit anti pmTOR, pp70S6k, p4EBP, LC3II (Cell Signaling, Euroclone, Milan, Italy), rabbit anti-pNFk β (Elabscience, Microtech, Naples, Italy), mouse anti pERK (Santa Cruz, Milan, Italy), and mouse ant-tubulin (Sigma-Aldrich, Milan, Italy). The bands were visualized using ECL (GE Healthcare, Amersham, Buckinghamshire, UK) with exposure times of 2–10 min. The band intensity was evaluated by integrating all the pixels of a band after subtraction of the background to calculate the average of the pixels surrounding the band [10].

4.7. Statistical Analysis

Statistical analysis and graphics were obtained from Graph Pad Prism (San Diego, CA, USA). The mean and standard deviation of the experiment was calculated; their significance was evaluated by Student's *t*-test, only accepting results that showed values of $p < 0.05$ as significant.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/ijms23073655/s1>.

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EDITED BY

Stefano Guandalini,
The University of Chicago, United States

REVIEWED BY

Josep Manyé,
Germans Trias i Pujol Health Science Research
Institute (IGTP), Spain
Imad Omar AL KASSAA,
Fonterra, New Zealand

*CORRESPONDENCE

Maria Vittoria Barone
✉ mv.barone@unina.it

[†]These authors have contributed equally to this work and share first authorship

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The protective role of *Lactobacillus rhamnosus* GG postbiotic on the alteration of autophagy and inflammation pathways induced by gliadin in intestinal models

Francesca Furone^{1†}, Claudia Bellomo^{1†}, Martina Carpinelli¹, Martina Nicoletti¹, Francesca Natasha Hewa-Munasinghe¹, Majed Mordaa¹, Roberta Mandile², Maria Vittoria Barone^{1,3*} and Merlin Nanayakkara¹

¹Department of Translational Medical Science (Section of Paediatrics), University of Naples Federico II, Naples, Italy, ²Department of Translational Medical Sciences, School of Medicine and Surgery, University of Naples Federico II, Naples, Italy, ³European Laboratory for the Investigation of Food Induced Diseases (ELFID), University of Naples Federico II, Naples, Italy

Celiac disease (CD) is an autoimmune enteropathy caused by an abnormal immune response to gliadin peptides in genetically predisposed individuals. For people with CD, the only available therapy thus far is the lifelong necessity for a gluten-free diet (GFD). Innovative therapies include probiotics and postbiotics as dietary supplements, both of which may benefit the host. Therefore, the present study aimed to investigate the possible beneficial effects of the postbiotic *Lactobacillus rhamnosus* GG (LGG) in preventing the effects induced by indigested gliadin peptides on the intestinal epithelium. In this study, these effects on the mTOR pathway, autophagic function, and inflammation have been evaluated. Furthermore, in this study, we stimulated the Caco-2 cells with the undigested gliadin peptide (P31-43) and with the crude gliadin peptic-tryptic peptides (PTG) and pretreated the samples with LGG postbiotics (ATCC 53103) (1×10^8). In this study, the effects induced by gliadin before and after pretreatment have also been investigated. The phosphorylation levels of mTOR, p70S6K, and p4EBP-1 were increased after treatment with PTG and P31-43, indicating that the intestinal epithelial cells responded to the gliadin peptides by activating the mTOR pathway. Moreover, in this study, an increase in the phosphorylation of NF- κ B was observed. Pretreatment with LGG postbiotic prevented both the activation of the mTOR pathway and the NF- κ B phosphorylation. In addition, P31-43 reduced LC3II staining, and the postbiotic treatment was able to prevent this reduction. Subsequently, to evaluate the inflammation in a more complex intestinal model, the intestinal organoids derived from celiac disease patient biopsies (GCD-CD) and controls (CTR) were cultured. Stimulation with peptide 31-43 in the CD intestinal organoids induced NF- κ B activation, and pretreatment with LGG postbiotic could prevent it. These data showed that the LGG postbiotic can prevent the P31-43-mediated increase in inflammation in both Caco-2 cells and in intestinal organoids derived from CD patients.

KEYWORDS

postbiotic, gliadin, organoids, inflammation, autophagy, p31-43

1. Introduction

Celiac disease (CD) is an autoimmune disease caused by the ingestion of gluten. In genetically predisposed subjects, there is an abnormal response to gluten proteins, generally causing enteropathy. The only therapy available to patients with celiac disease is a lifelong necessity for a gluten-free diet.

It is well known in the literature that dysbiosis is a common condition in celiac patients (1, 2). One of the early stages of the pathology is characterized by the activation of innate immunity by gliadin. In particular, the undigested peptide P31-43 (3), present in the gliadin fraction, has pleiotropic activity, such as innate immunity activation, inflammation, delay of vesicular trafficking, actin remodeling induction, and proliferation, at the biological level in Epidermal Growth Factor Receptor (EGFR)- and Extracellular Signal-Regulated Kinase (ERK)-dependent manners (4–6).

The mammalian target of rapamycin (mTOR) is the major regulator of several important cellular activities, including protein synthesis, autophagy, lysosomal function, and cellular metabolism. In addition, mTOR is a potential target for the treatment of microbial infections, inflammatory bowel disease, and colorectal cancer (7). Gliadin can both trigger the mTOR inflammatory response in celiac disease (8, 9) and activate the nuclear transcription factor- κ B (NF- κ B) pathway, leading to the overregulation of proinflammatory cytokines in the intestine of celiac patients (9–12).

A key role in maintaining cellular homeostasis or metabolic balance is played by autophagy, in which damaged cellular organelles, unwanted proteins, and the different cytoplasmic components get recycled. Dysregulation of autophagy leads to several diseases, including cancer, neurodegeneration, and microbial infections (13–18). Autophagy activation can suppress inflammatory responses following infection by preventing the production of proinflammatory cytokines (19, 20). Light chain protein 1–3, which is associated with microtubules (LC3, a mammalian homolog of yeast Atg8) is commonly used as an autophagosome membrane marker (21). P62, also known as SQSTM1, which is involved in autophagic flux, is also a marker of autophagy as it participates in the structure of the autophagosome and is effectively degraded by the autophagic pathway (22). Moreover, p62 acts as a positive regulator of the transcription factor NF- κ B (23).

