New Therapeutic Strategies in Celiac Disease

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1 Introduction

(a) *Celiac disease: epidemiology and pathogenesis*

Celiac disease (CD) in a chronic intestinal disorder with autoimmune treats that affects approximately 1 in 100 individuals worldwide in gluten consuming countries and is caused by a dysregulated immune response to gluten proteins of wheat and related proteins of barley and rye [1]. CD is characterized by a large spectrum of clinical presentations, with either gastrointestinal and extra-intestinal manifestations, though symptomless cases are not uncommon [2]. Both symptomatic and asymptomatic CD are characterized by the presence of HLA-risk genes, namely the DQ2- and DQ8-heterodimers encoding alleles, and serum positivity of anti-tissue transglutaminase (tTG2) antibodies. Gluten ingestion by CD patients causes a chronic inflammatory process that may lead to profound morphological changes characterized by villous atrophy and crypt hyperplasia and marked functional dysregulation [3]. However, there is a spectrum of histological alterations with at the mildest end only infiltration of the epithelium (potential CD). The diverse grade of enteropathy does not exactly match with clinical manifestations, as some patients with villous atrophy might be asymptomatic, whilst those with potential-CD may complain of severe symptoms [4].

A complex interaction between genetic, environmental and inflammatory pathways contributes to CD aetiology, with many of these factors not fully understood. As documented by several studies, CD is defined as a cell-mediated immune dis-

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ease, in which CD4+ T cells reactive to gluten peptides and restricted by HLA DQ2/DQ8 molecules have a central role, as reviewed in [5]. A step-forward in the understanding of CD pathogenesis came from the elucidation of the gluten peptide deamidation by tTG2, the CD-associated autoantigen [6]. Due to high contents of glutamine and proline, gluten proteins basically lack negatively charged amino acids, a mandatory condition to bind the HLA DQ2/DQ8 molecules. The tTG2-catalyzed deamidation of specific glutamine residues strongly increases the capability of digested gluten peptides to stimulate intestinal CD4+ T cells to produce inflammatory cytokines, mainly Interferon(IFN)-γ and Interleukin(IL)-21 [7]. To date, more than 50 different immunogenic peptides have been identified in all three gliadin families and in glutenins [8]. These gluten T cell epitopes are active both in children and adult celiac patients with no substantial differences in their immunogenicity [9, 10]. However, further investigations carried out on gut mucosal explants of either acute patients and murine models of CD-enteropathy demonstrated no direct involvement of gluten-specific CD4+ T lymphocytes in the epithelial destruction and mucosa histological changes [11]. Further evidences pinpointed the involvement of cytotoxic natural killer T cells (NKT) in the villous atrophy, through an inflammatory mechanism triggered by gluten but independent from a T cell receptor (TCR) mediated activation [11]. Notably, IL-15 cytokine, massively released by enterocytes of acute CD patients upon gluten exposure, is the main activator of NKT-mediated lysis of epithelium [11]. It was elegantly demonstrated that in the gut mucosa of acute CD patients, the adaptive anti-gluten immunity drives the massive expansion of intraepithelial NKT cells (IE-CTL), whilst IL-15 and gluten-stressed enterocytes synergistically drive their activation, by licencing to kill enterocytes [12]. Based on these relevant findings, HLA-DQ8 and IL-15 double transgenic mice have been recently developed, as model of CD-enteropathy [13]. The authors demonstrated that the overexpression of IL-15 in the epithelium and lamina propria, as well as IFN-γ released by anti-gluten CD4+ T cells resident in the lamina propria are required for the development of villous atrophy. By contrast, patients with potential CD, characterized by an anti-tTG positivity but a morphologically normal mucosa, have IE-CTL not fully activated or armed to destroy epithelial cells [12]. The same group demonstrated that viral infection, in particular a reovirus strain, is a key environmental factor contributing to the inflammatory processes that allow the loss of immune tolerance to dietary gluten proteins [14].

