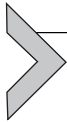

CHAPTER SIX

The gliadin p31–43 peptide: Inducer of multiple proinflammatory effects

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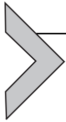
Abstract

Coeliac disease (CD) is the prototype of an inflammatory chronic disease induced by food. In this context, gliadin p31–43 peptide comes into the spotlight as an important player of the inflammatory/innate immune response to gliadin in CD. The p31–43 peptide is part of the p31–55 peptide from α-gliadins that remains undigested for a long time, and can be present in the small intestine after ingestion of a gluten-containing diet. Different biophysical methods and molecular dynamic simulations have shown that p31–43 spontaneously forms oligomeric nanostructures, whereas experimental approaches using *in vitro* assays, mouse models, and human duodenal tissues have shown that p31–43 is able to induce different forms of cellular stress by driving multiple inflammatory pathways. Increased proliferative activity of the epithelial cells in the crypts, enterocyte stress, activation of TG2, induction of Ca^{2+}, IL-15, and NFκB signaling, inhibition of CFTR, alteration of vesicular trafficking, and activation of the inflammasome platform are some of the biological effects of p31–43, which, in the presence of appropriate genetic susceptibility and environmental factors, may act together to drive CD.



1. Introduction

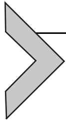
Coeliac disease (CD) is an immune-mediated enteropathy triggered in genetically susceptible individuals by a group of wheat proteins (commonly called gluten) and related prolamins from toxic cereals (Sollid and Jabri, 2013). It is characterized by a variable combination of gluten-induced symptoms, generation of CD-associated autoantibodies, and enteropathy (Husby et al., 2020; Lindfors et al., 2019). A central role in the pathogenesis of CD is played by the HLA-restricted gliadin-specific intestinal T cell response (Iversen and Sollid, 2020; Shan et al., 2002). The molecular basis of the interaction between gliadin peptides and HLA-DQ2.5 or HLA-DQ8 molecules, (*HLA-DQA1*05/HLA-DQB1*02:01* and *HLA-DQA1*03:01/HLA-DQB1*03:02*, respectively), as well as the role of transglutaminase 2 (TG2) in converting neutral glutamine into negatively charged residues, and consequently, strongly enhancing the peptide affinity to the HLA susceptibility alleles, have been well established (Molberg et al., 1998). Poor digestion together with deamidation can explain why strongly immunogenic peptides are generated, and several gliadin peptides that are preferentially recognized in the context of HLA molecules have been identified (Sollid et al., 2012, 2020). Furthermore, the T cell receptor expressed by gliadin-specific T cells has been characterized, and the rules governing

the molecular interactions with gliadin T cell epitopes have been established (Qiao et al., 2014). The cytokine pattern involved has also been defined, and a Th1 phenotype dominated by IFNγ has been attributed to intestinal gliadin-specific T cell clones expanded from the coeliac mucosa (Jabri and Sollid, 2017).

Despite such progress, a central question remains still unanswered, namely, why a proinflammatory T cell response is generated instead of a regulatory response, which normally promotes oral tolerance to dietary protein antigens. Dendritic cells play a central role in determining the outcome of the immune response (Mowat and Agace, 2014). In an inflamed environment enriched in cytokines such as IL-15 or type I interferons, T cells tend to acquire a proinflammatory phenotype. Recently, elegant studies have been conducted in mice demonstrating that mucosal inflammation due to reovirus infection may disrupt oral tolerance by suppressing the regulatory T cell (Treg) conversion and promoting Th1 immunity against gliadin (Bouziat et al., 2017).

The factors that create a proinflammatory environment in the CD intestine, leading to an expansion of gliadin-specific T cells in genetically susceptible individuals and further shifting them toward a proinflammatory phenotype, remain to be identified. Many mice-based studies as mentioned above as well as epidemiological data have suggested viral infections as one of such factors (Stene et al., 2006). However, a number of other factors may contribute to the generation of a "sterile" inflammation, such as metabolic, chemical, and physical factors, drugs, alterations of the mucosal barrier, and changes in the microbiota. Dietary proteins are also a candidate for this role: wheat proteins, such as ATIs (amylase trypsin inhibitors), have been shown to activate innate immunity through TLRs (Junker et al., 2012), but most of the evidence points to gliadin itself and in particular, a peptide from the N-terminal portion of α-gliadin, named peptide p31–43. In addition, the capacity to "stress" epithelial cells could play a decisive role for the initiation of the effector destructive phase of the disease. However, it remains to be explained why CD patients are particularly "sensitive" to gliadin. Both genetic and environmental factors have been advocated.

This review aims at discussing: (1) the available evidence in relation to the biological activity of p31–43, and particularly its proinflammatory properties and its ability to trigger innate immunity, (2) the mechanisms underlying such activity at the cellular level, and (3) the specific susceptibility of coeliac patients to such biological activities.



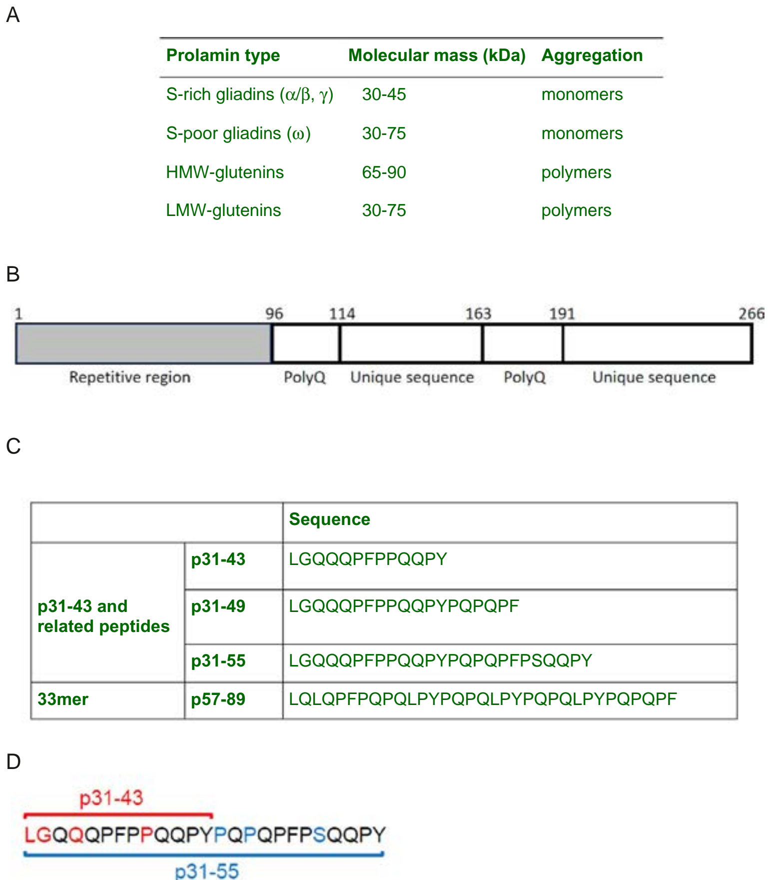
2. Wheat prolamins: A very particular group of proteins

Wheat is one of the most popular cereal crops worldwide, both in terms of production and utilization. Wheat flour contains the storage proteins present in the endosperm of the grain and is a major source of protein and dietary fibers important for human nutrition (Shewry, 2019).

As originally proposed by Osborne (1908), wheat proteins can be classified in groups according to their solubility in different solvents: albumins in water, globulins in saline solutions, and gliadins in 60–70% aqueous ethanol. The molecular mass of gliadins varies from about 30 to 75 kDa. The proteins that require more aggressive denaturing solvents in order to be solubilized are collectively termed glutenins. They have bigger structures with molecular masses ranging from 30 to 90 kDa, and can be divided into high (HMW) and low (LMW) molecular weight glutenins (Shewry and Halford, 2002) (Fig. 1A). Both gliadins and glutenins have an uncommonly high content of proline and glutamine residues, and for this reason, they are called prolamins. One of the most used classification of gliadins is based on electrophoresis in acidic conditions. α-, β-, γ-, and ω-gliadins are defined in decreasing order of mobility at pH = 3 (Bernardin et al., 1967).

Pioneering studies from Kasarda et al. (1967) characterized the specific ability of α-gliadins to self-assemble and form fibrils. After laborious biochemical techniques, Kasarda et al. (1967) and Bernardin et al. (1967) optimized a procedure to purify α-gliadins, and finally described the first complete sequence of a member of the α-gliadin type, namely, A-gliadin (Kasarda et al., 1984). This work was instrumental for our current knowledge on the molecular basis of the functional properties of wheat proteins. The gliadin sequence showed well-defined regions containing unique sequences, poly-glutamine domains, and repetitive sequences (Fig. 1B). The repetitive sequences in the N-terminal end are formed by motifs containing proline and glutamine residues, such as PQQPFPQQ (P: proline, Q: glutamine, F: phenylalanine) found in γ-gliadins. These sequences are responsible for many of the biochemical and immunochemical properties of these proteins (Shewry and Halford, 2002). As we discuss later, this region also contains the most studied peptides related with CD pathogenesis.

In addition to their solubility in different solvents, prolamins can be divided into gliadins and glutenins also based on their polymerization properties. Glutenins can polymerize by the formation of intermolecular disulfide bonds, whereas gliadins remain as monomers. In the presence of water,



A

Prolamin type	Molecular mass (kDa)	Aggregation
S-rich gliadins (α/β, γ)	30-45	monomers
S-poor gliadins (ω)	30-75	monomers
HMW-glutenins	65-90	polymers
LMW-glutenins	30-75	polymers

C

		Sequence
p31-43 and related peptides	p31-43	LGQQQPFPPQQPY
	p31-49	LGQQQPFPPQQPYPQPQPF
	p31-55	LGQQQPFPPQQPYPQPQPFPSQQPY
33mer	p57-89	LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF

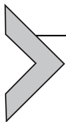
Fig. 1 Molecular characteristics of prolamins. (A) Classification of prolamins. (B) Structure of an α-type gliadin. The gray box account for the repetitive region where, the most studied peptides related to CD pathogenesis are allocated. (C) Sequence of common model peptides used in the study of pathogenic mechanisms in CD. (D) p31–55 has an almost duplicate sequence of p31–43. Panel (A): Adapted from Kasarda, D.D., et al., 1984. Nucleic acid (cDNA) and amino acid sequences of alpha-type gliadins from wheat (Triticum aestivum)*. Proc. Natl. Acad. Sci. U. S. A. 81 (15), 4712–4716. doi: 10.1073/pnas.81.15.4712.*

gliadins and glutenins form the viscoelastic structure called gluten, which is stabilized by non-covalent interactions and disulfide bond exchange, and determines the bread-making quality of wheat flour (Shewry, 2019; Shewry and Halford, 2002).

Furthermore, the purification of other components from wheat, barley, and rye (Field et al., 1982) allowed us to draw parallels between the proteins

derived from these cereals: gliadins, hordeins and secalins, respectively. Studies from the group of Shewry PR showed the relationship between the seed storage proteins from wheat, barley, and rye at the molecular level (Kreis et al., 1985) and in their immunochemical reactivity (Festenstein et al., 1984).

The product of gliadin digestion by pepsin and trypsin (commonly named as PT-gliadin) has been largely used in the investigation of the pathogenic mechanisms of CD. This enzymatic treatment, representing the gastric-pancreatic digestion, generates relatively large fragments, in accordance with the low digestibility of gluten compared to other dietary proteins. In order to characterize the remaining peptides after gluten digestion, Shan et al., (2002) analyzed the products generated after the treatment of α-gliadin with pepsin and trypsin by liquid chromatography coupled with tandem mass spectroscopy. The most representative fragment found was the one comprising residues 57–89, commonly referred to as the 33-mer. This peptide has been extensively used in research because it is highly stable to proteolytic digestion and contains a total of six T cell epitopes belonging to three distinct types: PFPQPQLPY, PQPQLPYPQ (three copies), and PYPQPQLPY (two copies). Additionally, as discussed above, the short peptide comprising residues 31–43 from α-gliadin (p31–43), which is not recognized by T cells, has also been studied. In conclusion, PT-gliadin and the synthetic peptides 33-mer and p31–43, as well as other related peptides (Fig. 1C), have been used for decades in the research on CD pathogenesis.



3. Early evidence of the toxic effects of gliadin peptides

The studies aimed at investigating the toxic effects of gliadin fragments were instrumental in generating the first insights on the mechanism of mucosal damage by gluten peptides (Browning and Trier, 1969). Intestinal biopsies were placed on a stainless-steel mesh on a Petri dish with the villus surface of the tissue specimens facing upwards. Using this approach, human mucosal biopsies could be cultivated for up to 24 h or even longer, in a medium under controlled atmosphere (95% O_2/5% CO_2) at 37 °C. These studies were conducted to evaluate the role of gliadin fractions, PT-gliadin, and later, synthetic peptides. Treated tissue pieces could be then used for histological, electron microscopy, immunochemical or physiological analysis. Although organ culture has some limitations, primarily due to the loss of connection with the vascular and lymphatic systems and because

gliadin fragments may access the interior of the tissue by a non-physiological manner, this technique allowed major advances in our understanding of CD pathology.

Falchuk et al. (1974) incubated intestinal biopsies from CD patients and healthy individuals with PT-gliadin or A-gliadin for 24–72 h and analyzed them by electron microscopy, finding that gluten fragments were toxic on microvilli at the ultrastructural level and produced changes in brush border enzymes.

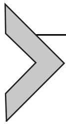
By peptic/tryptic digestion of whole gliadin and following several purification steps, Wieser et al. (1984, 1986) isolated a 53 amino acid long peptide (named B3142), which corresponds to the N-terminal sequence of A-gliadin comprising residues 3–55. This peptide showed toxic activity in organ-culture tests when samples from healthy individuals and CD patients were compared. Interestingly, a proline instead of a leucine residue was found at position 31. This is a known variation in the α-gliadin type sequence, and the one with leucine residue is most commonly used in the studies (Fig. 1C). Altogether, these studies showed that sequences with high content of proline and glutamine residues seem to be directly involved in gluten toxicity, and some motifs, such as QQQP, were initially described as necessary for the toxic effects of larger fragments.