Postbiotics are defined as functional bioactive compounds that are generated in a matrix during fermentation. They can be used to promote health (24) when administered in adequate amounts. The term “postbiotics” includes different preparations such as “metabiotics,” “metabolic probiotics,” “postbiotics,” “biological drugs,” or “pharmacobiotics.” They are small molecules that are the structural components of probiotic (symbiotic) microorganisms and/or their metabolites and/or signaling molecules with defined (known) chemical structures that can affect the microbiome and/or human metabolic and signaling pathways. On the contrary, the term “paraprobiotics” indicates the use of inactivated microbial cells or cell fractions that confer health benefits to the host (25, 26). The postbiotic components can be used as promising tools for both prevention and treatment strategies in gastrointestinal disorders, especially in infants and children (27).

Lactobacillus rhamnosus GG (LGG) has been identified as a potential probiotic strain because of its resistance to acid and bile, good growth characteristics, and ability to adhere to the intestinal epithelial layer (28, 29). Furthermore, LGG can produce both a biofilm that can mechanically protect the mucosa and different soluble factors that are beneficial to the gut by increasing intestinal crypt survival, decreasing intestinal epithelial apoptosis, and preserving cytoskeletal integrity (30). Moreover, as a postbiotic, LGG has protective effects on the cytoskeleton, apoptosis, and NF- κ B-mediated inflammation (31, 32).

In the present study, we analyzed mTOR activation and the autophagic pathway before and after the treatment with P31-43 and the Peptic Tryptic Product of gliadin (PTG) in Caco-2 cells, an intestinal epithelial cell line derived from colon adenocarcinoma. In this study, the role of the LGG postbiotic in the prevention of the P31-43 and PTG effects on Caco-2 cells was also investigated. Moreover, we evaluated the effects of P31-43 and the LGG postbiotic on the pNF- κ B mediated inflammatory pathway in both Caco-2 cells and intestinal organoids.

2. Materials and methods

2.1. Cell cultures and treatments

Human colon adenocarcinoma-derived cells (Caco-2) (Centro di Biotecnologie Avanzate, Genoa, Italy) were cultured for 5–6 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 units/ml of penicillin–streptomycin, and 1 mmol/L of glutamine (all products were purchased from Gibco Invitrogen, Milan, Italy) (4). Cell plates were incubated at 37°C in a humidified atmosphere (95% air and 5% carbon dioxide (CO₂)). The sequence of P31-43, an LPS-free synthetic peptide, was LGQQQPFPPQPY (Caslo > 95% purity, MALDI-TOF analysis; DK-2800 Kongens Lyngby, Denmark) and it was used at 10–100 µg/ml. The peptic–tryptic-digest of gliadin (PTG) was extracted from whole wheat (*Triticum aestivum* var. Sagittario) flour and then subjected to digestion (33). PTG was used at a concentration of 500 µg/ml.

2.2. Organoids

Fragments of duodenal mucosa from individual patients with gluten-containing-diet CD (GCD-CD) and controls (CTR) were obtained by standard esophagogastroduodenoscopy (EGDS) and were placed in an ice-cold 10-ml isolation buffer to separate the crypts (34) (Table 1). The isolation buffer consisted of 5.6 mmol/L Na₂HPO₄ (Sigma S7907; Sigma–Aldrich, Milan, Italy); 8.0 mmol/L KH₂PO₄ (Sigma P5655; Sigma–Aldrich, Milan, Italy); 96.2 mmol/L NaCl (Sigma S5886; Sigma–Aldrich, Milan, Italy); 1.6 mmol/L KCl (Sigma P5405; Sigma–Aldrich, Milan, Italy); 43.4 mmol/L sucrose (Fisher BP220-1; Thermo Fisher Scientific, Milan, Italy); and 54.9 mmol/L D-sorbitol (Fisher BP439-500; Thermo Fisher Scientific, Milan, Italy) in deionized water. Crypt units were isolated according to the protocol of Yuli Wang et al. (35) with minor modifications. After 60 min, enzymatic digestion with collagenase (2 mg/ml, Sigma–Aldrich Milan, Italy)

TABLE 1 Patient characteristics.

Characteristics	CONTROL = 3	GCD-CD = 4
Range age (years)	4–16	8–15
Sex	M = 2 F = 1	M = 1 F = 3
Biopsy (Marsh classification)*	T0	1 = T3C3 = T3 c/b
Serum AntiTG2 (U/mL)	0–1,9	>50
Anti-Endomysial Antibody (EMA)	Negative	Positive

Marsh classification* (40).

was performed on the biopsy samples in wash buffer (WB) containing penicillin (100 units ml^{-1}), streptomycin (0.1 mg ml^{-1}), l-glutamine (2 mM), and FBS (10%, vol/vol) in DMEM/F12 (all these products were purchased from Gibco Invitrogen, Milan, Italy) on ice for 30 min. The digest was filtered through a 70- μm strainer (Falcon, Germany), and the strainer was rinsed with an additional 10 ml of WB. The crypts were collected by centrifugation at 500g for 5 min. After discarding the supernatant to allow 3D growth in 48-well plates, the crypts were carefully resuspended in 40- μl of ice-cold Matrigel (Corning 356231 Milan, Italy). The plates were incubated in a cell culture incubator at 37°C and 5% CO_2 for 10 min to allow the Matrigel to solidify. Afterward, 300- μl of supplement-enriched cell culture medium (CM-S) was added to each well and replaced every other day (34).

Culture Medium with Supplements (CM-S) was prepared using 50% L-WRN conditioned medium (mouse l-cells expressing Wnt3a, R-spondin, and Noggin) (ATCC CRL-3276, Genoa, Italy) and 50% fresh primary culture media: advanced DMEM/F-12 (cat. 12634-010, Invitrogen Milan, Italy) 1-mM N-acetyl-l-cysteine (cat. A7250, Sigma, Milan, Italy), 1 $\times$ B-27[®] supplements (cat. 12587-010 Gibco, Milan, Italy), 50-ng/mL epidermal growth factor, (cat. PMG8041 Gibco Milan, Italy), 10-mM nicotinamide (cat. N0636, Sigma, Milan, Italy), 10-nM Leu15-gastrin I (cat. G9145 Sigma, Milan, Italy), 500-nM A8301 (inhibitor of ALK4/5/7, cat. 70024-90-7 Sigma, Milan, Italy), 10- μM SB202190 (p38 MAP kinase inhibitor, cat. S7076 Sigma, Milan, Italy), and 10- μM Y-27632 (p160 ROCK inhibitor; cat. 1254 Tocris, Milan, Italy).