All together these studies have dissected the complex inflammatory mechanism induced by gluten proteins in CD patients and have provided essential knowledge for the development of new therapeutic strategies alternative to the gluten free diet (GFD).

(b) *Biochemical and immunological properties of gluten proteins*

Gluten, a heterogeneous mix of water-insoluble proteins, can be considered as the "dough treasure" due to its unique visco-elasticity properties [15]. Gluten contains hundreds of high homologous proteins that are grouped in two large families, the gliadins and glutenins families, based on monomeric or polymeric structures,

respectively [15]. All gliadins and glutenins proteins are characterized by high contents of glutamine and proline (more than 50% of aminoacidic content, thus named prolamins). This peculiar amino acid composition constitutes a limitation for degradation by gastrointestinal (GI) proteases, indeed, the marked resistance to digestion results in the release into the gut lumen of large gluten fragments with a T-cell immunogenic potential [16].

(c) *Current therapy and unmet needs*

From a nutritional standpoint, gluten proteins are poor nutrients for humans, but necessary to give elastic properties to dough and high palatability to large food stuff products. However, a diet that excludes wheat, barley or rye cereals has accounted for an increased risk of nutrient deficiency and metabolic syndrome [17]. Furthermore, some patients encounter difficulty to maintain the cure over time, especially during travelling and social events, with compliance consequences. Moreover, a consistent number of gluten-free products have a high glycaemic index and are highly caloric, with a not negligible risk to develop obesity and cardiovascular disease over the time [17]. Last but not least, there are patients suffering of refractory CD characterized by unresponsiveness to the gluten exclusion diet with serious complications including high mortality risk [18]. Based on all these constrains and risk of nutritionally unbalanced diet based on gluten exclusion, there is an unmet need to develop valid and safe drug strategies to treat celiac disease.

(d) *Aims of new strategies: replacing GFD or treating non-responsive CD as addition to GFD?*

Gluten containing cereals are largely used in the diet worldwide, furthermore gluten is an ingredient very common as additive in many foodstuffs, creating substantial risk of "gluten free" food contamination that CD community daily meet. In order to solve these issues, numerous strategies are currently under investigation that are devoted to either in vitro detoxify gluten proteins, or to provide specific drugs that supplied as an oral pill, or systemically injected, may in vivo counteract the gluten immune toxicity.

As the exclusion diet, when strictly followed, guarantees the complete recovery of small intestinal damage, with resolution of all types of symptoms (both intestinal and extraintestinal), and disappearance of CD-antibodies, the main feature of a new therapy is to be as safe and effective as the GFD. However, it still under debate if alternative therapies, whenever efficacious and safe, have to be used to counteract occasional and inadvertent exposure to gluten, or to fully replace GFD.

2 Enzymatic Approaches Reducing Gluten Load by Wheat Flour Pre-treatment

A number of procedures based on enzymatic reaction are currently under investigation to obtain wheat (or barley and rye) flour with detoxified gluten proteins to prepare pasta and baked food with no immune toxicity for CD patients [19]. These novel approaches are designed to achieve the gluten proteins detoxification before the ingestion and include the pre-digestion of flour with proteases from a mixture of acidic microorganisms and a transamidation reaction with microbial tTG2 and methyl-lysine [20–24].

(a) *Bacterial and fungal proteases hydrolysis of wheat flour*

Gobbetti and co-workers exploited the use of selective sour dough *Lactobacilli* combined with fungal proteases to completely degrade proline rich proteins [20]. They found that gluten proteins were almost completely hydrolyzed after 24 hours of fermentation and lost the immunostimulatory activity, as assessed on celiac intestinal T cells. A clinical study reported that 60 days consumption of fully hydrolyzed baked goods was highly tolerated, as no immune activation or mucosal lesion were induced [21]. This approach, although it guarantees a total degradation of gluten immunogenic sequences, alters the dough viscoelasticity and, consequently, requires the flour being integrated with structuring agents, as hydrocolloids or gelatinized supplements.