Using organ cultures with peptides obtained after the digestion of A-gliadin with chymotrypsin, de Ritis et al. (1988) identified A-gliadin fragments comprising residues 1–30, 1–55, and 31–55 that had toxic effects, as assessed by the observed reduction in enterocyte height. On the contrary, the fragment comprising residues 56–68 did not show any toxic effects in that experimental system. However, while most of the peptides were isolated to a 98–99% purity, the chromatographic separation caused the partial contamination of p1–30 with p31–55. Therefore, the possibility that the toxic effects of p1–30 were due to the presence of p31–55 could not be excluded. These reports suggest that in organ culture assays, some of the peptides from the N-terminal region of α-type gliadins, particularly p31–55, exert toxic effects on the small intestine.

Moreover, the toxic activity of gliadin fractions has also been demonstrated *in vivo*, after instilling pure α-, β-, γ-, and ω-gliadins into the proximal small intestine in CD patients, which caused alterations in morphometric parameters of the mucosa after 6 h (Ciclitira et al., 1984). Further studies by the group of Ciclitira, using the same experimental approach, were the first to investigate the effects of synthetic peptides with sequences corresponding to amino acids 3–21, 31–49, and 202–220 of A-gliadin.

The assessment of morphometric parameters of enterocytes and the number of intraepithelial lymphocytes (IELs) 6h after the instillation of 200mg of each peptide showed significant histological changes only for p31–49 (Sturgess et al., 1994). In a second study, the authors compared the enterocyte height in duodenal samples from CD patients to that of healthy individuals and reported a significant reduction upon *in vivo* treatment with p31–49, but not with the other synthetic peptides (p3–21 or p202–220) (Shidrawi et al., 1995). These studies showed that, after instillation into the small intestine of CD patients, the p31–49 peptide is toxic *in vivo*.

The toxicity of the p31–55 fragment was also observed by Maiuri et al. (1996a) using organ cultures of intestinal biopsies from CD patients. In order to investigate the toxic activity of smaller synthetic fragments, two synthetic peptides covering the p31–55, p31–43, and p44–55 sequences were tested. While p31–43 produced the same effects (reduction of enterocyte height and induction of expression of HLA-DR in cells from the crypts) as p31–55 after 24h incubation, the concentration of p44–55 needed to obtain equivalent results higher. It is interesting to notice that the sequence of fragment p31–55 is almost identical to that of p31–43 (9 out of 12 residues are the same in the p44–55 sequence) (Fig. 1D). This work provided the first piece of evidence that p31–43, a shorter sequence (13-mer) from the originally recognized p31–55 (25-mer), was able to produce toxic effects on the small intestinal mucosa.



4. Biochemistry of p31–43

4.1 Structural analysis of p31–43

Flour from wheat has become one of the main dietary sources in human nutrition because wheat storage proteins have the unique property of forming a viscoelastic dough when they are combined with water and kneaded. During dough making, gluten proteins are the main determinants of dough properties, which eventually determine the structure and texture of the wheat flour-based baked foods. Therefore, the need to produce better products led to the crossing and selection of the better wheat varieties for each purpose for thousands of years, but more recently, molecular and structural studies have provided basic knowledge that can help us to understand the biochemical properties of these proteins. The conformation of prolamins has been studied by different techniques, but the first description of their secondary structure was provided by circular dichroism. Accordingly, the large repetitive regions of prolamins show an equilibrium between β-turn

and poly-proline II conformations, whereas the non-repetitive regions present α-helix or random conformations (Kasarda et al., 1967, 1968; Tatham et al., 1985). Using circular dichroism, Tatham et al. (1990) showed that peptides from the N-terminal region of α-gliadin (p3–55, p3–19, and p39–45) present β-reverse turns, which in water and isoosmotic saline buffers are in equilibrium with a poly-proline II structure.

Our group has recently analyzed the conformation of p31–43 using different biophysical approaches. Transmission electron microscopy showed that p31–43 spontaneously forms oligomeric nanostructures, whose size depends on the peptide concentration, whereas circular dichroism analysis showed that p31–43 presents a poly-proline II secondary structure (Gómez Castro et al., 2019). This peptide has hypochromic properties suggesting that it may undergo self-assembly in solution. The relationship between oligomer size and p31–43 concentration was also observed by atomic force microscopy. At lower concentrations (5 μM) (Fig. 2A and B), spherical nanostructures with different heights were observed, while some bigger oligomers, clusters, and precipitation were detected at 50–100 μM. The size of these structures correlated well with that from structures observed by electron microscopy. These findings suggest that p31–43 is able to self-assemble into oligomers, which interact with each other to generate bigger structures (Herrera et al., 2020).

In silico analysis, using coarse-grained molecular dynamics simulations, showed that p31–43 adopts a poly-proline II structure, before assembling spontaneously into small clusters and then, into larger structures. *In silico* modeling showed that isolated p31–43 peptides exhibit broad conformational dynamics with several poly-proline II components. Simulation of multiple replicas showed a spontaneous tendency for aggregation with a concomitant increase in the poly-proline II content (Fig. 2C) (Gómez Castro et al., 2019). The poly-proline II secondary structure of p31–43 was also confirmed by nuclear magnetic resonance experiments (Calvanese et al., 2019). The agreement of the superposition between structures of individual peptides within the oligomer identified by molecular dynamics simulations and experimentally determined structures was remarkable (Fig. 2D). Thus, different approaches support a model of p31–43 monomers in equilibrium with self-assembled structures, whereby isolated p31–43 peptides suffer minor conformational changes when passing from monomeric to oligomeric states. These findings further strengthen the idea that oligomers might work as reservoirs protecting p31–43 from degradation (Barrera et al., 2019).

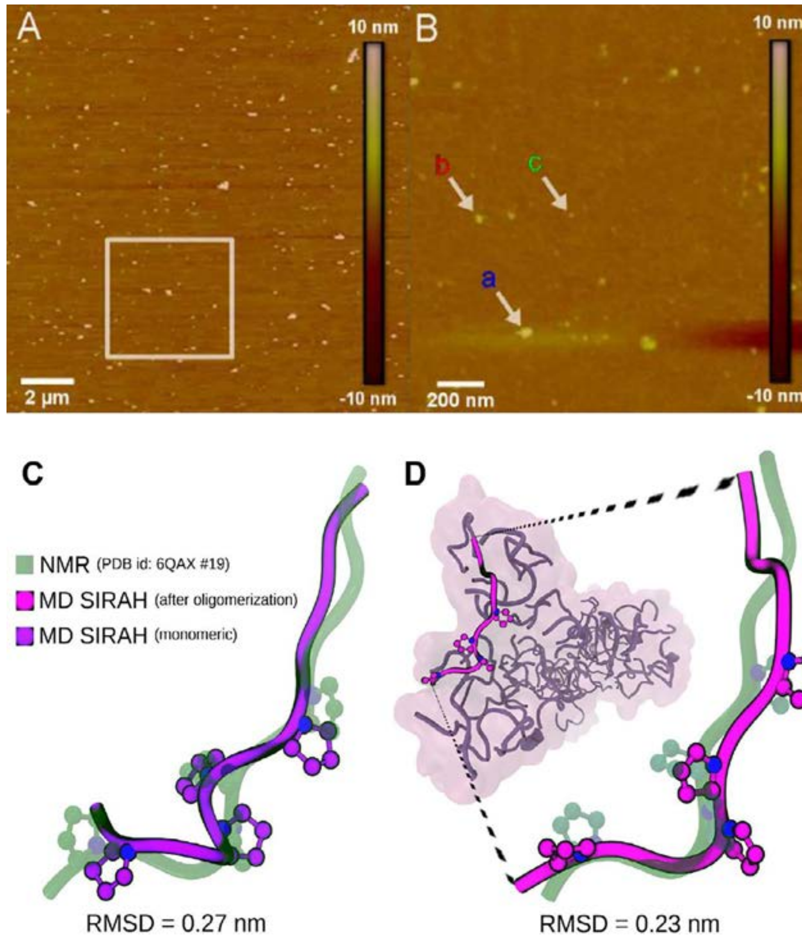


Fig. 2 Characterization of p31–43 structure and oligomer formation. (A) Analysis by Atomic Force Microscopy (AFM) of 5 μM gliadin p31–43 solutions in Milli Q water and deposited on mica surface. AFM images were obtained in tapping mode ($10 \times 10\,\mu m^2$). (B) Cross-section of the oligomers indicated in A showing representative different size oligomers: (a) blue: higher oligomers, (b) red: intermediate oligomers and (c) green: smaller oligomers. *Structural analysis of p31–43 conformation*. Structural alignments for the best matching solution between NMR and coarse-grained molecular dynamics p31–43 conformers. Low root mean square deviation (RMSD) values indicate the high degree of structural similarity observed between experiments and simulations. Similar conformations were obtained for two different simulation scenarios: (C) p31–43 in its monomeric form and (D) p31–43 after its oligomerization. *Panel (B): Adapted from Gomez Castro, M.F., Doctoral Thesis. Facultad de Ciencias Exactas. UNLP. May 2019; Gómez Castro, M.F., et al., 2019. P31-43 gliadin peptide forms oligomers and induces NLRP3 inflammasome/caspase 1-dependent mucosal damage in small intestine. Front. Immunol. 10 (Jan), 1–11. doi: 10.3389/fimmu.2019.00031. Panels (C and D): Prepared by Barrera E., PhD. Institut Pasteur Montevideo, Uruguay.*

Since this model proposes an equilibrium between isolated peptides and oligomers, free monomeric forms may also be able to cause toxic effects. Villella et al. (2019) published the first report describing the molecular effect of p31–43 monomers, which bind to the NBD1 subunit of the anion channel (cystic fibrosis transmembrane conductance regulator, CFTR), inhibit its function, and induce different inflammatory pathways (see Section 5.8).

Circular dichroism studies and molecular dynamics simulations of p31–43 over a wide pH range (3–8) did not show structural transitions, indicating that its conformation is not affected by changes in pH. Since p31–43 is an uncharged peptide, different types of intermolecular forces may be the key drivers for self-assembly. This model suggests that the aggregation process might contribute to the resistance properties of p31–43, and would not be affected by changes in pH along the gastrointestinal tract and in the small intestinal mucosa. Altogether, studies by circular dichroism, transmission electron microscopy, atomic force microscopy, and molecular dynamics simulations demonstrate that p31–43 is able to self-organize and form nanostructures (Herrera et al., 2020). Therefore, the toxic effects induced by p31–43 can be linked to either the monomeric form or to nanostructures, and could be related to the poly-proline II structure and the oligomerization properties of the peptide.

4.2 Gliadins are proteins difficult to digest

Several studies have approached the issue of gliadin digestibility in different systems. Using a sophisticated *in vitro* multi-compartment model that included oral, gastric, and duodenal phases of digestion, 25 peptides were obtained after the treatment of A-gliadin recombinant protein (Mamone et al., 2007). After the supplementation with intestinal BBM (Brush Border Membranes) containing additional enzymes, only two main peptides, a 25-mer (p31–55) and a 33-mer (p57–89), remained undigested. Overnight incubation of the synthetic p31–55 with BBM confirmed its high resistance to proteolytic enzymes. Surprisingly, in the same system, the immuno-active epitope for the adaptive T cell-mediated immune response (the 33-mer), was completely digested after BBM supplementation. This indicates that while A-gliadin can be extensively hydrolyzed *in vitro*, the peptide p31–55 remains unaffected. Interestingly, the digestion of cooked pasta, (*Triticum durum* semolina) in a similar system showed the persistence of several wheat-derived peptides, which were identified by liquid chromatography–mass spectrometry. Some of the peptides were

substantially modified, but the peptide p31–55 and its shortened form p31–43 were mostly intact (Gianfrani et al., 2015). These data indicate that p31–55, as well as p31–43, are particularly resistant to digestion by gastric, pancreatic, and BBM enzymes, and therefore, they can come into contact with the intestinal epithelium in our everyday life.

4.3 Passage across the epithelium and mechanisms of cell entry of gliadin peptides

The transport of gliadin peptides from the apical side through the intestinal epithelium occurs by a non-degradative pathway, which results in the release of intact peptides on the basal side. This route seems to be preferentially active in untreated CD patients, since a leakage through the paracellular route due to an increase in intestinal permeability does not explain this passage. In untreated CD patients, p31–49, as well as the 33-mer, appear intact on the basal side, whereas p57–68 is completely degraded during intestinal transport (Matysiak-Budnik et al., 2003).

In untreated CD patients, CD71 is overexpressed in the intestinal epithelium and binds luminal IgA or IgA anti-gliadin/gliadin complexes, which are endocytosed and transported to the basal side without lysosomal degradation (Matysiak-Budnik et al., 2008). This protected passage which is observed for p31–49 and may be active for p31–43 as well, can explain how these gliadin peptides can use a transcellular pathway to get into the tissue with no or minimal degradation.

Additional *in vitro* experiments showed that the undigested p31–43 and p57–68 peptides enter the cells by endocytosis. Their entrance into the cells requires a temperature of 37 °C and the presence of Ca^{2+} in the media (Barone et al., 2014). Experiments with inhibitors of receptor-mediated endocytosis, such as methyl-β-cyclodextrin, showed reduced entrance of p31–43 labeled with a fluorescent tag. On the other side, filipin, an inhibitor of lipid raft/caveolae-mediated endocytosis, was not able to block the entrance of p31–43. These inhibitors had the opposite effect on p57–68, indicating that both peptides enter intestinal epithelial cells by endocytosis, but only p57–68 enters the cells by lipid raft/caveolae-mediated endocytosis (Barone et al., 2014; Zimmermann et al., 2014). An additional difference between these peptides has been observed: whereas p31–43 is segregated in early endosomes after endocytosis, p57–68 is transported to late endosomes (Barone and Zimmer, 2016). Further studies have demonstrated that in a Caco-2 (human intestinal cell line) monolayer culture, p31–43 is translocated to the basal side (Barone and Zimmer, 2016), and the p31–43

transcytosis can be enhanced in the presence of anti-gliadin antibodies bound to the transferrin receptor (Mnard et al., 2012).