2.3. 2D organoid experiments

After growth in a three-dimensional (3D) culture, the organoids were seeded in a two-dimensional (2D) culture for the experiments because, in 3D culture, the spheroidal architecture of the organoids prevents access of exogenous compounds to the luminal epithelial surface. During the unfolding of the spherical organoid into a 2D plane, the tissue was obtained using Matrigel diluted at a ratio of 1:40 in phosphate-buffered saline (PBS) and cultured for 3 days. After 3 days of culture, the 2D organoids of GCD-CD and CTR were stimulated with P31-43 in the presence or absence of LGG postbiotic for 3 h with different concentrations of P31-43 (10, 20, 50, and 100 $\mu\text{g/ml}$). To obtain cell lysates, the plates were first treated with Cell Recovery Solution (354253 Corning[®], Milan Italy) to remove the Matrigel and were homogenized in tissue homogenization buffer containing 50-mM Tris-HCl

(pH 8), 150-mM NaCl, 5-mM MgCl_2 , 1% Triton, 0.5% sodium deoxycholate, 0.1% Sodium Dodecyl Sulfate (SDS, Biorad, Milan, Italy), 1 mM Biorad, Milan Italy Phenylmethylsulfonyl Fluoride (PMSF, Signma, Milan, Italy), 1-mM VO_4 , aprotinin, and Mixture of several Protease inhibitor (LAP, Roche, Microtech, Naples, Italy).

2.4. Bacterial growth conditions

In this study, to prepare the postbiotic, *L. rhamnosus* GG was cultured in DMEM supplemented with 10% fetal calf serum (FBS), low glucose (1 g/L D-glucose), and 1-mM glutamine to 10^9 CFU/ml as described in a previous study (36, 37). The bacterial culture was then centrifuged at 3,000g for 10 min—a speed that generally does not break up the bacterial bodies. The pH after incubation was ~ 7 . The supernatant (metabiotic) was filtered through a 0.22-mm filter to remove the bacterial bodies (9). This preparation (metabiotic) without the bacterial bodies was used for the experiments and is referred to as “postbiotic” throughout the text.

2.5. LC3 immunofluorescence staining

Caco-2 cells grown on sterile glass coverslips were pretreated for 2 h, ensuring that the cells were not pretreated with the postbiotics, and then stimulated with P3-43 and PTG for 1 h. In this study, 3% paraformaldehyde and 0.2% Triton (Biorad, Milan, Italy), were used for fixation and permeabilization, respectively, for 5 min at room temperature. The samples were then pretreated with 1% Bovine Serum Albumin, Sigma, Milan Italy (BSA, Sigma, Milan, Italy) for 30 min and stained with anti-LC3II antibody (Cell signaling, Milan, Italy) for 1 h at room temperature. An anti-rabbit Alexa-488 conjugated (Invitrogen, Milan, Italy) was used as a secondary antibody for 45 min at room temperature. The coverslips, after mounting on glass slides, were observed with a confocal microscope (LSM 510 Zeiss, Milan, Italy), and the images (63 $\times$ objective) were analyzed with AIS Zeiss software to evaluate the intensity of fluorescence (FI) (38).

2.6. Western blot

Untreated (NT) Caco-2 cells, after treatment with P31-43 and PTG for 1 h, and pre-treatment with LGG postbiotic, were washed twice with cold PBS and resuspended in lysis buffer containing 50-mM Tris-HCl [pH 7.4], 1-mM EDTA, 1-mM EGTA, 5-mM MgCl_2 , 150-mM NaCl, 1% Triton, 1-mM PMSF, 1-mM VO_4 , 100 $\times$ aprotinin, and 50 $\times$ LAP, all of which were purchased from Sigma, Milan, Italy, except for LAP, which was purchased from Roche, Milan, Italy.

The cell lysates were analyzed using SDS-PAGE with a standard running buffer containing 25-mM Tris, 192-mM glycine, and 0.1% SDS and transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany) using a transfer buffer containing 25-mM Tris, 192-mM glycine, 0.1% SDS, and 20% methanol, all of which were purchased from Sigma-Aldrich, Milan, Italy. The membranes were blocked with 5% skim milk powder (SKI400.500,

Microgem SRL, Naples, Italy) and probed with rabbit anti-pmTOR, pp70S6K, p4EBP, LC3II (Cell Signaling, Euroclone, Milan, Italy), rabbit anti-pNF- κ B (Elabscience, Microtech, Naples, Italy), mouse anti-pERK (Santa Cruz, Milan, Italy), and mouse anti-tubulin (Sigma-Aldrich, Milan, Italy). The bands were visualized using ECL (GE Healthcare, Amersham, Buckinghamshire, UK) with exposure times of 2–10 min. The band intensity was evaluated by integrating all the pixels of a band after subtraction of the background to calculate the average of the pixels surrounding the band (4).

2.7. Statistical analysis

GraphPad Prism (San Diego, CA) was used for statistical analysis and graphing. The mean and the standard deviation of the experiment were calculated, and their significance was evaluated by a Student's *t*-test. Means and SDs were considered statistically significant only when the results showed a *p*-value of < 0.05.