(b) *Transamidation of wheat flour*

tTG2 is a calcium dependent enzyme with a central physiological role in repairing tissue damages by catalyzing protein cross-linking through a transamidation reaction and formation of lysine-glutamine bonds. By using acyl-acceptor molecules, lysine or lysine methyl ester, as a substrate of tTG2 activity, two different research groups demonstrated that lysine-transamidated gliadin peptides lose binding affinity to HLA-DQ molecules, and consequently the capability to stimulate cognate T cells [22, 23]. A further study demonstrated that it is possible to detoxify whole wheat flour with a food-grade microbial transglutaminase (mTG), largely used by industry in order to ameliorate the texture of foods [24]. In a randomized single blinded study CD patients consumed for 90 days, 3.7 g/day of gluten in transamidated-flour bread slices. Compared to the control group eating untreated flour, a reduction of clinical, serological and gut mucosa histological relapses was observed in the experimental group. Furthermore, no volunteer completed the study in control group, by contrast, 14 out 35 completed the 90-days of transamidated gluten dietary treatment [24]. In a next phase 2 randomized double blinded study (NCT02472119), CD patients in remission were enrolled to consume for 90-days transamidated or unmodified bread and underwent endoscopy. Only a minority of volunteers (14.3%) ingesting modified bread and 57.1% ingesting regular bread presented villous atrophy and positive serology (anti-tTG2 and EMA) [25]. These clinical findings, combined with good baking properties and palatability of treated

flour, make this detoxification approach very attractive for CD dietary treatment [24, 25], Table 1.

3 Approaches Reducing Gluten Contact with the Immune System

(a) *Gluten sequestering*

In the recent years, an increasing attention has been paid to biochemical strategies that aim to sequester gluten proteins in the gut lumen, thus avoiding their interaction with the gut immune system. **Chitosan**, a biocompatible aminopolysaccharide was used to produce a supramolecular compound by in vitro assembling gluten proteins. The chitosan-gluten complex displayed a marked reduction of T cell and humoral immunogenicity in preclinical analysis [26].

Similarly, **AGY Gluten Sequestering** is a strategy based on wheat gliadin protein complexation by polyclonal antibody. A single phase 1 clinical study has been completed on biopsy-diagnosed CD patients on GFD for at least 6 months who were orally administered with two capsules of 500 mg each for 4 weeks (for a total of 1 g AGY per day) before meal. The trial showed that AGY was safe and induced an improvement in celiac-associated symptoms, measured by the health-related quality of life (HRQoL) questionnaire and did not increased intestinal permeability measure by lactulose/mannitol excretion ratio (LMER). The authors claimed that AGY-based therapy is designed to neutralize 5 g of gliadin [27], Table 1.

(b) *Glutenases, endopeptidases highly efficient in degrading gluten*

Because of the pronounced resistance of gluten proteins to the intragastric degradation due to the high content of prolamin and glutamine residues, large gluten fragments remain intact in the gut lumen, being potential stimulators for inflammatory T cells. In the last two decades, great efforts were devoted to design proteases highly efficient to degrade gluten at low pH and resistant to pepsin digestion, two conditions occurring in the gastric milieu. Since the pioneristic study by Khosla and co-workers in 2002, that reported bacterial prolyl endopeptidases (PEPs) highly efficient in degrading the most immunogenic gliadin peptides, the a-gliadin 33-mer [28], several "glutenases" have been described from a variety of sources including bacteria and fungi (*Aspergillus niger, Flavobacterium meningosepticum, Sphingomonas capsulate, Actinoallomurus*), plant (*barley*), or in vitro engineered recombinant proteins with a diverse bioactivity [29, 30].