Some works have highlighted the role of TG2 (transglutaminase 2) on p31–43 uptake. *In vitro* studies using Caco-2 cells showed that the interaction of anti-TG2 antibodies with TG2 at the cell surface reduced p31–43 uptake, but had no effect on p57–68 uptake (Martucciello et al., 2020). The uptake of EGF by Caco-2 cells was also affected negatively by antibodies against TG2, suggesting a general role of surface TG2 in endocytosis. However, p31–43 enters HEK 293 cells, which do not express TG2, indicating that TG2 may be a regulator of peptide uptake, but its presence is not necessary for internalization. The TG2 enzymatic activity does not seem to be involved in p31–43 uptake, since enzymatic inhibitors, such as the competitive substrate mono-dansyl-cadaverine (MDC) and the inhibitor cystamine, do not interfere with p31–43 internalization (Martucciello et al., 2020). Therefore, different *in vitro* assays showed that the cell entry of p31–43 is fast and protected.

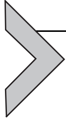
4.4 A receptor for p31–43 is missing

Several experimental approaches have sought a surface receptor for p31–43, but so far, no membrane protein has been identified as a potential p31–43 receptor/carrier (Paolella et al., 2018). This has led to the hypothesis that the entrance of p31–43 into the cell might involve a direct interaction with the cell membrane. This hypothesis is supported by several observations:

- p31–43, but not the immunogenic p57–68, can interact with a membrane-mimetic environment (Vilasi et al., 2010).
- The entrance of fluorescently labeled p31–43 into Caco-2 cells is not reduced by increasing the concentration of the unlabeled peptide, indicating that the peptide does not bind to a receptor (Paolella et al., 2018).
- p31–43 competes with HRS (Hepatocyte growth factor-Regulated tyrosine kinase Substrate), a regulator of endocytosis (see Section 5.7) present on the cytosolic side of endosomes (Vilasi et al., 2010), suggesting that p31–43 is able to penetrate the cell membrane or escape from endocytic vesicles.

This result is not entirely surprising, since many naturally occurring bioactive peptides can interact with the membranes directly and do not need a receptor to enter the cells. These peptides, defined as cell-penetrating peptides (CPPs), use endocytic pathways and then, are able to escape from vesicles (Bechara and Sagan, 2013). As described in Section 4.1, p31–43

presents a poly-proline II helical secondary structure and forms oligomers with a well-defined compact conformation, and these are characteristics shared by other CPPs (Franz et al., 2016; Herrera et al., 2020; Oba et al., 2019). Altogether, these observations strengthen the hypothesis that p31–43, owing to its peculiar sequence and structure, can enter the cells without a receptor.



5. Signaling pathways and pathological effects induced by p31–43

5.1 Activation of innate immunity

Innate immunity has been recognized as a necessary component in the development of CD (Kim et al., 2015). Under homeostatic conditions, regulatory mechanisms in the intestinal mucosa keep an adequate balance between immunity against pathogens and tolerance toward innocuous stimuli (Mowat, 2018). However, several innate pathways could become interconnected and synergize to potentiate local inflammation, crossing the threshold for the full development of an adaptative gluten-specific response through the activation of *lamina propria* $CD4^+$ Th1 cells.

Different experimental approaches, using organ culture assays, *in vitro* assays, and animal models, have demonstrated the biological effects elicited by p31–43 that lead to the induction of innate immunity mechanisms, such as IL-15 and IFNα upregulation (Maiuri et al., 2003; Nanayakkara et al., 2018).

As mentioned before, the study by Maiuri et al. (1996b) was the first demonstration of the toxic effects of p31–43 in an organ culture assay. Incubation of duodenal biopsies for 24 h with 1 mg/mL p31–43 caused a reduction of enterocyte height and an upregulation of HLA class II molecules.

Further investigations from Maiuri et al. (2003), using the same approach, were aimed at assessing the toxicity of p31–43 in comparison with two other immunogenic peptides derived from the sequence of the 33-mer, namely, p57–68 (QLQPFPQPQLPY), and p62–75 (PQPQLPYPQPQLPY), their deamidated forms, and a non-related peptide. Toxic effects were observed when the intestinal tissue from CD patients was treated with 20 μg/mL p31–43, but not with the other gliadin or control peptides tested. The TUNEL reaction showed increased enterocyte death after incubation with p31–43 incubation for 24 h. Evidence for the activation of innate mechanisms was provided by the upregulation of IL-15, ICAM1, and

HLA molecules, and the activation of dendritic cells as determined by the expression of CD83. Some of these effects were further increased when the biopsies were incubated for 3 h with p31–43 and then, with one of the other α-gliadin immunogenic peptide (p57–68) for 21 h, indicating that the first innate response step primes the system for a subsequent more robust adaptative response (Maiuri et al., 2003).

5.2 p31–43 is linked to IL-15Rα/EGFR expression

IL-15 is at the crossroad between innate immunity and p31–43 effects (Jabri and Abadie, 2015). IL-15 belongs to a family of cytokines (also including IL-2, IL-4, IL-7, IL-9, and IL-21), which share part of a heterotrimeric receptor, providing specificity for IL-15 through the α chain. IL-15 binding to its receptor leads to the phosphorylation of Jak molecules and STAT5, driving T cell and NK cell expansion. In T lymphocytes, IL-15 triggers other signaling pathways as well, including activation of distinct protein kinases, such as Src, Lck, Syk, PI3K, and MAPK (Budagian et al., 2006).

In active CD, IL-15 and its receptor are overexpressed in duodenal tissue (Bernardo et al., 2008; Maiuri et al., 2000). This cytokine plays multiple roles in inducing changes on epithelial cells, IELs, $CD4^+$ and $CD8^+$ T lymphocytes, and dendritic cells from the *lamina propria* of the small intestine (Maiuri et al., 2000; Mention et al., 2003; Meresse et al., 2004). Immunofluorescence analysis of sections of duodenal tissues from CD patients treated with 20 μg/mL p31–43 for 3 h showed increased expression of IL-15 (Maiuri et al., 2003). *In vitro* stimulation of Caco-2 cells with gliadin-derived p31–43, but not p57–68, upregulated the mRNA levels of IL-15 after overnight incubation but not after shorter incubation times (3 or 6 h). Upregulation of IL-15 surface expression was also observed in p31–43-treated Caco-2 cells by flow cytometry (Barone et al., 2011). Furthermore, western blot analysis of membrane fractions of Caco-2 cells treated with p31–43 showed an increment in the expression of IL-15 and its receptor IL-15Rα (see Section 5.2). This is linked to the biological activity of IL-15 bound to its membrane receptor (Duitman et al., 2008), which, in addition to the already mentioned effects on T and NK cells, affects the expression of other molecules, such as stress markers (see Section 5.5). The *in vitro* induction of IL-15 was also reported by Vincentini et al. (2015), who analyzed the protein extracts of T84 cells (human intestinal epithelial cells) stimulated with 40 μg/mL p31–43 for 24 h. Furthermore p31–43 activated the IL-15 downstream effector STAT5 in epithelial cells and fibroblasts from

CD patients (Lania et al., 2019; Nanayakkara et al., 2013). Interestingly, IL-15 induced cell proliferation in the intestine, indicating that innate immunity and enterocyte proliferation are closely related (Reinecker et al., 1996). Evidence obtained from Caco-2 cells and intestinal biopsies from CD patients have demonstrated that IL-15 and EGF cooperate in inducing enterocyte proliferation (see Section 5.2).

In untreated CD patients, the intestinal mucosa suffers from chronic and aggressive damage, and an increase in proliferative activity is part of the strategy to remodel the mucosa and control tissue destruction (Przemioslo et al., 1995). Different studies carried on cell lines and biopsies from untreated and potential CD patients showed that among the multiple pathways that might contribute to induce proliferation, p31–43 acts in an EGFR-mediated manner. The effect mediated by p31–43 was specific for epithelial cells found in the crypts of CD patients, and it was not observed in tissues from healthy subjects (Barone and Zimmer, 2016; Barone et al., 2007; Nanayakkara et al., 2013).

5.3 NFκB and MAPK signaling driven by p31–43

The NFκB pathway is considered the masterpiece of the inflammatory response. *In vitro* studies using Caco-2 showed that incubation with 50 μg/mL p31–43 for 45 min increased the expression of p65 NFκB. In parallel, the increased levels of ERK phosphorylation showed that the MAPK pathway was also activated (Capozzi et al., 2013). Thus, these pathways were rapidly induced by p31–43 *in vitro*. The NFκB pathway is activated in the intestinal mucosa of untreated CD patients, and remains active even during the remission phase. This observation suggests that following the induction of the inflammatory response by gluten peptides, other mechanisms maintain an ongoing inflammatory process in the intestinal mucosa of CD patients regardless of the further gluten intake (Castellanos-Rubio and Bilbao, 2018). NFκB and MAPK pathways were also found active in fibroblasts derived from the skin and small intestine of CD patients. This is remarkable since the skin fibroblasts are at a significant distance from the tissue with the active inflammatory response (Lania et al., 2019). Interestingly none of the effects on NFκB or MAPK signaling were induced by the immunogenic peptide p57–68. Remarkably, p31–43 could activate the MAPK and NFκB pathways in cells from control subjects, suggesting that p31–43 "*per se*" has the ability to induce inflammation. Studies using

synthetic peptides containing alanine replacements along the p31–43 sequence demonstrated that the reported effects are specific for this peptide (Lania et al., 2019).

5.4 Cellular stress caused by p31–43

The production of reactive oxygen species (ROS) and ER-stress are routes for cellular distress leading to inflammation. The production of ROS by gliadins was demonstrated by different *in vitro* assays (Monguzzi et al., 2019; Orlando et al., 2019; Rivabene et al., 1999), *ex vivo* assays using duodenal biopsies (Monguzzi et al., 2019) and *in vivo* studies using the *C. elegans* model (Lim et al., 2018). Interestingly, Maiuri et al. (2010) found that when T84 cells were treated with 20 μg/mL p31–43 for 24 h, the peptide was accumulated in vesicles, a result that is consistent with the induction of oxidative stress as early as 3 h post-treatment. These effects were not observed when a scrambled-sequence p31–43 peptide, the p57–68 and p62–75 gliadin peptides, or a peptide with unrelated sequence were used instead. These authors also showed that incubation of duodenal biopsies from CD patients but not from healthy controls with p31–43 for 3 h induced ROS through the activation of the peroxisome proliferator-activated receptor (PPARγ).

CD is an example of a pathology driven by specific $CD4^+$ Th1 cells. These T cells preferentially recognize gluten peptides that have been deamidated by the enzyme TG2. TG2 catalyzes peptide deamidation by converting specific glutamine residues to glutamate at certain positions. Negatively charged anchor residues are responsible for the preferential binding of deamidated gluten peptides to the disease-associated HLA alleles, DQ2 and DQ8. This enzymatic activity generates one of the best characterized post-translational modification on neoepitopes as a way to break T cell tolerance to self-antigens and promote autoimmunity (Sollid and Jabri, 2013).

TG2 is a Ca^{2+}-dependent enzyme with multiple physiological roles (mitochondria function, cell adhesion, migration, and phagocytosis, among others), which explains why it appears as a mediator in so many human diseases, such as inflammatory disorders (rheumatoid arthritis, lupus, uveitis), neurological diseases (Alzheimer's disease, Parkinson's disease, Huntington's disease), and cancer (Szondy et al., 2017). The expression of TG2 gene is regulated by the canonical NFκB pathway; consequently, it can be induced by proinflammatory cytokines such as TNFα. In addition, TG2 can activate

NFκB by blocking the inhibitory function of IκBα *via* polymer formation (Lee et al., 2004), leading to a complex cross-regulation between TG2 activity and the NFκB pathway, a circuit that plays a role in inflammation and cancer (Mehta et al., 2010). Importantly, the combination of TNFα and IFNγ produce a strong synergistic effect on TG2 expression in human cell lines and duodenal biopsies of untreated CD patients (Bayardo et al., 2012).

In the enterocytes of untreated CD patients, the levels of TG2 are increased in the brush border, cytoplasm, and also in the extracellular matrix (Caputo et al., 2013; Esposito, 2003; Hansson et al., 2002; Martucciello et al., 2020), TG2 is predominantly localized inside the cells, but following an unconventional secretion route, which depends on TG2 binding to phosphoinositides on endosomal membranes, it is delivered in the recycling endosomes and becomes secreted (Zemskov et al., 2011). Although TG2 has been observed in different cellular locations, the precise site where gliadin deamidation and cross-linking takes place has not been properly defined. Since appropriate Ca^{2+} levels are critical for TG2 activity, the low Ca^{2+} concentration inside the cell, as well as in the extracellular space, keeps intracellular and secreted TG2 in an inactive conformation (Siegel et al., 2008). Recent work from Sollid's group, showed that TG2 can be released into the lumen from shed enterocytes, implying that deamidation can occur in the intestinal lumen prior to peptide internalization to the tissue (Iversen et al., 2020).

There is a complex connection between Ca^{2+} levels and TG2 function, which links mitochondria and the ER. D'Eletto et al. (2018) showed that TG2 interacts with GRP75 (glucose-regulated protein 75) in mitochondria-associated membranes, creating a close contact between the ER and mitochondria. These physical bridges promote the transport of Ca^{2+} from the endoplasmic reticulum to mitochondria. Therefore, TG2 also participates in the regulation of intracellular Ca^{2+}.

Taking into account that Ca^{2+} levels are critical for many aspects of cellular physiology but particularly for TG2 function, the effect of p31–43 on Ca^{2+} mobilization was assessed on Caco-2 cells. *In vitro* assays showed that in Caco-2 cells, low amounts of p31–43, such as 0.2 μg/mL, were able to mobilize Ca^{2+} from the ER and the mitochondria. The consequent increase in cytosolic Ca^{2+} levels led to the activation of the TG2 enzymatic function in the cytosol (Caputo et al., 2012). When Caco-2 cells were treated with 20 μg/mL p31–43 for 24 h, an increase was observed in the expression of TG2, as well as in that of the glucose-regulated protein-(GRP)78 and of the CCAAT/enhancer binding protein-homologous protein, which are

both biochemical markers of ER-stress. None of these effects was observed for p57–68 (Caputo et al., 2012). Further work in Caco-2 cells, showed that p31–43, but not p57–68, could bind to Ca^{2+}-activated TG2 (Villella et al., 2019). p31–43 was found covalently bound to TG2, promoting the formation of a supramolecular complex involving TG2 and the NBD1 subunit of CFTR channel (see Section 5.8). Thus, activation of TG2 may sustain the p31–43-mediated inhibition of CFTR function through the formation of covalent links between p31–43, CFTR, and TG2, generating a trimolecular complex. Therefore, p31–43 can cause a cascade of different events, including ROS induction, an ER-response, and the mobilization of intracellular Ca^{2+} from ER store, activating TG2, which would then participate in the inflammatory response, as well in the deamidation of immunogenic gliadin peptides and the formation of TG2/gliadin complexes, inducing the formation of anti-TG2 autoantibodies (Iversen and Sollid, 2020).