3. Results

3.1. Gliadin peptides induced mTOR activity and the phosphorylation of two key regulators, p70S6K, and 4E-BP

The target of rapamycin (mTOR) in mammals is a serine/threonine kinase protein that responds to intracellular and extracellular signals to modulate growth and proliferation. In this study, in gliadin-responding Caco-2 cells, the effects of gliadin peptide P31-43 and PTG (the peptic-tryptic digested gliadin product) on the mTOR and the autophagic pathways were evaluated. In this study, to determine their effect on the mTOR pathway, the effects of gliadin peptides on Caco-2 cells were analyzed before and after the treatment with PTG and P31-43. In particular, the active/phosphorylated form of mTOR, the downstream components p70S6K, and the binding protein eIF4E (4EBP) were evaluated by Western blot analysis. Treatment with both P31-43 and PTG induced and increased the activation of mTOR, p70S6K, and the binding protein eIF4E. **Figures 1A, B** show the activation of mTOR (P31-43: 1.05 ± 0.19 , $p < 0.05$; PTG: 0.65 ± 0.08 , $p < 0.05$); **Figures 1A–C** show the activation of p70S6K (P31-43: 0.4 ± 0.1 , $p < 0.05$; PTG: 0.09 ± 0.01 , $p < 0.05$); and **Figures 1D, E** show the activation of p4EBP (P31-43: 0.97 ± 0.19 , $p < 0.05$; PTG: 0.83 ± 0.06 , $p < 0.01$) after treatment with P31-43 and PTG. Moreover, the pretreatment with LGG postbiotic could prevent the activation of the mTOR pathway induced by P31-43 and PTG. Furthermore, pmTOR (**Figures 1A, B**) (LGG, P31-43: 0.4 ± 0.05 , $p < 0.01$; LGG, PTG: 0.15 ± 0.02 , $p < 0.01$), p70S6K (**Figures 1A–C**) (LGG, P31-43: 0.6 ± 0.07 , $p < 0.01$; LGG, PTG: 0.4 ± 0.07 , $p < 0.01$), and p4EBP (**Figures 1D, E**) (LGG, P31-43: 0.13 ± 0.04 , $p < 0.05$; LGG, PTG: 0.02 ± 0.03 , $p < 0.01$) were significantly reduced in the presence of LGG postbiotic.

3.2. Gliadin peptides deregulated the autophagy pathway by reducing the levels of LC3II and increasing the levels of p62

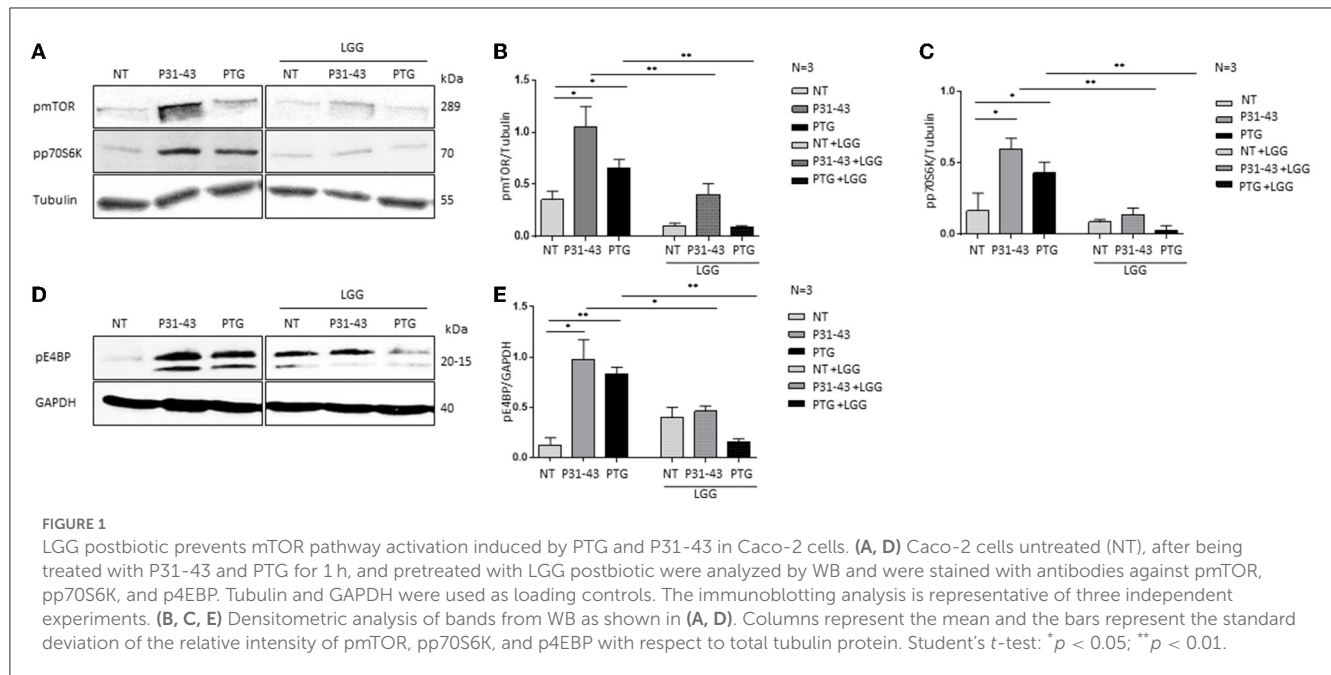
When mTOR is dephosphorylated, autophagy is induced, whereas the opposite happens when mTOR is phosphorylated (9, 39). Therefore, in this study, the effects of gliadin peptides P31-43 and PTG on the autophagy pathway were evaluated in Caco-2 cells (**Figure 2**). Autophagy was studied by evaluating the levels of LC3II and p62 protein in Caco-2 cells by Western blot before and after the treatment with P31-43 and PTG as shown in **Figure 2**. We observed that the treatment after 24 h with P31-43 and PTG reduced the LC3II levels (**Figures 2A, B**) (P31-43: 0.08 ± 0.02 , $p < 0.05$; PTG: 0.18 ± 0.04 , $p < 0.05$) and increased the p62 levels (**Figures 2C, D**) (P31-43: 2.5 ± 0.15 , $p < 0.01$; PTG: 2.3 ± 0.08 , $p < 0.01$), indicating that gliadin peptides reduce autophagy and its progression. The pre-treatment with LGG postbiotic prevented gliadin-induced effects on the LC3II and p62 levels. LGG postbiotic increased the LC3II levels (**Figures 2A, B**) (LGG, P31-43: 0.26 ± 0.02 , $p < 0.01$; LGG, PTG: 0.51 ± 0.04 , $p < 0.01$) and reduced the p62 levels (**Figures 2C, D**) (LGG, P31-43: 1.2 ± 0.08 , $p < 0.01$; LGG, PTG: 0.75 ± 0.15 , $p < 0.01$) in a significant way. These data were also confirmed by immunofluorescence analysis (**Figures 2E, F**) (LGG, P31-43: $1,072 \pm 112.6$, $p < 0.001$; LGG, PTG: $1,846 \pm 212.3$, $p < 0.001$).