Latiglutenase–ALV003. A combination strategy has been proposed by Khosla, based on a glutamine-specific endoprotease derived from germinating barley (**EP-B2**), active under gastric condition, and on a prolyl endopeptidase from *Sphingomonas* (**SC-PEP**), that synergises with pancreatic proteases in duodenum

Table 1 Ended or current therapies targeting gluten load

Therapeutic strategy	Mechanism of action	Status	Clinical Trial Identifier	Publications	Outcome measures	Clinical trials results
mTG-transamidation	Gluten peptides transamidation	Phase 2	NCT02472119	25	Histopathological changes, serum antibodies, symptoms	A minor percentage of patients ingesting transamidated flour rusks showed positive serology (29% vs. 57%), histological damage (14% vs. 57%), and symptoms than patients taking unmodified flour
AGY	Gluten sequestring (egg yolk-derived anti-gliadin antibody)	Phase 1	NCT01765647	27	Safety and tolerability	AGY is safe and induces an improvement in celiac-associated symptoms, does not increase intestinal permeability (by LMER)
		Phase 2	NCT03707730	Not provided	Safety, symptoms, gut permeability	Not available
Latiglutenase (formerly ALV003)	Gluten proteolysis	Phase 1	NCT00626184 NCT00669825	32	Safety and tolerability	Latiglutenase is well tolerated by patients with celiac disease and healthy individuals
		Phase 2	NCT03585478	Not Provided	Villous height/crypth depth (Vh:Cd), symptom severity	Latiglutenase induces a histological damage protection
		Phase 2 (recruiting)	NCT04839575	Not Provided	Symptom scores	Reduction of symptoms severity
		Phase 2a	NCT00959114	33	Intestinal mucosal morphology, intestinal intraepithelial lymphocyte density/phenotype, serology	Latiglutenase attenuates gluten-induced mucosal injury in celiac disease patients
		Phase 2a	NCT01255696			
		Phase 2b	NCT01917630	34	Safety, intestinal mucosal morphology (Vh:Cd) ratio, intestinal intraepithelial lymphocyte density, serology	Latiglutenase does not improve histology and symptom scores in symptomatic patients
				35		Symptom improvement in seropositive and symptomatic patients

(continued)

Table 1 (continued)

Therapeutic strategy	Mechanism of action	Status	Clinical Trial Identifier	Publications	Outcome measures	Clinical trials results
AN-PEP	Gluten proteolysis	Phase 2	NCT00810654	38	Histopathological changes, serum antibodies, gluten-reactive T cells, symptoms	AN-PEP is well tolerated
		NA (Not Applicable)	NCT01335503	37	Gluten monitoring by ELISA, western blot, HPLC	AN-PEP enhances gluten digestion in the stomach of healthy volunteers
		Phase 4 (recruiting)	NCT04788797	Not Provided	Detection of gliadin immunogenic peptides (GIP) in stool and urine	Not available
TAK-062 (formely PvP001/PvP002)	Gluten proteolysis	Phase 1	NCT03594331	Not provided	Adverse events, efficacy	Not available
		Phase 1	NCT03701555	40	Safety, tolerability, pharmacokinetics, gluten degradation activity	TAK-062 is well tolerated and efficient in degrading gluten
Endopeptidase E40	Gluten proteolysis	Preclinical	NA	41	Detection of glutenasic activity by western blot, HPLC, ELISA, celiac intestinal T cells	E40 degrades gluten peptides in gastric condition and in the absence of pepsin
Larazotide (AT-1001)	Blocking epithelial permeability	Phase 3 (recruiting)	NCT03569007	Not provided	Safety, symptom severity	Not available
		Phase 1b	NCT00386165	45	Safety, pharmacokinetic, intestinal permeability	AT-1001 is well tolerated, reduces intestinal pro-inflammatory cytokine production, gastrointestinal symptoms in celiacs after gluten exposure
		Phase 2b	NCT00492960	46	Intestinal permeability changes (by LAMA), clinical symptoms and serology	AT-1001 is well tolerated, reduces gastrointestinal symptoms in celiac patients undergoing gluten challenge, no effect on intestinal permeability
		Phase 2	NCT00362856	47		
		Phase 2B	NCT01396213	48	Safety and tolerability, Symptom (CeD GSRS)	AT-1001 reduces signs and symptoms in celiac patients on a GFD