5.5 Expression of stress molecules: The case of MICA

MHC class I chain-related A (MICA) proteins are non-classical MHC class I polymorphic proteins, which, in the gastrointestinal epithelium, are conformationally stable without being bound to conventional MHC class I peptides. Therefore, MICA molecules have no role in conventional antigen presentation, but their functions are associated with responses under cellular stress (Groh et al., 1996). Unlike the case of MHC class I genes, the MICA promoter region contains elements found in the genes of heat shock proteins. Under conditions of cellular stress, such as thermal, oxidative stress, viral infections, and tumor transformation, MICA expression is upregulated (Groh et al., 1996; Perera et al., 2007; Raulet, 2003). The surface expression of MICA has been shown to be a signal for enterocyte killing by interacting with the NKG2D receptor constitutively expressed on NK cells, $CD8^+$ α/β T cells, and intestinal intraepithelial γ/δ T cells (Hüe et al., 2004; Meresse et al., 2004; Raulet, 2003). NKG2D expression and signaling, as well as MICA expression (Hüe et al., 2004), are upregulated by IL-15.

In the intestinal mucosa during active CD, MICA associated to the intracellular compartment was redistributed to the cell surface (Hüe et al., 2004; Meresse et al., 2004). Remarkably, the increased MICA levels returned to normal under gluten-free diet, highlighting the importance of the signals derived from gliadin peptides in the direct or indirect, through cellular stress, triggering of MICA expression (Allegretti et al., 2013). It has been reported

that the gliadin peptides p31–49 (Hüe et al., 2004) and p31–43 (Allegretti et al., 2013) increase MICA expression. Together, these observations suggest that stress conditions induce the expression of MICA in enterocytes, while inflammation and IL-15 potentiate the cytotoxicity activity of some IEL subsets. These findings support a key role for MICA/NKG2D in the activation of intraepithelial immunity in response to enterocyte damage (Hüe et al., 2004; Meresse et al., 2004). Since p31–43 can promote ER-stress, which may induce MICA overexpression (Allegretti et al., 2013) and IL-15 (Barone et al., 2011; Maiuri et al., 2003), this may be one way by which p31–43 mediate enterocyte cytotoxicity. More recently, Jabri's group reported that in CD, additional enterocyte markers can modulate the specificity of the IEL response, in particular, that of some of the TCRγ/δ^{+} subsets (Mayassi et al., 2019).

5.6 Induction of mediators of inflammation: *In vitro* findings

It has been found that several proinflammatory cytokines and chemokines that play a key role in the orchestration of the inflammatory response in the intestinal mucosa, promoting the recruitment and activation of immune cells, are produced as consequence of p31–43 stimulation.

The production of key inflammatory mediators was evaluated *in vitro* using T84 cells (human intestinal epithelial cells), stimulated with 40 μg/mL p31–43 for 3 h in a transwell co-culture system in the presence of peripheral blood mononuclear cells (PBMC) (Vincentini et al., 2015). Quantitative ELISA in samples from the culture supernatant showed increased production of IL-1β, and also of the inflammatory chemokines CCL2, CCL3 (MIP1α), and CCL4 (MIP1β), which are major chemoattractant for monocytes, macrophages, and dendritic cells.

The inflammatory effects of p31–43 were also observed in mast cells. Using LAD2 cells, a human mast cell line, Frossi et al. (2017) demonstrated that 100 μg/mL p31–43 was able to induce degranulation, assessed by the release of β-hexosaminidase. Interestingly, flow cytometry analysis showed that p31–43 binds to LAD2 cells and activates the NFκB pathway. After incubation for 24 h, p31–43 was found to induce the expression of cytokines and chemokines in the culture supernatant, particularly that of IL-6, TNFα, IL-17, CXCL8, CCL2, but not of IL-1β, IL12p70, or IL-10. These effects were not observed for other dietary proteins (rice and soya proteins) nor for the immunogenic gliadin peptides p57–68 (QLQPFPQPQLPY) and p62–75 (PQPQLPYPQPQLPY). It is noteworthy that after incubation

with p31–43, mast cells isolated from duodenal biopsies of CD patients with severe histological changes (as partial or total villous atrophy) exhibited significantly higher histamine release compared with cells isolated from control patients (Frossi et al., 2017).

Undoubtedly, type I IFNs play a critical role in the amplification of the inflammatory response in the coeliac lesion. In duodenal biopsies of CD patients, who were either untreated or in remission, and under a gluten-free diet, but not in those from healthy controls, incubation with p31–43 upregulated the IFNα pathway (induction of IFNα and MxA proteins, as well as of IFNα mRNA 7 and 17), suggesting that p31–43 may contribute to the expansion of the effect of this pathway in the small intestine of patients with CD (Nanayakkara et al., 2018). Studies with mouse models confirmed the rapid induction of type I IFNs after p31–43 treatment, as well as the fact that type I IFN signaling is relevant for the intestinal pathology, since p31–43-dependent enteropathy was not observed in IFNαR deficient mice (Araya et al., 2016; Gómez Castro et al., 2019). Thus, type I IFNs also play a role in the loss of oral gluten tolerance in CD patients (Kim et al., 2015; Monteleone et al., 2001).

Since a growing body of evidence suggests that gliadin peptides induce an innate response in the intestine, Thomas et al. (2006) performed studies on small intestinal segments in mice. PT-gliadins and the 33-mer and p31–43 peptides induced zonulin release and increased the intestinal permeability, as assessed by a reduction in the transepithelial resistance. These effects were dependent on MyD88, but TLR2 or TLR4 were not required. The authors also observed that incubation of primary macrophages from C57BL/6 mice with 100 μg/mL of p31–43 for 3 h led to the production of proinflammatory cytokines (TNFα, IL-12 p35/p40, IL-6, and IFNβ) and chemokines (CXCL10 and MCP-5). The requirement of MyD88 for some of the biological effects mediated by p31–43 was also documented by Frossi et al. (2017). These authors found that murine mast cells from wild-type, but not from MyD88-deficient mice, challenged *in vitro* with 100 μg/mL p31–43 for 24 h produced proinflammatory cytokines, including IL-6, IL-17, TNFα, and MCP-1.

The induction of a plethora of proinflammatory cytokines and chemokines by p31–43 observed by *in vitro* tests using human cell lines, isolated intestinal human cells, or mouse models highlights the relevance of the role of p31–43 for the generation of a strong proinflammatory setting, necessary for the full development of a specific adaptive response against gluten-derived peptides.

5.7 Consequences of vesicular trafficking delay on signaling pathways

In Caco-2 cells (Barone et al., 2007, 2010; Maiuri et al., 2010) and enterocytes isolated from biopsies of the small intestine (Barone et al., 2007; Zimmer et al., 2010). Both p31–43 and p57–68 gliadin peptides have been observed to enter the cells by endocytosis and interact with the vesicular system. However, while the p57–68 passes from early to late endosomes in a short time, p31–43 remains localized in early vesicles, slowing down their motility and maturation toward late endosomes (Barone et al., 2007; Zimmer et al., 2010). In fact, using two very different experimental approaches, namely, pulse-chase immunofluorescence experiments (Barone et al., 2007) and electron microscopy (Zimmer et al., 2010), it has been demonstrated that in intestinal biopsies of CD patients, p31–43 trafficking is delayed at the stage of early vesicles (Barone and Zimmer, 2016). Therefore, p31–43, but not p57–68, slows the maturation of early vesicles carrying EGFR (Epidermal Growth factor receptor) and, presumably, other receptors, which can then remain active for longer periods of time. This is a well-known effect by which vesicular trafficking regulates the signaling of extracellular receptors and *vice versa* (Ceresa and Schmid, 2000).

Vesicular trafficking is regulated by complex mechanisms involving several molecules, such as Hepatocyte growth factor-regulated tyrosine kinase (HRS), which is a key protein for endocytic maturation because it recruits clathrin to early endosomes (Raiborg et al., 2001). HRS is located on early vesicles and the multivesicular body, which participates in the ubiquitination of proteins destined for lysosomes. HRS can be phosphorylated in cells treated with growth factors or cytokines, and can also become ubiquitinated (Komada and Kitamura, 1995). These post-translational modifications are necessary for the efficient HRS-mediated sorting of various proteins for degradation. Interestingly, p31–43 shares a strong amino acid sequence similarity with a small domain of HRS rich in proline/glutamine, which is necessary for the localization of HRS on the membrane of vesicles. Although gliadins are known to contain domains rich in glutamine and proline, the high homology of p31–43 with the HRS domain is restricted to this peptide (Barone et al., 2010). Experiments carried out in HRS-EGFP transfected Caco-2 cells, showed that p31–43, but not p57–68, colocalizes with HRS on the membrane of endocytic vesicles, mediating the translocation of HRS from the vesicular membrane into the cytosol, suggesting that p31–43 interferes with HRS-mediated early endosome maturation (Barone et al., 2010).

Growth factor receptors come to the scene when the consequences of the vesicular trafficking delay need to be considered. The activity of growth factor receptors is influenced by feedback mechanisms that regulate their degradation (Burke et al., 2001; Wilde et al., 1999). In cells in which HRS has been silenced, mutated, or dislocated, EGFR (Epidermal Growth Factor Receptor), which mediates the most potent mitogen signal in epithelial cells (Marshall, 1995), and other tyrosine kinase receptors, can be recycled on the cell surface extending their lifespan (Raiborg and Stenmark, 2002; Raiborg et al., 2008).

A delayed transition from early to late endocytosis, with a consequent delayed inactivation of EGFR, has been observed in enterocytes of intestinal biopsies from CD patients treated with p31–43 (Barone and Zimmer, 2016; Barone et al., 2007). In this context, p31–43 increased the proliferation of crypt enterocytes in CD patients, but not in healthy controls, an effect that was prevented by EGFR inhibitors. In fact, p31–43 slows down the trafficking of early vesicles that carrying EGFR, which therefore, remains active for longer time (Barone et al., 2007). Moreover, pulse and chase experiments with EGF-Alexa in intestinal biopsies of untreated patients, showed that p31–43 treatment delayed the trafficking of vesicles carrying the EGF/EGFR complex (Barone and Zimmer, 2016; Barone et al., 2007) (Fig. 3).

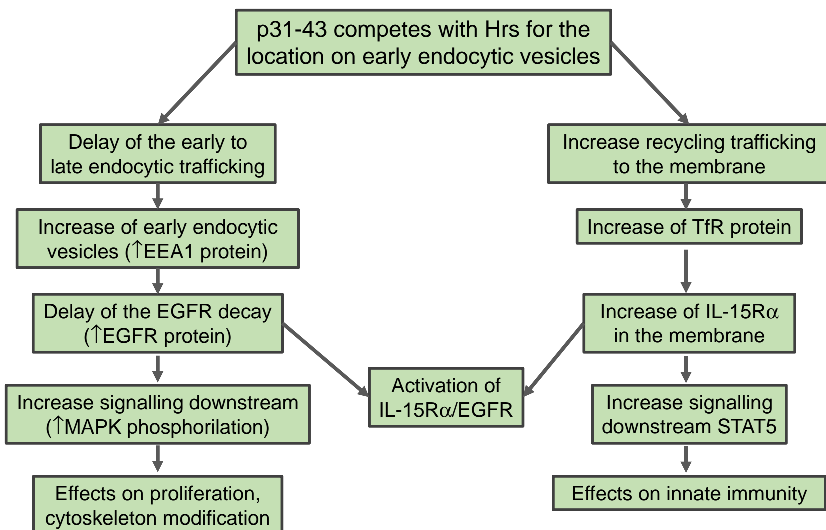


Fig. 3 Effects of p31–43 on endocytic trafficking. Cascade of events affected by p31–43 on early and recycling endocytic trafficking and biological consequences.

The biological effects of trafficking delay depend on the array of receptors present on the cell. The interplay between different receptors is a known phenomenon in biology, and EGFR is especially prone to form complex network with other receptors, including cytokine receptors (Sanchez-Guerrero et al., 2013). One such complex having multiple functions in the intestinal mucosa is the one formed between EGFR and IL-15Rα. The EGFR/IL-15Rα complex is crucial for the proliferation of crypt enterocytes. In Caco-2 cells, the presence of p31–43 increased complex formation between EGFR and IL-15Rα (Nanayakkara et al., 2013). Each of the ligands (EGF of IL-15) can initiate signaling by the EGFR/IL-15Rα complex. Furthermore, EGF can activate the transcription of IL-15, and IL-15 the transcription of EGF (Nanayakkara et al., 2013). The proliferation of cells induced by p31–43 can be prevented by treatment with IL-15 and EGFR specific antibodies in duodenal biopsies from CD patients, or by specific antibodies or siRNA in Caco-2 cells (Barone et al., 2011; Nanayakkara et al., 2013). These findings demonstrate the interplay between p31–43 and EGF/IL-15, occurring at different levels (Figs. 3 and 4A).

The delay to transition from early to late vesicles due to HRS silencing or deletion can interfere with the degradation of tyrosine kinase receptors through recycling. Interestingly, cytokine receptors are recycled to the cell surface, whereas tyrosine kinase receptors are mainly degraded in late vesicles. Therefore, the trafficking blockade induced by the presence of p31–43 or an HRS malfunction can also interfere also with the function of cytokine receptors. As a consequence of this process, an increasing amount of transferrin receptor (TfR) and IL-15Rα accumulates on the cell surface upon treatment with p31–43 (Barone et al., 2011; Lania et al., 2019). IL-15 is known to be localized in the recycling endocytic vesicles and in the Golgi complex (Fehniger and Caligiuri, 2001; Gaggero et al., 1999). Since p31–43 delays the maturation of these vesicles to lysosomes, IL-15 accumulation has also been observed (Barone et al., 2007, 2010; Zimmer et al., 2010). p31–43 also increases the number of recycling vesicles carrying IL-15, as well as the amount of IL-15 and its receptor on the surface of Caco-2 cells, but not the amount of the cytoplasmic or secreted protein (Barone et al., 2011).