3.3. Postbiotic *L. rhamnosus* GG prevented inflammatory marker NF- κ B phosphorylation after treatment with P31-43 and PTG

Figures 3A, B shows that both P31-43 and PTG can activate the NF- κ B signaling pathway (P31-43: $p < 0.01$; PTG: $p < 0.01$). In particular, the effect of PTG on mTOR has also been studied (8). We tested the effect of LGG postbiotic on the increase in inflammatory markers induced by the treatment of cells with P31-43 and PTG. In this regard, Caco-2 cells were pretreated with LGG postbiotic for 1 h and then stimulated with PTG and P31-43, and the activation of the NF- κ B signaling pathway was detected by Western blot analysis. Our data showed an increase in the phosphorylation levels of NF- κ B after stimulation with P31-43 (**Figures 3A, B**) (0.3 ± 0.01 , $p < 0.01$) and PTG (**Figures 3A, B**) (0.36 ± 0.03 , $p < 0.01$). The LGG postbiotic was able to reduce this phosphorylation. In fact, LGG postbiotic was able to prevent NF- κ B phosphorylation induced by P31-43 (**Figures 3A, B**) (0.06 ± 0.02 , $p < 0.001$) and by PTG (**Figures 3A, B**) (0.04 ± 0.01 , $p < 0.01$).

3.4. In the GCD-CD organoid, pretreatment with postbiotic *L. rhamnosus* GG reduced NF- κ B phosphorylation

In addition, this study used intestinal organoids derived from biopsies of CD patients and control subjects to assess the effects of LGG postbiotic in a more complex cell model (**Table 1**) (40).



The organoids were derived from intestinal staminal cells and were cultured for 4 weeks in a 3D culture before being cultured to allow treatment on the apical part of the epithelial cells.

The intestinal organoids were derived from GCD-CD and CTR intestinal biopsies and tested for the inflammatory marker pNF- κ B (34). Compared to CTR organoids, pNF- κ B was increased in GCD-CD organoids, as evaluated by Western blot analysis (34). The CTR organoids were not inflamed, and the LGG treatment was not effective, whereas the pNF- κ B levels were not altered before or after the treatment with LGG (Figures 4A, B). On the contrary, as shown in Figure 4, treatment with LGG supernatant was able to prevent the NF- κ B phosphorylation present in GCD-CD (C-D). Treatment of GCD-CD organoids with LGG supernatant was able to reduce the levels of pNF- κ B from 0.38 ± 0.083 to 0.20 ± 0.04 , and the reduction was statistically significant ($p < 0.05$).

3.5. In GCD-CD organoids, postbiotic *L. rhamnosus* GG reduced NF- κ B phosphorylation after treatment with P31-43

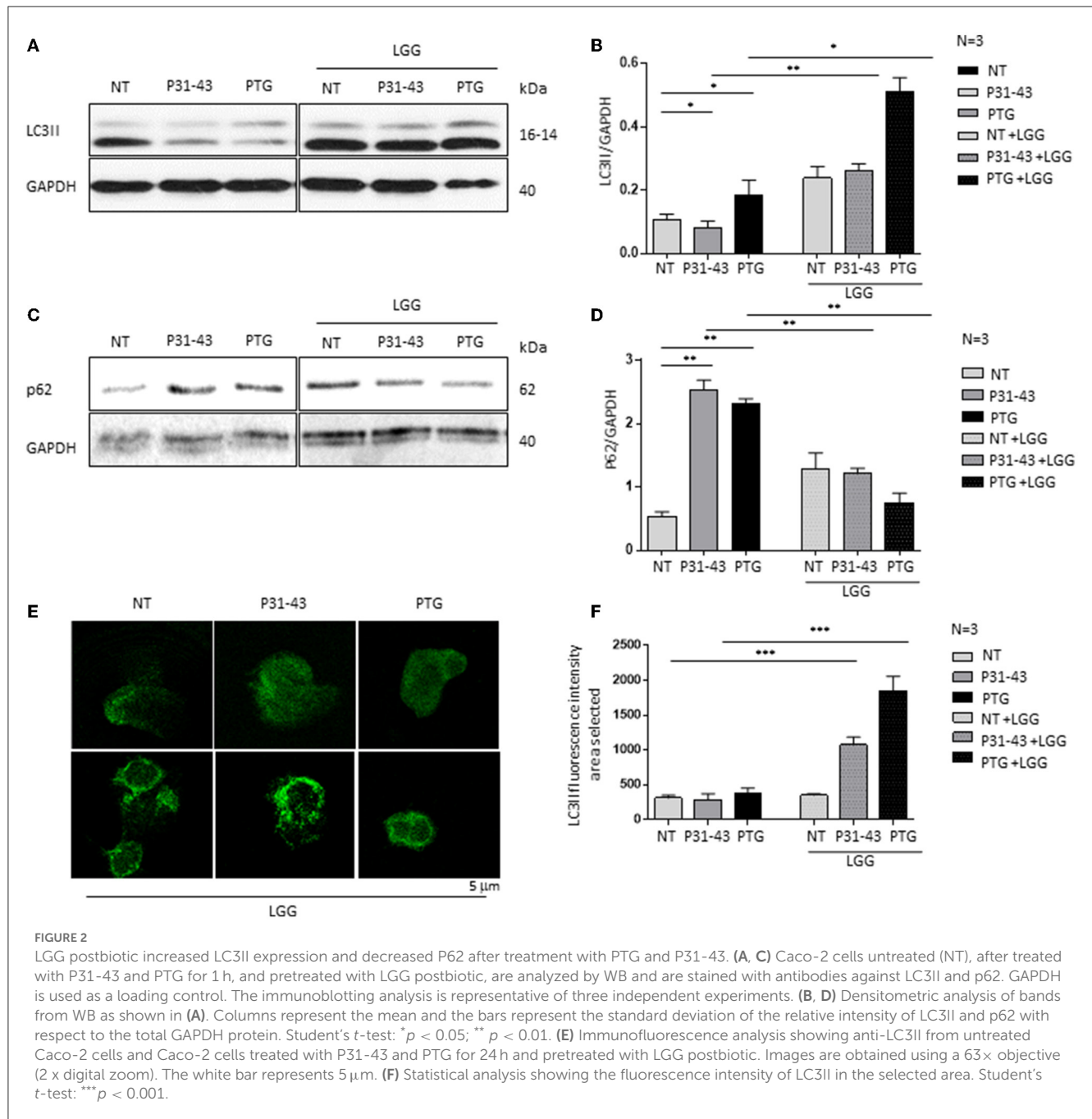
In this study, to investigate the effect of P31-43 peptide on pNF- κ B (3), GCD-CD organoids were stimulated with P31-43 peptide at a concentration (10 μ g/ml) that is inactive on CTR organoids as shown in Figures 5A, B. CTR organoids responded to the P31-43 treatment only at the high concentration (100 μ g/ml) (Figures 5A, B) (0.67 ± 0.16 , $p < 0.05$). Therefore, in this study, a low concentration of P31-43 peptide (10 μ g/ml) was used to challenge GCD-CD organoids as shown in Figures 5C, D. Under these conditions, there was an increase in the expression of the inflammatory marker pNF- κ B that was statistically significant (0.65 ± 0.05 , $p < 0.05$). Pretreatment of GCD-CD organoids with LGG supernatant could prevent the increase of NF- κ B phosphorylation