milieu [31]. This enzyme mixture was able to proteolyse complex gluten proteins in bread, as indicated by in vitro and in vivo (rat) experimental systems that simulated human gastric digestion [31]. After phase 1 studies proving that the new enzyme combination was well tolerated by CD patients and healthy individuals [32], a first phase 2a study performed in adult CD patients demonstrated that Latiglutenase-IMGX003 (formerly **ALV003**) may prevent the mucosa lesion induced by a 6-weeks daily gluten consumption (2 g/day), assessed by detection of intraepithelial CD8+ lymphocytes infiltration and villous height/crypt depth (VH: CrD) ratio, compared to placebo group. However, no differences in gluten-triggered gastrointestinal and extra-intestinal symptoms were observed between the two treatment groups [33]. A second phase 2a study done in a larger cohort reported that Latiglutenase significantly attenuates clinical manifestation severity in anti-tTG2 seropositive CD patients on GFD [34, 35]. Currently, two additional phase 2a/b studies are ongoing. The first (a single centre, randomized, double-blind, placebo controlled/gluten challenge, NCT03585478) has completed the recruitment phase whilst the second one (multicentre, prospective, randomized, double-blind, placebo-controlled/crossover study, NCT04243551) aims to assess the efficacy and safety of latiglutenase treatment in symptomatic CD patients on GDF occasionally exposed to gluten (Table 1).

AN-PEP. PEPs derived from *Aspergillus Niger* (**AN-PEP**) were shown to efficiently degrade gluten to small non-immunogenic peptides in low and high caloric meals. The glutenasic activity was assessed by either a dynamic in vitro system that closely mimics the human gastrointestinal tract [36], and in vivo in healthy volunteers through a catheter used for meal infusion and for aspiration of gastric-duodenal juices [37]. In a randomized double-blind placebo-controlled pilot study in which CD patients consumed toast containing approximately 7 g of gluten daily for 2 weeks, it was demonstrated that AN-PEP was well tolerated. By contrast, this study failed to assess drug efficacy in preventing gluten immune toxicity as, no sign of duodenal immune activation, duodenal morphological changes, as well as, serum antibody positivity (primary study endpoints) were reported in placebo control group [38], Table 1.

Kuma030–TAK-062. Since the above described pioneristic studies, other proteolytic enzymes are currently under investigations for their ability to degrade gluten under gastric conditions and be suitable as oral enzyme supplementation for treatment of gluten intolerance. A third-generation enzyme, computationally designed and molecular engineered as recombinant protein, the **Kuma030** (now **TAK-062**) was reported to be efficient in catalyzing in vitro the digestion of gluten proteins in the stomach acidic condition, by degrading more than 90% of gluten protein load [39]. In a phase 1 single-centre study (NCT03701555), TAK-062 efficacy in gluten degradation was evaluated either in vitro by a dynamic gastric model (DGM) and in vivo in healthy and CD patients. The DGM simulated gastric digestion of two different meals containing 3–9 g gluten, and TAK-062 (100 and 300 mg) in the presence of pepsin (0.6 g/ml) showed a marked gluten degradation up to 99% within 10 minutes. In vivo experiments showed that in volunteers orally

administered with homogenized meals added with gluten proteins (1, 3, 6, and 9 g), and a single scaling dose of TAK-062 (100, 300, 900 mg), the intragastric residual gliadin immunogenic peptides, quantified by competitive R5 and G12 ELISA, were almost totally degraded within 20–65 min. Furthermore, TAK062 was well tolerated, as assessed by pharmacokinetics and symptoms evaluation [40], Table 1.