The mechanisms that regulate vesicular trafficking are also important for the response to viral infections. TLR7 is one of the endosomal receptors that interact with the viral genome (specifically, ssRNA, occurring in rotavirus infection), and is regulated by endosomal trafficking (Heil et al., 2003; Pane et al., 2014). HRS is also a key factor in the trafficking of endosomal

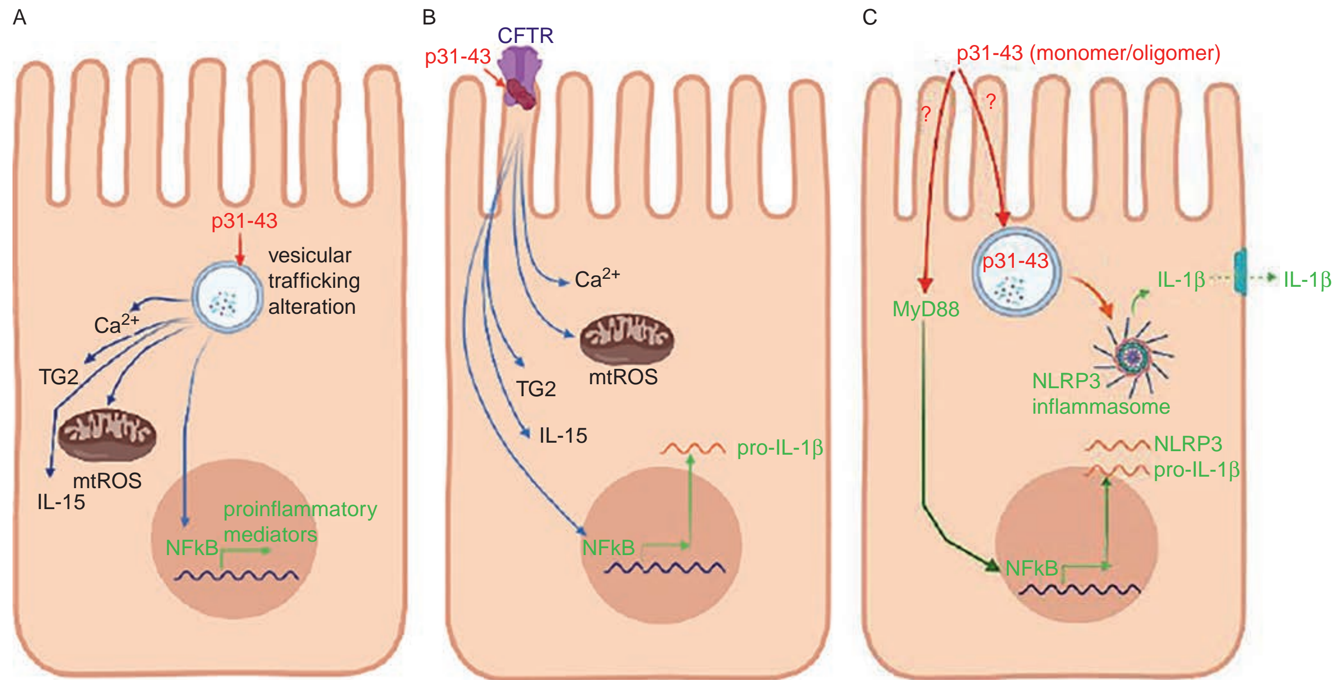
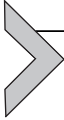


Fig. 4 Schematic representation of multiple proinflammatory effects driven by gliadin p31–43 peptide in enterocytes. (A) Gliadin p31–43 peptide causes alteration on vesicular trafficking leading to vesicular stress associated to multiple effects such as increased intracellular Ca^{2+} concentration, activation of TG2, alteration of mitochondria function and release of ROS, with a parallel NFκB activation and upregulation of IL-15 and proinflammatory mediators. (B) Gliadin p31–43 peptide binds to the NBD1 domain of CFTR, keeping it in inactive conformation, thus blocking CFTR function as an anion channel. As a consequence, TG2 is activated and forms a tripartite complex that stabilizes p31–43 binding to CFTR. CFTR inhibition leads to impaired endosomal trafficking, cytoskeleton disassembly, NFκB activation, IL-15 production, and inflammasome activation resulting in IL-1β secretion. (C) Mucosal damage induced by p31–43 requires MyD88. The activation of this pathway leads to upregulation of proinflammatory genes, among them NLRP3 and pro-IL-1β. In addition, p31–43 causes vesicular stress, which may release signals leading to assembly and activation of the NLRP3 inflammasome. Active caspase-1 produces mature IL-1β and N term GSDMD which oligomerizes and forms membrane pores. These pores allow the release of IL-1β and alarmins, and can cause cell death by pyroptosis. Mechanisms of p31–43 cell entry, accumulation in vesicles and activation of MyD88 pathway are still unknown.

receptors such as TLR7, and is necessary for the ubiquitin-dependent targeting to the lysosomes (Chiang et al., 2012). In fact, the early/late delay of the maturation of the vesicular system, observed in Caco-2 cells upon treatment with p31–43 or HSR silencing, produces effects relevant for the TLR7 pathway, such as the delay in trafficking of the TLR7 in the early vesicles, the increase in complex TLR7/MyD88 formation, the increase in the levels of type I IFN and MxA proteins, and the activation of NFκB (Nanayakkara et al., 2018). In addition, the viral ligand loxrubine, which is specific for TLR7, and p31–43 activate the same signaling pathways that depend on TLR7 activation, and cooperate in inducing the activation of the type I IFN pathway (Nanayakkara et al., 2018).

5.8 A new link: p31–43 and the ion channel cystic fibrosis transmembrane conductance regulator (CFTR)

Cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-activated Cl^- and HCO_3^- conducting channel expressed in epithelial cells. The structure of CFTR presents two transmembrane domains (TMD1 and TMD2), two nucleotide-binding domains (NBD1 and NBD2) and a regulatory domain. Studies by Villella et al. (2019), demonstrated that p31–43, but not p57–68, binds to the NBD1 subunit of CFTR, reducing its ATPase activity. *In silico* modeling predicted that p31–43 interacts with NBD1 by binding at the boundary between NBD1 and TMD1, stabilizing the inactive conformation of CFTR. Loss of CFTR function causes an increase in ROS generation and a persistent activation of TG2, leading to increased nuclear translocation of NFκB. The activation of the NFκB pathway induces the transcription of proinflammatory cytokines, such as IL-17A, IL-21, and IL-15, which are closely related with different effector mechanisms in CD pathogenesis, as well as of components of the NLRP3 inflammasome pathway, which, upon activation, leads to the production of active IL-1β. These results were obtained *in vitro*, in assays using Caco-2 cells or PBMC from CD patients, as well as *in vivo*, in mouse models (Villella et al., 2019). The use of a compound maintaining CFTR in the active conformation prevented these p31–43 effects. Collectively, these findings showed that p31–43 can alter CFTR function, with multiple biological consequences for the pathogenesis of CD and, importantly, for other processes associated with the inflammatory response that may have an impact in other tissues as well (Fig. 4B).



6. Use of experimental mice models to unveil the *in vivo* effects of p31–43

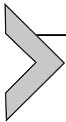
Experimental mouse models have been developed to increase our understanding of the pathogenic mechanisms of gliadin peptides. Initial work from Troncone and Ferguson (1991) showed that mouse models were useful to investigate the immune response in the small intestine and to assess the pathogenic mechanisms underlying CD. Further work, using more complex models based on transgenic mice, were aimed at dissecting the mechanisms of mucosal damage involving the whole immune response (Ryan, 2015). Some of these models investigated the role of HLA molecules (Black et al., 2002; Marietta et al., 2004), IL-15 and T cell activation (Korneychuk et al., 2015; Yokoyama et al., 2011), the role of TG2 (Dafik et al., 2012), regulatory T cells (Du Pré et al., 2011; Galipeau et al., 2011; Van Leeuwen et al., 2017), the effect of microbiota (Caminero et al., 2019), and viral infections (Bouziat et al., 2017). All this information helped building the most complex mouse system, which allows drawing a more complete picture of CD pathology, incorporating most of the aspects related to IL-15, T cell activation, and IELs cytotoxicity (Abadie et al., 2020). Since the model developed by Bana Jabri's group recapitulates innate and adaptive mechanisms playing a role in CD pathogenesis, it could be also a very useful tool for the preclinical validation of therapeutic interventions.

The main goal of the various therapeutic strategies is the restoration of gluten tolerance in CD patients. Freitag et al. (2020) demonstrated the efficacy of nanoparticles encapsulating gliadin proteins to reduce gliadin-specific T cell proliferation, the production of inflammatory cytokines and circulating gliadin-specific antibodies, in three different mouse models (C57BL/6, RAG-deficient and huDQ8-transgenic). In addition, FoxP3 levels and the expression of genes associated with the regulatory response were enhanced. These findings in mice suggest an interesting potential strategy for therapeutic intervention in patients.

These mouse models were developed to assess the pathogenic mechanisms underlying CD, but they were mainly focused on the analysis of the gluten-specific response, and as a consequence, the role of innate immunity has been given less attention.

To evaluate whether p31–43 is able to drive innate mechanisms in the intestinal mucosa *in vivo*, a mouse model of enteropathy based on the

intraluminal delivery of p31–43 by a surgical procedure was developed (Araya et al., 2016). This study showed that p31–43 reduces the villus height/crypt depth (V/C) ratio and increases the number of intraepithelial lymphocytes (IELs) (the most common morphological parameters used to evaluate changes in the mucosa of the small intestine). Experiments using deficient mice showed that these changes were dependent on MyD88 and type I IFNs, but not on TLR4. Inflammatory mediators (IFNγ, IFNβ, and CXCL10) were upregulated in the intestinal mucosa 4 h after p31–43 treatment. Later, to avoid the inflammation caused by the surgical procedure, we demonstrated the toxic effects of p31–43 by intragastric delivery (Gómez Castro et al., 2019). In this model, the pathology in the upper small intestine, characterized by a reduced V/C ratio and a higher number of IELs, was observed 16 h after intragastric administration of 10 μg of p31–43. Treated mice also showed increased levels of IFNβ mRNA in the mucosa 4 h after treatment. Importantly, the effects of p31–43 on the intestinal mucosa were sequence specific, since synthetic peptides with an inverted (YPQQPFPPQQQGL) or scrambled sequence (YQPLFQPQGPQPQ) did not show any effect even at a 10 times higher dose. The structure of p31–43 described in Section 4.1, markedly different to that of the scrambled or inverted peptides, may be responsible for such biological effects. Together, these results indicate that p31–43 activates *in vivo* innate immune pathways, such as type I IFNs dependent inflammation, and particularly, the inflammasome pathways (see Section 7).



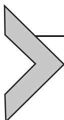
7. Inflammasome activation by p31–43

Inflammasomes are cytosolic macromolecular complexes that assemble upon sensing components from pathogens or host damage-associated molecular patterns (DAMPs). The assembly of the inflammasome triggers the activation of caspase-1, which subsequently, induces the cleavage and maturation of the proinflammatory cytokines IL-1β and IL-18. Caspase-1 also cleaves gasdermin D (GSDMD), which oligomerizes and forms pores in the cellular membrane, leading to a programmed cell death called pyroptosis.

Evidence of inflammasome activation by gliadins came first from studies by Harris et al. (2010), which showed that incubation of PBMC from CD patients with PT-gliadins led to the release of IL-1β. Then, Palová-Jelínková et al. (2013) demonstrated the activation of the NLRP3 inflammasome by PT-gliadins. In this study, treatment of PBMC from CD patients with

PT-gliadins induced IL-1β and IL-1α production, while treatment with the caspase-1 inhibitor *Z*-YVAD-fmk reduced the release of IL-1β but not of IL-1α. Blocking K^+ efflux by incubating with a high extracellular K^+ concentration or by inhibiting K^+ channels, using quinidine or glybenclamide, suppressed IL-1β production. Experiments using cells from deficient mice showed that maximal production of IL-1β induced by PT-gliadins required TLR4/MyD88 signaling and was NLRP3- and ASC-dependent. Since these studies highlight the induction of inflammatory mediators by PT-gliadins through the inflammasome, later work aimed at determining directly whether p31–43 could activate the inflammasome platform.

In vitro assays, using THP1-ASC-GFP reporter cells, showed that p31–43 promotes ASC speck formation and the assembly of the inflammasome multimolecular platform (Gómez Castro et al., 2019). Importantly, intragastric administration of p31–43 to wild-type mice induced activation of NLRP3, caspase-1, IL-1β, and GSDMD. *In vivo* inhibition of caspase-1 precluded the activation of IL-1β and GSDMD upon p31–43 treatment, demonstrating the rapid induction of the NLRP3 inflammasome (Gómez Castro et al., 2019; Ruera et al., 2020). Our work demonstrated that activation of the inflammasome by p31–43 depends on NLRP3, ASC, and caspase-1, but the underlying molecular mechanism is unknown. As described above, p31–43 has a number of different toxic effects, known to produce various types of cellular stress, such as ER-stress and alterations of the mitochondria, which may promote NLRP3 activation. In contrast with other rather selective inflammasomes, NLRP3 is activated by a range of stimuli, including bacterial toxins (e.g., pore-forming toxins), host metabolites (e.g., uric acid and cholesterol crystals), and environmental irritants (e.g., asbestos and silica) (Swanson et al., 2019), which make difficult to identify the molecular connection between p31–43 and NLRP3 inflammasome assembly and activation. However, the particular conformation of p31–43 and its tendency to form oligomers are likely to be responsible for NLRP3 inflammasome activation (Fig. 4C).



8. A modern view of the role of p31–43 in CD pathogenesis

Here, we have reviewed literature of more than 20 years, describing the multiple proinflammatory effects of gliadin in general, and the gliadin p31–43 peptide in particular, in several cellular systems, mouse models,

and intestinal tissues from CD patients. Subsequently, the question to be answered after having collected evidence from different experimental approaches is: why p31–43 affects only CD patients?