induced by P31-43 in a statistically significant way (Figures 5C, D) (LGG, P31-43: 0.3 ± 0.03 , $p < 0.05$).

4. Discussion

Postbiotics could be a potential tool for prevention strategies and/or the treatment of dysbiosis or gastrointestinal disorders with less significant side effects, especially in infants and children (29). The postbiotic concept is based on the observation that the beneficial effects of probiotics are mediated by the secretion of various metabolites. According to Zólkiewicz et al. (41), “postbiotics include any substance released or produced through the metabolic activity of the microorganism that exerts a beneficial effect on the host, directly or indirectly.” [SIC] Although postbiotics do not contain live microorganisms, they exert a beneficial effect on health through mechanisms similar to those that are characteristic of probiotics, while minimizing the risks associated with ingesting live microorganisms (42). Although there are a few studies that directly compare substances belonging to both probiotic and postbiotic groups, the postbiotic groups tend to be free of serious side effects while the probiotic groups maintain a similar efficacy (43).

In this study, the benefits of the postbiotic LGG were investigated against the effects induced by P31-43 and PTG on the intestinal epithelium. The intestinal epithelial cell models used in this study included both secondary lines, such as Caco-2 cells, and primary lines, such as intestinal organoids. To assess the effects of gliadin peptide P31-43, in particular, the pathways of mTOR and autophagy were evaluated in addition to the activation of inflammation by determining the phosphorylated state of NF- κ B. The mTOR protein is a tyrosine kinase that can regulate cell proliferation and inhibit the autophagy pathway. It is modulated by stimuli, such as growth factors and nutrients that promote cell growth and proliferation. The autophagy pathway is active

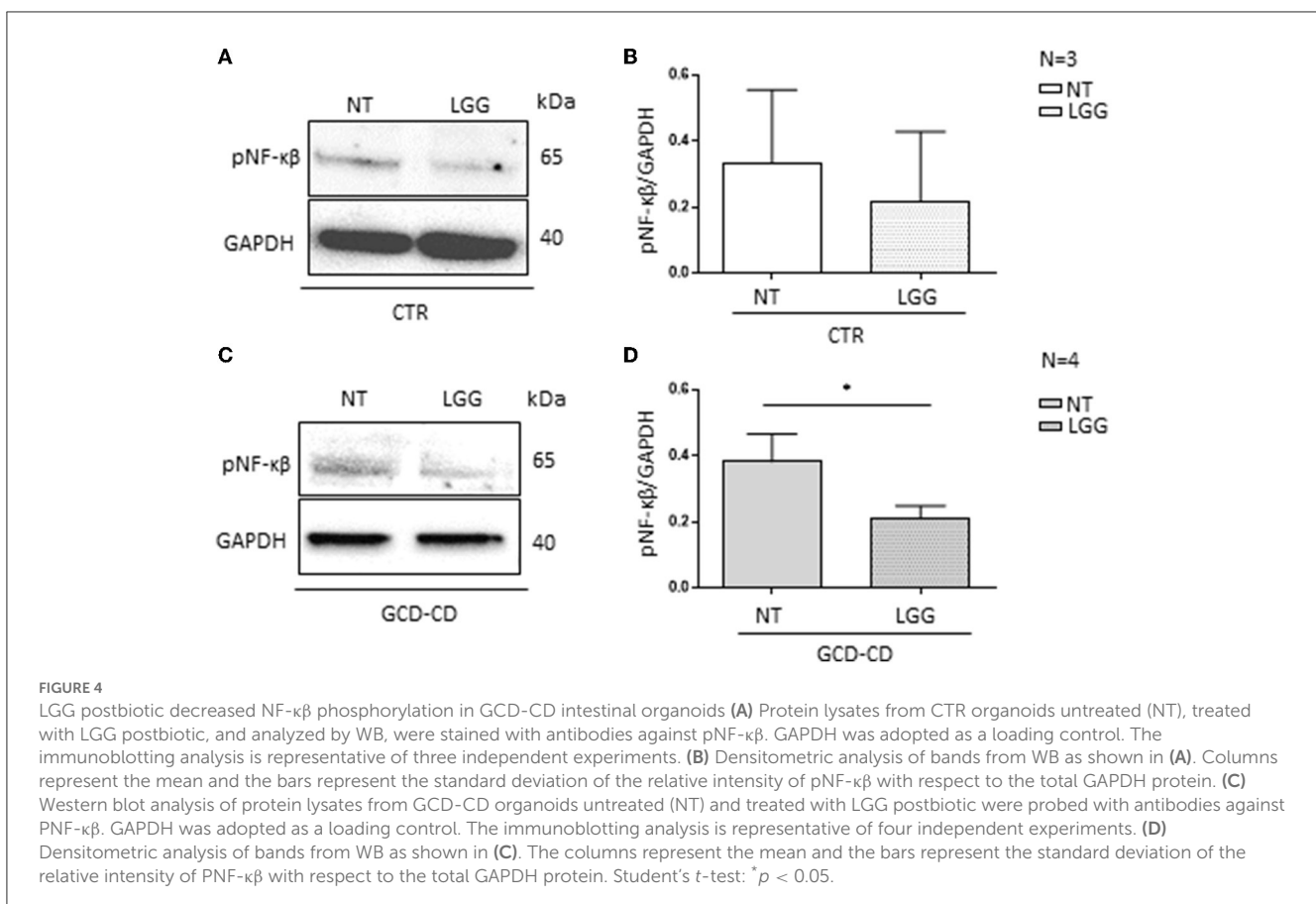
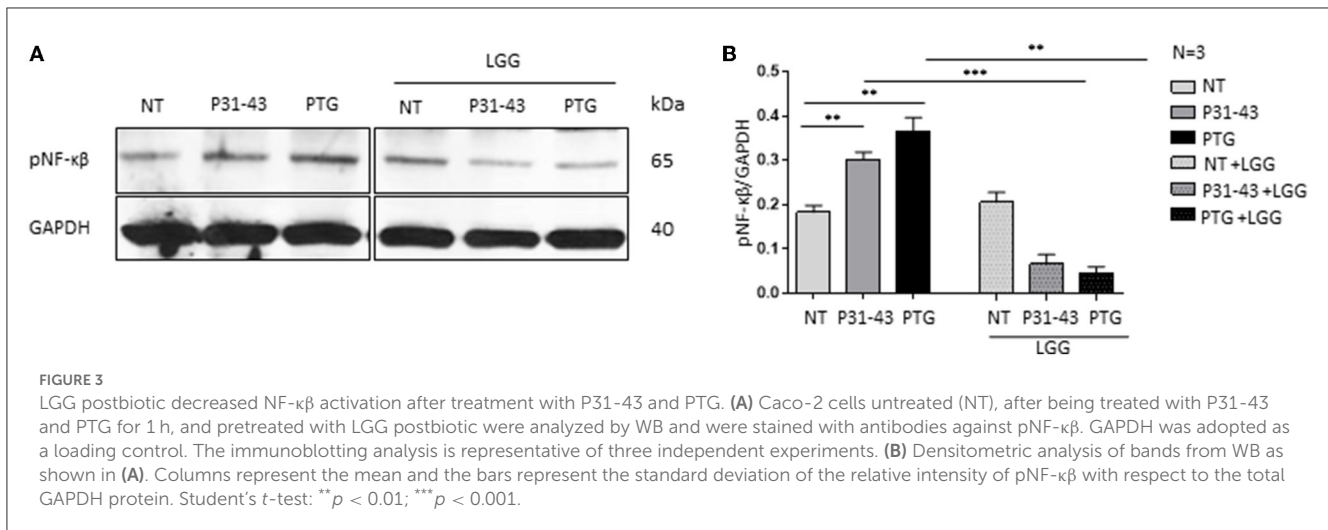