E40. A recent study reported a novel protease of microbial origin, the endoproptease-40 (**E40**) recombinantly produced in *Streptomyces lividans*. Similarly to the previous proteases, E40 showed a high resistance to pepsin digestion at gastric pH and marked proteolytic activity, demonstrated by the extensive degradation of whole gliadin proteins and of the known immunogenic peptides of alfa-gliadin of 33-aminoacid length [41] as tested by SDS gel electrophoresis, mass spectroscopy, and functional assay with celiac intestinal T cells. Of note, E40 degraded gluten within 30 min of incubation at low gliadin:enzyme weight ratio (20:1) and in the absence of pepsin. Altogether, these studies showed that glutenases are highly promising approaches, Table 1. However, further studies are needed to evaluate: (i) the maximal amount of complex food containing gluten proteins within lipid and starch matrices that may be digested, (ii) if the glutenase-based pills are able to protect patients with CD from an occasional inadvertent gluten exposure or from a long-term consumption of a regular gluten containing meal.

4 Approaches Affecting Intestinal Permeability: Zonulin Receptor Agonist

Larazotide acetate–AT1001. Very little is known about the mechanisms trough gluten peptides resulting from GI digestion cross the epithelial layer before encountering immune cells. In non-inflamed condition, the intestinal epithelium is almost impermeable, due to the intercellular tight junctions. An increased permeability has been reported in gut mucosa of CD patients that could partially be mediated by the gluten-triggered upregulation of zonulin [42]. A zonulin receptor peptide agonist that blocks zonulin binding to specific receptor on enterocytes has been largely investigated as potential drug for CD treatment [43, 44]. Phase 1 and 2 studies have shown that Larazotide-AT1001 successfully passed the safety analysis and was well tolerated. With the exception of the pilot clinical studies [45–47] in which an oral supplementation of Larazotide acetate was given for three times daily to patients challenged with 2.5 g gluten for 2 weeks, the last published trial was designed to assess its beneficial effect to ameliorate health condition in symptomatic patients on gluten free diet [48], Table 1.

Other clinical studies are necessary to investigate the Larazotide acetate efficiency to prevent gluten immune toxicity in CD patients, although recently the nature of AT1001 has been questioned [49].

5 Approaches Based on Immunomodulation

(a) *Gluten tolerization by nanoparticles oral supplementation*

For the treatment of CD, different therapeutic approaches based on immunomodulation could be used targeting T cells reactive to gluten proteins and restoring the immune tolerance. One of these potential therapeutic approaches involves the use of "*Tolerogenic Immune Modifying Nanoparticles*" encapsulating gliadin protein (**TIMP-GLIA**, now **TAK-101**), small particles formed with a food grade polymer (poly-lactide-co-glycolide-PLGA) containing inside wheat gliadin [50]. It was shown that TIMP-GLIA nanoparticles are able to induce tolerance to gliadin in mouse model of CD, after systemic administration, with a reduction in enteropathy and inflammatory cytokine production [50]. To date, after a phase 1 study (NCT03486990) that assessed the safety and tolerability of TAK-101 in CD patients without any serious side effects, a phase 2 randomized, double-blind, placebo-controlled study (NCT03738475) has been completed. In this phase 2 trial, a cohort of 34 CD patients on GFD received TIMP-GLIA or placebo treatment and, after 7 days, consumed gluten for 14 days, as following: 12 g for the first 3 days followed by 6 g for the next 11 days [51]. The group of CD subjects treated with TIMP-GLIA, at day 6 of gluten challenge, showed a significant decrease of gliadin-specific T cells response (change from baseline in circulating gliadin-specific, IFNγ–producing cells) and reduced number of circulating α4β7+ CD4+ T cells, TCRγδ+ T cells and memory effector Th cells, compared to placebo group [51]. Another phase 2 study (NCT04530123) is ongoing, although it has not yet recruited patients, to evaluate the optimal dosing of TIMP-GLIA for the administration to CD patients during gluten challenge, Table 2.