The answer may reside in the constitutive alterations present in celiac subjects. These involve several biological pathways, such as signaling/proliferation, stress/innate immune response and inflammation (Barone et al., 2014; Dieterich et al., 2020; Lania et al., 2019). A notable example is the NFκB pathway, constitutively altered in CD, with more than 20 components of the pathway increased in biopsies from patients on a GFD (Fernandez-jimenez et al., 2014; Lania et al., 2019). The constitutive inflammatory condition in CD cells is confirmed both in skin and intestinal fibroblasts from CD patients on a GFD with an increase of the activation of NFκB and MAPK1 (Lania et al., 2019). One possible candidate as responsible for this condition of inflammatory environment is the alteration of vesicular trafficking. Interestingly in fact, inducing a delay of the endocytic trafficking by silencing the HRS protein, produces at cellular level the same alterations that p31–43 does. Moreover, many non-HLA loci and e-qtl effects found related to CD are involved in the regulation of various stages of the vesicular trafficking (Lania et al., 2019). Finally, recent studies on intestinal organoids, derived from CD patients at the remission phase of the disease, indicate that constitutive alterations of the intestinal epithelial cells are present also in absence of gluten (Dieterich et al., 2020). Taken all together these data indicate that in CD constitutive alterations exist that renders the cells more sensitive to the proinflammatory activity of p31–43.

The variety of signaling pathways that can be activated by p31–43, such as the NFκB and MAPK pathways, Ca^{2+} mobilization, TG2 activation, inhibition of CFTR, and NLRP3 inflammasome activation, that have been reviewed here, suggest that p31–43 is able to alter cell biology in different ways. The particular biophysical characteristics of p31–43 likely play a role in the toxic effects described, and particularly in the activation of the inflammasome. Inflammation represents the common ground for several multifactorial diseases, such as rheumatic disease, type 2 diabetes, obesity, inflammatory bowel diseases, cardiovascular and neurodegenerative diseases, asthma, and cancer (Furman et al., 2019; King et al., 2020; Singh et al., 2018). Environmental factors, such as infections, western diet, but also the constituents of wheat proteins, amylase trypsin inhibitors (ATIs), and p31–43 can be considered important items in the list of drivers of the inflammatory reaction (Barone et al., 2014; Spisni et al., 2019). Although p31–43 may induce inflammation in normal tissues, CD patients seem to be

particularly susceptible and react even to lower dose (Lania et al., 2019). Interestingly, combinations of proinflammatory factors may have synergistic effects. Thus, gliadins and viruses may cooperate to induce the activation of innate immunity and inflammatory pathways, which create a micro-environment with a lower threshold for the emergence and the amplification of gliadin-specific T cells (Lindfors et al., 2019; Nanayakkara et al., 2018).

To examine the long-lasting effects of inflammation even when it has apparently subsided, information gained from genomic studies must be considered. In addition to the HLA locus, 43 other loci are known to contribute to 40% of CD susceptibility. The non-HLA loci are mainly SNPs, which have also been associated with other autoimmune diseases. However, the most relevant finding is that the vast majority of CD-associated SNPs correspond to non-coding regions, thus, a simple connection with a protein function cannot be established (Withoff et al., 2016). For this reason, to link the genetic with the disease mechanism, additional information is required on the role of the particular SNPs on gene expression and how they are regulated, as well as on the affected pathways.

One example demonstrating such complexity was found by Castellanos-Rubio et al. (2016) and Castellanos-Rubio and Ghosh (2019) who studied long non-coding (lnc)RNAs. Since SNPs may localize in non-coding regions and affect the expression of genes by different mechanisms, the role of lncRNAs has received significant attention. lncRNA13 binds to a nuclear ribonucleoprotein, hnRNPD, and controls the expression of proinflammatory genes. Appropriate stimulation reduced the levels of lncRNA13, allowing the expression of the repressed genes. Interestingly, the lncRNA13 levels are significantly decreased in biopsy samples from the small intestine of CD patients, and a SNP variant of lncRNA13, found in CD patients, has less affinity to hnRNPD, leading to poor control of the repressed proinflammatory genes, and possibly contributing to the underlying chronic inflammation.

To increase the complexity, epigenetic changes, such as DNA methylation, may also contribute to the mechanisms that lead to the disease or, once the disease has been initiated, establish a chronic disorder. As an example, the evaluation of the methylome of the epithelial and immune cell populations of duodenal biopsies in CD patients and healthy individuals, showed that these cell populations present different methylation signatures, which may have a broad effect on the phenotype in a cell type-specific manner (Fernandez-Jimenez et al., 2019).

In addition, the analysis of the effects of SNPs requires taking into account the microenvironment and cell-cell contacts in the tissue. Thus, genetic and epigenetic studies have shown that not only T cells are mandatory to execute the tissue damage in CD but neutrophils, monocytes, enterocytes, and B cells also contribute to the whole picture of CD pathogenesis (Withoff et al., 2016). The use of new technologies and *in silico* analysis has begun to tackle the problem of the underlying complexity, and SNPs and epigenetic changes may be used in the future as biomarkers of the disease. Studies assessing whether p31–43 affects in the context of some particular SNPs or drives epigenetic changes on duodenal tissue may provide further insight on the understanding of the effects of this peptide on CD patients.

Since many of the biological effects reported for p31–43 are not completely understood, further investigations are required to determine the mechanisms of action of this peptide at the molecular and subcellular levels. We just begin to understand the relative contribution of genetic predisposition and environmental factors, such as viral infections (Fig. 5).

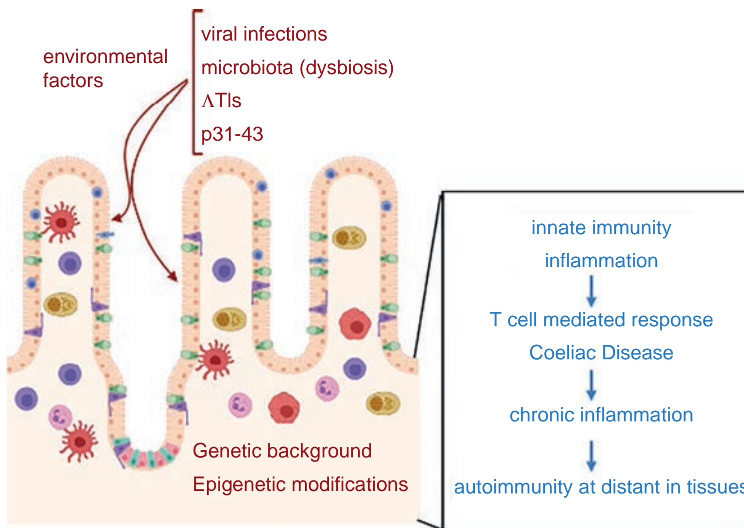
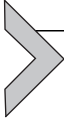


Fig. 5 Drivers of innate response in small intestine and its long term consequences. Environmental factors such as viral infections, changes in microbiota (dysbiosis), amylase trypsin inhibitors (ATIs) and p31–43, as the proposed stimuli driving the inflammatory response that promote the development of adaptive response in genetically predisposed individuals leading to Coeliac disease. Further chronic inflammation may be responsible to initiate or potentiate autoimmunity in different tissues.

Altogether, knowledge of the molecular mechanisms underlying the p31–43 action will have important implications in terms of both prevention and therapy, and will help us to understand the triggering events in CD-associated autoimmune conditions.



9. Conclusions

CD is the prototype of a chronic inflammatory disorder induced by dietary components. Cells in tissues from CD patients show increased sensitivity to the inflammatory insult from wheat-derived peptides. This is probably due to several layers of susceptibility factors: genes, non-coding regions, and epigenetic modifications. In this context, p31–43 comes into the spotlight as an important player in CD pathogenesis. Its particular conformation and its ability to induce different forms of cellular stress drive multiple inflammatory pathways, which, in the presence of appropriate susceptibility and environmental factors, may act together to drive the disease.

References

Abadie, V., et al., 2020. IL-15, gluten and HLA-DQ8 drive tissue destruction in coeliac disease. Nature 578 (7796), 600–604. https://doi.org/10.1038/s41586-020-2003-8.

Allegretti, Y.L., et al., 2013. Broad MICA/B expression in the small bowel mucosa: a link between cellular stress and celiac disease. PLoS One 8 (9), e73658. https://doi.org/10.1371/journal.pone.0073658.

Araya, R.E., et al., 2016. Mechanisms of innate immune activation by gluten peptide p31-43 in mice. Am. J. Physiol. Gastrointest. Liver Physiol. 311 (1), G40–G49. https://doi.org/10.1152/ajpgi.00435.2015.

Barone, M.V., Zimmer, K.P., 2016. Endocytosis and transcytosis of gliadin peptides. Mol. Cell. Pediatr. 3 (1), 4–8. https://doi.org/10.1186/s40348-015-0029-z.

Barone, M.V., et al., 2007. Growth factor-like activity of gliadin, an alimentary protein: implications for coeliac disease. Gut 56 (4), 480–488. https://doi.org/10.1136/gut.2005.086637.

Barone, M.V., et al., 2010. Gliadin peptide P31-43 localises to endocytic vesicles and interferes with their maturation. PLoS One 5 (8), e17039. https://doi.org/10.1371/journal.pone.0012246.

Barone, M.V., et al., 2011. Gliadin-mediated proliferation and innate immune activation in celiac disease are due to alterations in vesicular trafficking. PLoS One 6 (2), e17039. https://doi.org/10.1371/journal.pone.0017039.

Barone, M.V., Troncone, R., Auricchio, S., 2014. Gliadin peptides as triggers of the proliferative and stress/innate immune response of the celiac small intestinal mucosa. Int. J. Mol. Sci. 15 (11), 20518–20537. https://doi.org/10.3390/ijms151120518.

Barrera, E., Chirdo, F., Pantano, S., 2019. P31-43 gliadin peptide forms oligomers and induces NLRP3 inflammasome/caspase 1-dependent mucosal damage in small intestine. Commentary. Front. Immunol. https://doi.org/10.3389/fimmu.2019.02792.

Bayardo, M., et al., 2012. Transglutaminase 2 expression is enhanced synergistically by interferon-γ and tumour necrosis factor-α in human small intestine. Clin. Exp. Immunol. 168 (1), 95–104. https://doi.org/10.1111/j.1365-2249.2011.04545.x.

Bechara, C., Sagan, S., 2013. Cell-penetrating peptides: 20 years later, where do we stand? FEBS Lett. 587 (12), 1693–1702. https://doi.org/10.1016/j.febslet.2013.04.031.
Bernardin, J.E., Kasarda, D.D., Mecham, D.K., 1967. Preparation and characterization of alpha-gliadin. J. Biol. Chem. 242 (3), 445–450.
Bernardo, D., et al., 2008. Higher constitutive IL15Rα expression and lower IL-15 response threshold in coeliac disease patients. Clin. Exp. Immunol. 154 (1), 64–73.
Black, K.E., Murray, J.A., David, C.S., 2002. HLA-DQ determines the response to exogenous wheat proteins: a model of gluten sensitivity in transgenic knockout mice. J. Immunol. 169 (10), 5595–5600. https://doi.org/10.4049/jimmunol.169.10.5595.
Bouziat, R., et al., 2017. Reovirus infection triggers inflammatory responses to dietary antigens and development of celiac disease. Science 356 (6333), 44–50. https://doi.org/10.1126/science.aah5298.
Browning, T.H., Trier, J.S., 1969. Organ culture of mucosal biopsies of human small intestine. J. Clin. Invest. 48 (8), 1423–1432. https://doi.org/10.1172/JCI106108.
Budagian, V., et al., 2006. IL-15/IL-15 receptor biology: a guided tour through an expanding universe. Cytokine Growth Factor Rev. 17 (4), 259–280. https://doi.org/10.1016/j.cytogfr.2006.05.001.
Burke, P., Schooler, K., Wiley, H.S., 2001. Regulation of epidermal growth factor receptor signaling by endocytosis and intracellular trafficking. Mol. Biol. Cell 12 (6), 1897–1910. https://doi.org/10.1091/mbc.12.6.1897.
Calvanese, L., et al., 2019. Structural insights on P31-43, a gliadin peptide able to promote an innate but not an adaptive response in celiac disease. J. Pept. Sci. 25 (5), e3161. https://doi.org/10.1002/psc.3161.
Caminero, A., et al., 2019. Mechanisms by which gut microorganisms influence food sensitivities. Nat. Rev. Gastroenterol. Hepatol. 16 (1), 7–18. https://doi.org/10.1038/s41575-018-0064-z. Springer US.
Capozzi, A., et al., 2013. Modulatory effect of gliadin peptide 10-mer on epithelial intestinal CACO-2 cell inflammatory response. PLoS One 8 (6), e66561. https://doi.org/10.1371/journal.pone.0066561.
Caputo, I., et al., 2012. Gliadin peptides induce tissue transglutaminase activation and ER-stress through Ca2+ mobilization in Caco-2 cells. PLoS One 7 (9), e45209. https://doi.org/10.1371/journal.pone.0045209.
Caputo, I., et al., 2013. Anti-tissue transglutaminase antibodies activate intracellular tissue transglutaminase by modulating cytosolic Ca2+ homeostasis. Amino Acids 44 (1), 251–260. https://doi.org/10.1007/s00726-011-1120-y.
Castellanos-Rubio, A., Bilbao, J.R., 2018. Profiling celiac disease-related transcriptional changes. 1st edn. In: International Review of Cell and Molecular Biology, first ed. Elsevier Inc, https://doi.org/10.1016/bs.ircmb.2017.07.003.
Castellanos-Rubio, A., Ghosh, S., 2019. Disease-associated SNPs in inflammation-related lncRNAs. Front. Immunol. 10 (Mar), 1–9. https://doi.org/10.3389/fimmu.2019.00420.
Castellanos-Rubio, A., et al., 2016. A long noncoding RNA associated with susceptibility to celiac disease. Science 352 (6281), 91–95. https://doi.org/10.1126/science.aad0467.
Ceresa, B.P., Schmid, S.L., 2000. Regulation of signal transduction by endocytosis. Curr. Opin. Cell Biol. 12 (2), 204–210. https://doi.org/10.1016/S0955-0674(99)00077-0.
Chiang, C.Y., et al., 2012. Cofactors required for TLR7- and TLR9-dependent innate immune responses. Cell Host Microbe 11 (3), 306–318. https://doi.org/10.1016/j.chom.2012.02.002. Elsevier Inc.
Ciclitira, P.J., Evans, D.J., Fagg, N.L., Lennox, E.S., Dowling, R., 1984. Clinical testing of gliadin fractions in coeliac patients. Clin. Sci. (Lond.) 66 (3), 357–364.