when mTOR is dephosphorylated or inactivated. This pathway is a signaling pathway closely associated with cell regeneration; it is involved in the process of self-digestion that is activated to remove damaged macromolecules and organelles to maintain cellular homeostasis. Activation of mTOR and subsequent inhibition of the autophagic pathway cause the induction of inflammation markers, including NF- κ B (39).

Specifically, gliadin-responsive Caco-2 cells were used as one of the study models to test the effects of gliadin peptides on the mTOR-induced signaling pathway and the autophagic pathway. Both peptides, P31-43 and PTG, could activate mTOR and reduce the signaling pathway by activating E4BP and p70S6K proteins. Once activated, mTOR induces the downregulation of autophagy

(7). In this study, the autophagic pathway was investigated after treatment with P31-43 and PTG, and it was shown that, in the presence of P31-43 and PTG, there was a reduction in LC3II vesicles and autophagic flow to lysosomes was decreased, as evidenced by the increase in p62. These results suggest that gliadin peptides were able to perturb autophagy.

The effect of probiotics has been studied in patients with CD, showing *in vivo* that they are able to reduce the symptoms of CD patients with GFD. In the acute phase of the disease, probiotics do not influence intestinal lesions (44, 45). On the contrary, postbiotics have not been studied *in vivo* in CD; although studied *in vitro*, postbiotics have been shown to prevent gliadin-induced effects (9, 26, 31, 44). In this study, the ability of LGG postbiotic to



prevent the effects of gliadin and peptide 31-43 on the pathways of mTOR, autophagy, and inflammation in Caco-2 cells and organoids derived from CD patients as study models was focused on to test the preventive role of LGG postbiotic. Pretreatment with LGG postbiotic was able to reduce the levels of pmTOR, pE4BP, and p70S6K proteins in Caco-2 cells induced by PTG and P31-43. In this study, similar results were obtained using *Lactobacillus Paracasei* postbiotic (9). All these data show that, through different

probiotic strains, postbiotics can have activity on gliadin effects on epithelial cells.

Activation of the mTOR pathway can reduce autophagy in several systems (39). In this study, a reduction of autophagic vesicles and their flux toward lysosomes was found in the presence of P31-43 and PTG. The effect of PTG on mTOR was also described in a previous study (9). In this study, it has been shown that the pretreatment of Caco-2 cells with LGG postbiotic before