(b) *Gluten peptide-based immunomodulatory strategies*

Nexvax2, is a potential desensitizing vaccine for CD treatment that consists of a combo-peptide that includes three gluten peptides of 15–16 amino acid length responsible for the immune reaction elicited by gluten ingestion in the great majority of CD patients [9, 10]. Multicentre phase 1 clinical studies (NCT00879749, NCT02528799, NCT03543540) assessed the safety, tolerability and bioactivity of Nexvax2 [52–54].

Moreover, to further investigate the effect of Nexvax2, a phase 2 clinical trial (NCT03644069) has been started on adult HLA-DQ2.5 CD patients. Despite the encouraging results on safety and tolerability of Nexvax2 obtained in the phases I, as indicated by a press release of the Company ImmusanT, the study has been discontinued since the compound did not provide protection against symptoms induced by gluten exposure compared with placebo.

(c) *tTG2 inhibitors*

Inhibitors of tissue transglutaminase type 2 (tTG2) have been designed to prevent the deamidation of gliadin peptides, a key post-translational step conferring high immunogenicity/immunotoxicity to gluten proteins [5, 6, 8]. Several gluten-

mimetic peptides were developed to block the activity of tTG2, and among these, three gluten peptides analogues: **ZED1098, ZED1219, and ZED1227** that covalently bind with the cysteine in the active site and irreversibly block the enzyme. In particular, following the preclinical data on mouse models showing the reduced intestinal inflammation by the inhibitor ZED1227 [55], a phase 1 clinical trial proved its safety. A phase 2a, double-blind, randomised, placebo-controlled, dose-finding study was carried out (EUDRA CT 2017-002241-30) to test the efficacy and tolerability of a 6-week treatment with ZED1227 capsules vs. placebo, Table 2, in subjects with well-controlled CD during gluten challenge; to date, the results have not yet been published.

(d) *Anti-IL15 therapy*

The widely demonstrated over-expression of IL15 in the intestinal mucosa of CD patients makes of great scientific interest the development of drugs targeting the inhibition of IL15 production and/or its signalling pathways. **AMG714** is the first human monoclonal antibody anti-IL15, developed by Amgen, a biotechnology company, tested for the treatment of CD, being able to block all forms of IL15 stopping its activities. This drug was investigated in two phase IIa randomized, double-blind, placebo-controlled studies. One study (NCT02637141 and EUDRA CT 2015-003647-19) was conducted on 64 CD patients on GFD for at least 12 months, that received 150 mg or 300 mg AMG174 or placebo for a total of six doses, with subcutaneous injections every 2 weeks for 10 weeks, during a gluten challenge (2–4 g daily, for 2–12 weeks). AMG 714 did not prevent mucosal damage due to gluten challenge, though the density of intraepithelial T lymphocytes (IELs) was less increased in the group treated with the highest dose. Moreover, no serious adverse effects were observed in the study [56]. In the other phase 2a trial (NCT02633020), AMG174 was investigated in patients with refractory CD type 2 of which 19 received seven intravenous doses over 10 weeks (8 mg/kg) and 9 received placebo. Ameliorative effects by AMG174 were observed on the symptomatology, whereas no difference between drug and placebo groups was found in the reduction of aberrant IELs from baseline [57], Table 2.

(e) *Blocking cell gut migration (anti-CCR9/α4β7 integrin)*

Aimed to specifically block gut migration of gluten reactive T cells in CD patients, other therapeutic approaches were investigated, as CCR9 receptor antagonist CCX282-B, and α4β7 integrin antagonist PTG-100. A randomized, double-blind, placebo-controlled, phase 2 study (NCT00540657) tested the CCX282-B (250 mg capsule, twice daily for 13 weeks) in mitigating the effects of gluten ingestion in 90 patients, on GFD for at least 24 months, in terms of mucosal damage, serology and symptoms, Table 2. The study has already been completed, although the publication of the results is still pending.