D'Eletto, M., et al., 2018. Transglutaminase type 2 regulates ER-mitochondria contact sites by interacting with GRP75. Cell Rep. 25 (13), 3573–3581.e4. https://doi.org/10.1016/j.celrep.2018.11.094. ElsevierCompany.

Dafik, L., et al., 2012. Activation and inhibition of transglutaminase 2 in mice. PLoS One 7 (2), 1–7. https://doi.org/10.1371/journal.pone.0030642.

de Ritis, G., et al., 1988. In vitro (organ culture) studies of the toxicity of specific A-gliadin peptides in celiac disease. Gastroenterology 94 (1), 41–49. https://doi.org/10.1016/0016-5085(88)90607-5. Elsevier Inc.

Dieterich, W., Neurath, M.F., Zopf, Y., 2020. Intestinal ex vivo organoid culture reveals altered programmed crypt stem cells in patients with celiac disease. Sci. Rep. 10 (1), 1–10. https://doi.org/10.1038/s41598-020-60521-5.

Du Pré, M.F., et al., 2011. Tolerance to ingested deamidated gliadin in mice is maintained by splenic, type 1 regulatory T cells. Gastroenterology 141 (2). https://doi.org/10.1053/j.gastro.2011.04.048. AGA Institute American Gastroenterological Association.

Duitman, E.H., et al., 2008. How a cytokine is chaperoned through the secretory pathway by complexing with its own receptor: lessons from interleukin-15 (IL-15)/IL-15 receptor α. Mol. Cell. Biol. 28 (15), 4851–4861. https://doi.org/10.1128/mcb.02178-07.

Esposito, C., 2003. Expression and enzymatic activity of small intestinal tissue transglutaminase in celiac disease. Am. J. Gastroenterol. 98 (8), 1813–1820. https://doi.org/10.1016/s0002-9270(03)00431-3.

Falchuk, Z.M., et al., 1974. An in vitro model of gluten sensitive enteropathy. Effect of gliadin on intestinal epithelial cells of patients with gluten sensitive enteropathy in organ culture. J. Clin. Investig. 53 (2), 487–500. https://doi.org/10.1172/JCI107582.

Fehniger, T.A., Caligiuri, M.A., 2001. Interleukin 15: biology and relevance to human disease. Blood 97 (1), 14–32. https://doi.org/10.1182/blood.V97.1.14.

Fernandez-jimenez, N., et al., 2014. Coregulation and modulation of NFκB-related genes in celiac disease: uncovered aspects of gut mucosal inflammation. Hum. Mol. Genet. 23 (5), 1298–1310. https://doi.org/10.1093/hmg/ddt520.

Fernandez-Jimenez, N., et al., 2019. The methylome of the celiac intestinal epithelium harbours genotype-independent alterations in the HLA region. Sci. Rep. 9 (1), 1–13. https://doi.org/10.1038/s41598-018-37746-6.

Festenstein, G.N., Hay, F.C., Miflin, B.J., Shewry, P., 1984. Immunochemical studies on barley seed storage proteins: the specificity of an antibody to "C" hordein and its reaction with prolamins from other cereals. Planta 162 (6), 524–531.

Field, J.M., et al., 1982. The purification and characterization of homologous high molecular weight storage proteins from grain of wheat, rye and barley. Theor. Appl. Genet. 62 (4), 329–336. https://doi.org/10.1007/BF00275097.

Franz, J., et al., 2016. SAP(E)—a cell-penetrating polyproline helix at lipid interfaces. Biochim. Biophys. Acta, Biomembr. 1858 (9), 2028–2034. https://doi.org/10.1016/j.bbamem.2016.05.021. Elsevier B.V.

Freitag, T.L., et al., 2020. Gliadin nanoparticles induce immune tolerance to gliadin in mouse models of celiac disease. Gastroenterology 158 (6), 1667–1681.e12. https://doi.org/10.1053/j.gastro.2020.01.045. The American Gastroenterological Association.

Frossi, B., et al., 2017. Mast cells are associated with the onset and progression of celiac disease. J. Allergy Clin. Immunol. 139 (4), 1266–1274.e1. https://doi.org/10.1016/j.jaci.2016.08.011. Elsevier Ltd.

Furman, D., et al., 2019. Chronic inflammation in the etiology of disease across the life span. Nat. Med. 25 (12), 1822–1832. https://doi.org/10.1038/s41591-019-0675-0. Springer US.

Gaggero, A., et al., 1999. Differential intracellular trafficking, secretion and endosomal localization of two IL-15 isoforms. Eur. J. Immunol. 29 (4), 1265–1274. https://doi.org/10.1002/(sici)1521-4141(199904)29:04<1265::aid-immu1265>3.0.co;2-v.

Galipeau, H.J., et al., 2011. Sensitization to gliadin induces moderate enteropathy and insulitis in nonobese diabetic-DQ8 mice. J. Immunol. 187 (8), 4338–4346. https://doi.org/10.4049/jimmunol.1100854.

Gianfrani, C., et al., 2015. Extensive in vitro gastrointestinal digestion markedly reduces the immune-toxicity of Triticum monococcum wheat: implication for celiac disease. Mol. Nutr. Food Res. 59 (9), 1844–1854. https://doi.org/10.1002/mnfr.201500126.

Gómez Castro, M.F., et al., 2019. P31-43 gliadin peptide forms oligomers and induces NLRP3 inflammasome/caspase 1-dependent mucosal damage in small intestine. Front. Immunol. 10 (Jan), 1–11. https://doi.org/10.3389/fimmu.2019.00031.

Groh, V., et al., 1996. Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. Proc. Natl. Acad. Sci. U. S. A. 93 (22), 12445–12450. https://doi.org/10.1073/pnas.93.22.12445.

Hansson, T., et al., 2002. Transforming growth factor-β (TGF-β) and tissue transglutaminase expression in the small intestine in children with coeliac disease. Scand. J. Immunol. 56 (5), 530–537. https://doi.org/10.1046/j.1365-3083.2002.01157.x.

Harris, K.M., Fasano, A., Mann, D.L., 2010. Monocytes differentiated with IL-15 support Th17 and Th1 responses to wheat gliadin: implications for celiac disease. Clin. Immunol. 135 (3), 430–439. https://doi.org/10.1016/j.clim.2010.01.003. Elsevier Inc.

Heil, F., et al., 2003. The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. Eur. J. Immunol. 33 (11), 2987–2997. https://doi.org/10.1002/eji.200324238.

Herrera, M.G., et al., 2020. Structural conformation and self-assembly process of p31-43 gliadin peptide in aqueous solution. Implications for celiac disease. FEBS J. 287 (10), 2134–2149. https://doi.org/10.1111/febs.15109.

Hüe, S., et al., 2004. A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. Immunity 21 (3), 367–377. https://doi.org/10.1016/j.immuni.2004.06.018.

Husby, S., et al., 2020. European society paediatric gastroenterology, hepatology and nutrition guidelines for diagnosing coeliac disease 2020. J. Pediatr. Gastroenterol. Nutr. 70 (1), 141–156. https://doi.org/10.1097/MPG.0000000000002497.

Iversen, R., et al., 2020. Evidence that pathogenic transglutaminase 2 in celiac disease derives from enterocytes. Gastroenterology 159 (2), 788–790. https://doi.org/10.1053/j.gastro.2020.04.018. The American Gastroenterological Association.

Iversen, R., Sollid, L.M., 2020. Autoimmunity provoked by foreign antigens: in celiac disease, exogenous gluten drives T cell-B cell interactions that cause autoimmunity. Science 368 (6487), 132–133. https://doi.org/10.1126/science.aay3037.

Jabri, B., Abadie, V., 2015. IL-15 functions as a danger signal to regulate tissue-resident T cells and tissue destruction. Nat. Rev. Immunol. 15 (12), 771–783. https://doi.org/10.1038/nri3919. Nature Publishing Group.

Jabri, B., Sollid, L.M., 2017. T cells in celiac disease. J. Immunol. 198 (8), 3005–3014. https://doi.org/10.4049/jimmunol.1601693.

Junker, Y., et al., 2012. Wheat amylase trypsin inhibitors drive intestinal inflammation via activation of toll-like receptor 4. J. Exp. Med. 209 (13), 2395–2408. https://doi.org/10.1084/jem.20102660.

Kasarda, D.D., Bernardin, J.E., Thomas, R.S., 1967. Reversible aggregation of α-gliadin to fibrils. Science 155 (3759), 203–205. https://doi.org/10.1126/science.155.3759.203.

Kasarda, D.D., Bernardin, J.E., Gaffield, W., 1968. Circular dichroism and optical rotatory dispersion of alpha-gliadin. Biochemistry 7 (11), 3950–3957.

Kasarda, D.D., et al., 1984. Nucleic acid (cDNA) and amino acid sequences of alpha-type gliadins from wheat (Triticum aestivum). Proc. Natl. Acad. Sci. U. S. A. 81 (15), 4712–4716. https://doi.org/10.1073/pnas.81.15.4712.

Kim, S.M., Mayassi, T., Jabri, B., 2015. Innate immunity: actuating the gears of celiac disease pathogenesis. Best Pract. Res. Clin. Gastroenterol. 29 (3), 425–435. https://doi.org/10.1016/j.bpg.2015.05.001. Elsevier Ltd.

King, J.A., et al., 2020. Incidence of celiac disease is increasing over time: a systematic review and meta-analysis. Am. J. Gastroenterol. 115 (4), 507–525. https://doi.org/10.14309/ajg.0000000000000523.

Komada, M., Kitamura, N., 1995. Growth factor-induced tyrosine phosphorylation of Hrs, a novel 115-kilodalton protein with a structurally conserved putative zinc finger domain. Mol. Cell. Biol. 15 (11), 6213–6221. https://doi.org/10.1128/mcb.15.11.6213.

Korneychuk, N., Meresse, B., Cerf-Bensussan, N., 2015. Lessons from rodent models in celiac disease. Mucosal Immunol. 8 (1), 18–28. https://doi.org/10.1038/mi.2014.102. Nature Publishing Group.

Kreis, M., et al., 1985. Molecular evolution of the seed storage proteins of barley, rye and wheat. J. Mol. Biol. 183 (3), 499–502. https://doi.org/10.1016/0022-2836(85)90017-8.

Lania, G., et al., 2019. Constitutive alterations in vesicular trafficking increase the sensitivity of cells from celiac disease patients to gliadin. Commun. Biol. 2 (1), 1–13. https://doi.org/10.1038/s42003-019-0443-1. Springer US.

Lee, J., et al., 2004. Transglutaminase 2 induces nuclear factor-κB activation via a novel pathway in BV-2 microglia. J. Biol. Chem. 279 (51), 53725–53735. https://doi.org/10.1074/jbc.M407627200.

Lim, S.D., et al., 2018. Gliadin intake induces oxidative-stress responses in Caenorhabditis elegans. Biochem. Biophys. Res. Commun. 503 (3), 2139–2145. https://doi.org/10.1016/j.bbrc.2018.08.002. Elsevier Ltd.

Lindfors, K., et al., 2019. Coeliac disease. Nat. Rev. Dis. Primers. 5, 1. https://doi.org/10.1038/s41572-018-0054-z. Springer US.

Maiuri, L., Picarelli, A., et al., 1996a. Definition of the initial immunologic modifications upon in vitro gliadin challenge in the small intestine of celiac patients. Gastroenterology 110 (5), 1368–1378. https://doi.org/10.1053/gast.1996.v110.pm8613040.

Maiuri, L., Troncone, R., et al., 1996b. In vitro activities of A-gliadin-related synthetic peptides: damaging effect on the atrophic coeliac mucosa and activation of mucosal immune response in the treated coeliac mucosa. Scand. J. Gastroenterol. 31 (3), 247–253. https://doi.org/10.3109/00365529609004874.

Maiuri, L., et al., 2000. Interleukin 15 mediates epithelial changes in celiac disease. Gastroenterology 119 (4), 996–1006. https://doi.org/10.1053/gast.2000.18149.

Maiuri, L., et al., 2003. Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease. Lancet 362 (9377), 30–37. https://doi.org/10.1016/S0140-6736(03)13803-2.

Maiuri, L., et al., 2010. Lysosomal accumulation of gliadin p31-43 peptide induces oxidative stress and tissue transglutaminase-mediated PPARγ downregulation in intestinal epithelial cells and coeliac mucosa. Gut 59 (3), 311–319. https://doi.org/10.1136/gut.2009.183608.

Mamone, G., et al., 2007. Identification of a peptide from α-gliadin resistant to digestive enzymes: implications for celiac disease. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 855 (2 Spec. Iss), 236–241. https://doi.org/10.1016/j.jchromb.2007.05.009.

Marietta, E., et al., 2004. A new model for dermatitis herpetiformis that uses HLA-DQ8 transgenic NOD mice. J. Clin. Investig. 114 (8), 1090–1097. https://doi.org/10.1172/JCI200421055.

Marshall, M.S., 1995. Ras target proteins in eukaryotic cells. FASEB J. 9 (13), 1311–1318. https://doi.org/10.1096/fasebj.9.13.7557021.

Martucciello, S., et al., 2020. Interplay between type 2 transglutaminase (Tg2), gliadin peptide 31-43 and anti-tg2 antibodies in celiac disease. Int. J. Mol. Sci. 21 (10). https://doi.org/10.3390/ijms21103673.

Matysiak-Budnik, T., et al., 2003. Alterations of the intestinal transport and processing of gliadin peptides in celiac disease. Gastroenterology 125 (3), 696–707. https://doi.org/10.1016/S0016-5085(03)01049-7.

Matysiak-Budnik, T., et al., 2008. Secretory IgA mediates retrotranscytosis of intact gliadin peptides via the transferrin receptor in celiac disease. Exp. Med. 205 (1), 143–154. https://doi.org/10.1084/jem.20071204.