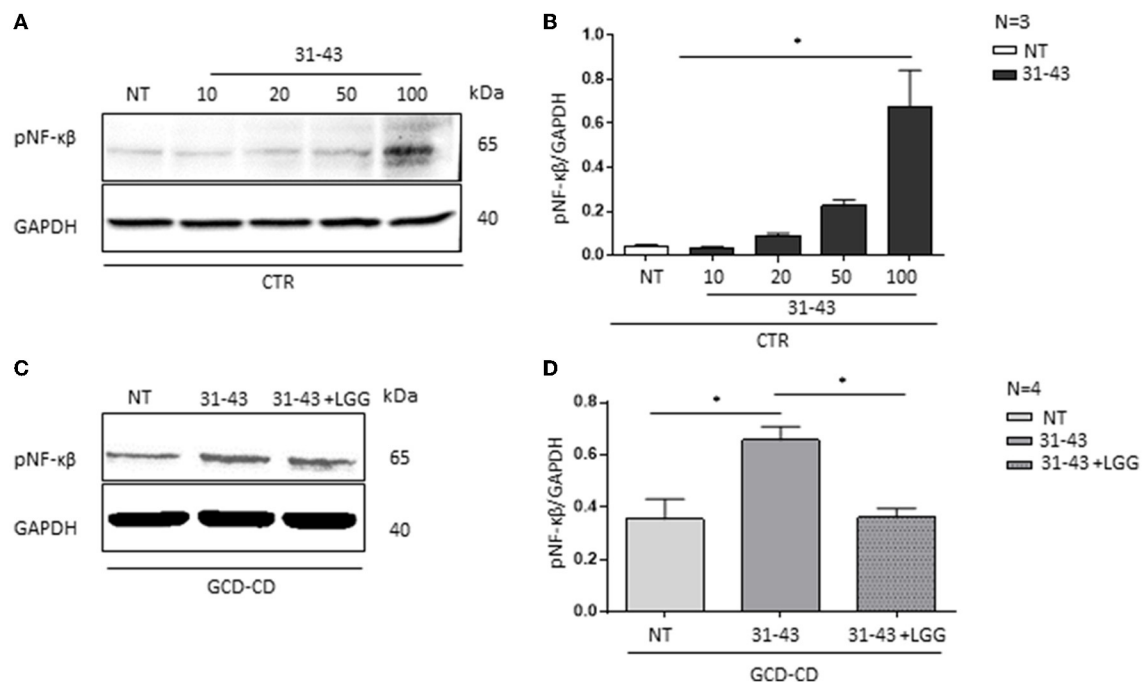


FIGURE 5

LGG postbiotic decreased NF-κβ activation and prevented P31-43 effects in GCD-CD organoids. (A) Western blot analysis of CTR organoids untreated (NT) and treated for 3 h with different concentrations of P31-43 (10, 20, 50, and 100 μg/ml) were blotted with antibodies against pNF-κβ. GAPDH was adopted as a loading control. The immunoblotting analysis is representative of three independent experiments. (B) Densitometric analysis of bands from WB as shown in (A). Columns represent the mean and the bars represent the standard deviation of the relative intensity of pNF-κβ with respect to the total GAPDH protein. Student's *t*-test: **p* < 0.05 (C) Western blot analysis of protein lysates from GCD-CD organoids untreated (NT), treated with P31-43 for 3 h, and pretreated with LGG postbiotic were blotted with antibodies against pNF-κβ. GAPDH was adopted as a loading control. The immunoblotting analysis is representative of four independent experiments. (D) Densitometric analysis of bands from WB as shown in (C). Columns represent the mean and the bars are the standard deviation of the relative intensity of pNF-κβ with respect to the total GAPDH protein. Student's *t*-test: **p* < 0.05.

P31-43 and PTG was able to induce an increase in autophagic vesicles with an increase in LC3 and a decrease in the flow of the autophagosomes to lysosomes with a decrease in p62.

Activation of the mTOR pathway is strictly linked to inflammation (8, 9, 39). In this study, the inflammation induced by gliadin peptides was studied in the presence of LGG postbiotic. We confirmed that both P31-43 and PTG cause inflammation by increasing NF-κβ phosphorylation (9, 34). Pretreatment of LGG postbiotic can prevent the pro-inflammatory effects induced by gliadin peptides on NF-κβ.

Organoids derived from intestinal staminal cells can be used to study the intestinal epithelium in several diseases (46, 47). In this study, organoids were derived from controls and CD biopsies, and it was observed that, without any treatment, the CD organoids are inflamed and more sensitive to P31-43 stimuli compared to CTR organoids. We have shown that CTR organoids respond to the gliadin peptide P31-43 by increasing NF-κβ, but only at a high concentration, the same concentration that is active in Caco-2 cells. In contrast, in CD organoids, P31-43 was still able to increase inflammation at lower concentrations, for example, at least 10 times lower than in controls (34). LGG postbiotic was able to prevent the increase in NF-κβ induced by P31-43 in CD organoids. These data indicate that LGG postbiotic can prevent the effects of P31-43 and PTG on intestinal epithelium and that organoids derived from CD patients may be a good model to study inflammation in CD.

Intestinal organoids can develop different cell types such as epithelial cells of the crypts and the villi, goblet cells, Paneth cells, and enterochromaffin cells. In this study, organoids that were composed mainly of epithelial cells were used in the presence of a few Paneth cells (34).

In the literature, there are several reports describing alterations of the enteroendocrine cells (48) in the intestinal mucosa of CD during the active phase of the disease, in addition to reports of decreased numbers of goblet cells (49). Organoids may be a good model to study not only the effects of gliadin or other proinflammatory agents on epithelial cells but also on other cell types present in the intestinal epithelium. The use of patient-derived organoids to model CD pathogenesis *in vitro* may be a novel tool to further study CD treatment and prevention.

Pre-clinical studies on primary tissues *in vitro* are a good basis for planning clinical trials in CD patients to prevent the pro-inflammatory effects of gliadin peptides.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human samples were reviewed and approved by the Ethical Committee of the Federico II University of Naples. Ethical approval: 115/09/ESPROT. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

Author contributions

CB and FF performed Western blot experiments and contributed to data analysis. MNi, MC, FNH-M, and MM took care of the biobank and cell culture. RM led the clinical recruitment of patients. MVB led the study conception, design, performance, and wrote the text. MNa led the study conception and design, performed experiments and was involved in the planning, and supervision of the work. All authors have read and approved the published version of the manuscript.

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(4) the decision to submit the manuscript for the publication. The authors have no other conflicts of interest directly relevant to the content of this manuscript, which remains their sole responsibility. The work was also supported by a project funded by the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.3—Call for Proposals No. 341 of 15 March 2022 of the Italian Ministry of University and Research funded by the European Union—Next Generation EU; Award No. Project code PE000000003, Concession Decree No. 1550 of 11 October 2022 adopted by the Italian Ministry of University and Research, CUP D93C22000890001, Project title ON Foods—Research and innovation network on food and nutrition sustainability, safety and security—Working ON foods.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Dottorando	Dr.ssa Claudia Bellomo
Tutor	Prof.ssa Maria Vittoria Barone
Coordinatore	Prof. Francesco Beguinot
Corso di Dottorato in	Dottorato di Ricerca in Medicina Clinica e Sperimentale
Ciclo	37°
Codice borsa e n.	DOT1318210-1 (Bellomo)
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