Mayassi, T., et al., 2019. Chronic inflammation permanently reshapes tissue-resident immunity in celiac disease. Cell 176 (5), 967–981.e19. https://doi.org/10.1016/j.cell.2018.12.039. Elsevier Inc.

Mehta, K., Kumar, A., Kim, H.I., 2010. Transglutaminase 2: a multi-tasking protein in the complex circuitry of inflammation and cancer. Biochem. Pharmacol. 80 (12), 1921–1929. https://doi.org/10.1016/j.bcp.2010.06.029. Elsevier Inc.

Mention, J.J., et al., 2003. Interleukin 15: a key to disrupted intraepithelial lymphocyte homeostasis and lymphomagenesis in celiac disease. Gastroenterology 125 (3), 730–745. https://doi.org/10.1016/S0016-5085(03)01047-3.

Meresse, B., et al., 2004. Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. Immunity 21 (3), 357–366. https://doi.org/10.1016/j.immuni.2004.06.020.

Mnard, S., et al., 2012. Paracellular versus transcellular intestinal permeability to gliadin peptides in active celiac disease. Am. J. Pathol. 180 (2), 608–615. https://doi.org/10.1016/j.ajpath.2011.10.019. Elsevier Inc.

Molberg, O., Mcadam, S.N., Körner, R., Quarsten, H., Kristiansen, C., Madsen, L., Fugger, L., Scott, H., Norén, O., Roepstorff, P., Lundin, K.E., Sjöström, H., Sollid, L., 1998. Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. Nat. Med. 4 (6), 713–717.

Monguzzi, E., et al., 2019. Gliadin effect on the oxidative balance and DNA damage: an in-vitro, ex-vivo study. Dig. Liver Dis. 51 (1), 47–54. https://doi.org/10.1016/j.dld.2018.06.020. Editrice Gastroenterologica Italiana.

Monteleone, G., et al., 2001. Role of interferon α in promoting T helper cell type 1 responses in the small intestine in coeliac disease. Gut 48 (3), 425–429. https://doi.org/10.1136/gut.48.3.425.

Mowat, A.M.I., 2018. To respond or not to respond—a personal perspective of intestinal tolerance. Nat. Rev. Immunol. 18 (6), 405–415. https://doi.org/10.1038/s41577-018-0002-x. Springer US.

Mowat, A.M., Agace, W.W., 2014. Regional specialization within the intestinal immune system. Nat. Rev. Immunol. 14 (10), 667–685. https://doi.org/10.1038/nri3738.

Nanayakkara, M., et al., 2013. An undigested gliadin peptide activates innate immunity and proliferative signaling in enterocytes: the role in celiac disease. Am. J. Clin. Nutr. 98 (4), 1123–1135. https://doi.org/10.3945/ajcn.112.054544.

Nanayakkara, M., et al., 2018. P31-43, an undigested gliadin peptide, mimics and enhances the innate immune response to viruses and interferes with endocytic trafficking: a role in celiac disease. Sci. Rep. 8 (1), 1–12. https://doi.org/10.1038/s41598-018-28830-y. Springer US.

Oba, M., et al., 2019. Secondary structures and cell-penetrating abilities of arginine-rich peptide foldamers. Sci. Rep. 9 (1), 1–9. https://doi.org/10.1038/s41598-018-38063-8. Springer US.

Orlando, A., et al., 2019. An in vitro study on mitochondrial compensatory response induced by gliadin peptides in caco-2 cells. Int. J. Mol. Sci. 20 (8), 1862. https://doi.org/10.3390/ijms20081862.

Osborne, T., 1908. Our present knowledge of plant proteins. Science 28, 417–427.

Palová-Jelínková, L., et al., 2013. Pepsin digest of wheat gliadin fraction increases production of IL-1β via TLR4/MyD88/TRIF/MAPK/NF-κB signaling pathway and an NLRP3 inflammasome activation. PLoS One 8 (4), e62426. https://doi.org/10.1371/journal.pone.0062426.

Pane, J.A., Webster, N.L., Coulson, B.S., 2014. Rotavirus activates lymphocytes from non-obese diabetic mice by triggering toll-like receptor 7 signaling and interferon production in plasmacytoid dendritic cells. PLoS Pathog. 10 (3), 1003998. https://doi.org/10.1371/journal.ppat.1003998.

Paolella, G., et al., 2018. The toxic alpha-gliadin peptide 31–43 enters cells without a surface membrane receptor. Cell Biol. Int. 42 (1), 112–120. https://doi.org/10.1002/cbin.10874.

Perera, L., et al., 2007. Expression of nonclassical class I molecules by intestinal epithelial cells. Inflamm. Bowel Dis. 13 (3), 298–307. https://doi.org/10.1002/ibd.20026.

Przemioslo, R., et al., 1995. Analysis of crypt cell proliferation in coeliac disease using MI-B1 antibody shows an increase in growth fraction. Gut 36 (1), 22–27. https://doi.org/10.1136/gut.36.1.22.

Qiao, S.W., et al., 2014. Biased usage and preferred pairing of α- and β-chains of TCRs specific for an immunodominant gluten epitope in coeliac disease. Int. Immunol. 26 (1), 13–19. https://doi.org/10.1093/intimm/dxt037.

Raiborg, C., Stenmark, H., 2002. Hrs and endocytic sorting of ubiquitinated membrane proteins. Cell Struct. Funct. 27 (6), 403–408. https://doi.org/10.1247/csf.27.403.

Raiborg, C., et al., 2001. Hrs recruits clathrin to early endosomes. EMBO J. 20 (17), 5008–5021. https://doi.org/10.1093/emboj/20.17.5008.

Raiborg, C., et al., 2008. Differential functions of Hrs and ESCRT proteins in endocytic membrane trafficking. Exp. Cell Res. 314 (4), 801–813. https://doi.org/10.1016/j.yexcr.2007.10.014.

Raulet, D.H., 2003. Roles of the NKG2D immunoreceptor and its ligands. Nat. Rev. Immunol. 3 (10), 781–790. https://doi.org/10.1038/nri1199.

Reinecker, H.C., et al., 1996. Intestinal epithelial cells both express and respond to interleukin 15. Gastroenterology 111 (6), 1706–1713. https://doi.org/10.1016/S0016-5085(96)70036-7.

Rivabene, R., Mancini, E., De Vincenzi, M., 1999. In vitro cytotoxic effect of wheat gliadin-derived peptides on the Caco-2 intestinal cell line is associated with intracellular oxidative imbalance: implications for coeliac disease. Biochim. Biophys. Acta Mol. basis Dis. 1453 (1), 152–160. https://doi.org/10.1016/S0925-4439(98)00095-7.

Ruera, C.N., Miculán, E., Pérez, F., Ducca, G., Carasi, P., Chirdo, F.G., 2020. Sterile Inflammation drives multiple programmed cell death pathways in the gut. J. Leukoc. Biol. https://doi.org/10.1002/JLB.3MA0820-660R.

Ryan, A.W., 2015. Celiac disease. Methods Mol. Biol. 1326. https://doi.org/10.1007/978-1-4939-2839-2.

Sanchez-Guerrero, E., Jo, S.R., Chong, B.H., Khachigian, L., 2013. EGFR and the complexity of receptor crosstalk in the cardiovascular system. Curr. Mol. Med. 13 (1), 3–12.

Shan, L., Molberg, Ø., Parrot, I., Hausch, F., Filiz, F., Gray, G.M., Sollid, L.M., Khosla, C., 2002. Structural basis for gluten intolerance in celiac sprue. Science 297 (5590), 2275–2279. https://doi.org/10.1126/science.1074129.

Shewry, P., 2019. What is gluten—why is it special? Front. Nutr. 6 (July), 1–10. https://doi.org/10.3389/fnut.2019.00101.

Shewry, P.R., Halford, N.G., 2002. Cereal seed storage proteins: structures, properties and role in grain utilization. J. Exp. Bot. 53 (370), 947–958. https://doi.org/10.1093/jexbot/53.370.947.

Shidrawi, R.G., et al., 1995. In vitro toxicity of gluten peptides in coeliac disease assessed by organ culture. Scand. J. Gastroenterol. 30 (8), 758–763. https://doi.org/10.3109/00365529509096324.

Siegel, M., et al., 2008. Extracellular transglutaminase 2 is catalytically inactive, but is transiently activated upon tissue injury. PLoS One 3 (3), e1861. https://doi.org/10.1371/journal.pone.0001861.

Singh, P., et al., 2018. Global prevalence of celiac disease: systematic review and meta-analysis. Clin. Gastroenterol. Hepatol. 16 (6), 823–836.e2. https://doi.org/10.1016/j.cgh.2017.06.037.

Sollid, L.M., Jabri, B., 2013. Triggers and drivers of autoimmunity: lessons from coeliac disease. Nat. Rev. Immunol. 13 (4), 294–302. https://doi.org/10.1038/nri3407. Nature Publishing Group.

Sollid, L.M., et al., 2012. Nomenclature and listing of celiac disease relevant gluten T-cell epitopes restricted by HLA-DQ molecules. Immunogenetics 64 (6), 455–460. https://doi.org/10.1007/s00251-012-0599-z.

Sollid, L.M., et al., 2020. Update 2020: nomenclature and listing of celiac disease–relevant gluten epitopes recognized by CD4+ T cells. Immunogenetics 72 (1–2), 85–88. https://doi.org/10.1007/s00251-019-01141-w.

Spisni, E., et al., 2019. Differential physiological responses elicited by ancient and heritage wheat cultivars compared to modern ones. Nutrients 11 (12), 1–20. https://doi.org/10.3390/nu11122879.

Stene, L.C., et al., 2006. Rotavirus infection frequency and risk of celiac disease autoimmunity in early childhood: a longitudinal study. Am. J. Gastroenterol. 101 (10), 2333–2340. https://doi.org/10.1111/j.1572-0241.2006.00741.x.

Sturgess, R., Day, P., Ellis, H.J., Lundin, K.E., Gjertsen, H.A., Kontakou, M., Ciclitira, P., 1994. Wheat peptide challenge in coeliac disease. Lancet 343 (8900), 758–761.

Swanson, K.V., Deng, M., Ting, J.P.Y., 2019. The NLRP3 inflammasome: molecular activation and regulation to therapeutics. Nat. Rev. Immunol. 19 (8), 477–489. https://doi.org/10.1038/s41577-019-0165-0. Springer US.

Szondy, Z., et al., 2017. Transglutaminase 2 in human diseases. BioMedicine (France) 7 (3), 1–13. https://doi.org/10.1051/bmdcn/2017070315.

Tatham, A.S., Drake, A.F., Shewry, P.R., 1985. A conformational study of a glutamine- and proline-rich cereal seed protein, C hordein. Biochem. J. 226 (2), 557–562. https://doi.org/10.1042/bj2260557.

Tatham, A.S., et al., 1990. Conformational studies of peptides corresponding to the coeliac-activating regions of wheat α-gliadin. Biochem. J. 270 (2), 313–318. https://doi.org/10.1042/bj2700313.

Thomas, K.E., et al., 2006. Gliadin stimulation of murine macrophage inflammatory gene expression and intestinal permeability are MyD88-dependent: role of the innate immune response in celiac disease. J. Immunol. 176 (4), 2512–2521. https://doi.org/10.4049/jimmunol.176.4.2512.

Troncone, R., Ferguson, A., 1991. Animal model of gluten induced enteropathy in mice. Gut 32 (8), 871–875.

Van Leeuwen, M.A., et al., 2017. Macrophage-mediated gliadin degradation and concomitant IL-27 production drive IL-10- and IFN-γ 3-secreting Tr1-like-cell differentiation in a murine model for gluten tolerance. Mucosal Immunol. 10 (3), 635–649. https://doi.org/10.1038/mi.2016.76.

Vilasi, S., et al., 2010. Interaction of "toxic" and "immunogenic" A-gliadin peptides with a membrane-mimetic environment. J. Mol. Recognit. 23 (3), 322–328. https://doi.org/10.1002/jmr.987.

Villella, V.R., et al., 2019. A pathogenic role for cystic fibrosis transmembrane conductance regulator in celiac disease. EMBO J. 38 (2), 1–22. https://doi.org/10.15252/embj.2018100101.

Vincentini, O., et al., 2015. Gliadin-dependent cytokine production in a bidimensional cellular model of celiac intestinal mucosa. Clin. Exp. Med. 15 (4), 447–454. https://doi.org/10.1007/s10238-014-0325-2. Springer Milan.

Wieser, H., Belitz, H.D., Ashkenazi, A., 1984. Amino-acid sequence of the coeliac active gliadin peptide B 3142. Z. Lebensm. Unters. Forsch. 179 (5), 371–376. https://doi.org/10.1007/BF01043432.

Wieser, H., et al., 1986. Coeliac activity of the gliadin peptides CT-1 and CT-2. Z. Lebensm. Unters. Forsch. 182 (2), 115–117. https://doi.org/10.1007/BF01454241.

Wilde, A., et al., 1999. EGF receptor signaling stimulates SRC kinase phosphorylation of clathrin, influencing clathrin redistribution and EGF uptake. Cell 96 (5), 677–687. https://doi.org/10.1016/S0092-8674(00)80578-4.

Withoff, S., et al., 2016. Understanding celiac disease by genomics. Trends Genet. 32 (5), 295–308. https://doi.org/10.1016/j.tig.2016.02.003. Elsevier Ltd.

Yokoyama, S., et al., 2011. Transgenic mice that overexpress human IL-15 in enterocytes recapitulate both B and T cell-mediated pathologic manifestations of celiac disease. J. Clin. Immunol. 31 (6), 1038–1044. https://doi.org/10.1007/s10875-011-9586-7.

Zemskov, E.A., et al., 2011. Unconventional secretion of tissue transglutaminase involves phospholipid-dependent delivery into recycling endosomes. PLoS One 6 (4), e19414. https://doi.org/10.1371/journal.pone.0019414.

Zimmer, K.P., et al., 2010. Endocytotic segregation of gliadin peptide 31-49 in enterocytes. Gut 59 (3), 300–310. https://doi.org/10.1136/gut.2008.169656.

Zimmermann, C., et al., 2014. Epithelial transport of immunogenic and toxic gliadin peptides in vitro. PLoS One 9 (11), 1–17. https://doi.org/10.1371/journal.pone.0113932.