To test PT-100, a phase 1b randomized, double-bind, placebo-controlled study is, to date, in recruitment status (NCT04524221). The clinical trial will evaluate the safety and efficacy of PTG-100 in preventing gluten-induced inflammatory injury to the small intestine in 30 CD patients, to whom will be administrated either

Table 2 Ended or current therapies targeting immuno stimulatory properties

Therapeutic strategy	Mechanism of action	Status	Clinical Trial Identifier	Publications	Outcome measures	Clinical trials results
TAK-101 (TIMP-GLIA)	Gluten tolerization	Phase 1	NCT03486990	51	Safety, tolerability, pharmacokinetics in celiac patients; circulating gliadin-specific T cells after 3- and 14-days gluten oral challenge (by ELISA and ELISPOT), intestinal mucosal morphology (Vh:Cd, and IELs), symptoms	TAK-101 was well tolerated and prevents gluten-induced immune activation
		Phase 2	NCT03738475			
		Phase 2 (Not yet recruiting)	NCT04530123	Not provided	Circulating gliadin-specific T cells after 3- and 14-days gluten oral challenge (by ELISA and ELISPOT)	Not available
ZED1227	tTG2 inhibitor	Phase 2a	EUDRA CT 2017–002,241-30	Not provided	Safety, efficacy and tolerability of a 6-week treatment in subjects with well-controlled CD during gluten challenge. Small-bowel mucosal morphology, inflammation, serology, adverse events	Not available
AMG 714 (PRV-015)	Human monoclonal antibody against interleukin 15	Phase 2b (Recruiting)	NCT04424927	Not provided	Symptoms, IELs density, PRV-015 and anti-PRV-015 antibodies serum concentrations	Not available
		Phase 2a	NCT02637141	56	Levels of serum antibodies; % IELs by flow cytometry and IHC; VH:CD Ratio and Marsh score	AMG 714 does not improve VH:CD ratio, affects the intraepithelial

(continued)

Table 2 (continued)

Therapeutic strategy	Mechanism of action	Status	Clinical Trial Identifier	Publications	Outcome measures	Clinical trials results
						lymphocyte density and symptoms
		Phase 2a	NCT02633020	57	Levels of serum antibodies; % IELs by flow cytometry and IHC; VH:CD Ratio and Marsh score	AMG 714 in patients with RCDII does not reduce the number of aberrant intraepithelial lymphocytes, effects on symptoms
CCX282-B	CCR9 antagonist	Phase 2	NCT00540657	Not provided	Mucosal damage (Vh:Cd) and inflammation, serology and symptoms after gluten ingestion in treated celiacs	Not available
PT-100	Anti-integrin $\alpha 4\beta 7$	Phase 1	NCT04524221	Not provided	Mucosal damage (Vh:Cd), intraepithelial lymphocyte density, serology and symptoms after gluten ingestion in treated celiacs	Not available
Hookworm Necator Americanus	Gluten tolerization	Phase 2a	NCT00671138	58	Duodenal histology, quantification of α-gliadin peptide (QE65)-specific systemic interferon-γ-producing cells (by ELISPOT) pre- and post-wheat challenge	Hookworm infection does not induce an improvement in histological damage
		Phase1/2	NCT01661933	60	Safety and efficacy; Duodenal histology (VH:CD Ratio and Marsh score), % IELs, levels of serum antibodies post low-dose of gluten challenge	Hookworm infections promote tolerance and stabilize or improve all tested indices of gluten toxicity in CeD subjects

(continued)

Table 2 (continued)

Therapeutic strategy	Mechanism of action	Status	Clinical Trial Identifier	Publications	Outcome measures	Clinical trials results
VIVOMIXX®	Probiotic supplementation	Phase 4	NCT04160767	Not provided	Vitamin B6, B12, 25' hydroxy vitamin D, folic acid and omocystein serum levels and inflammatory markers, metabolomics on stool samples in the CD patients on GFD	Not available

placebo or PTG-100 (600 mg taken twice daily in capsule form), for 42 days. They will also receive gluten challenge, a cookie or equivalent, twice daily. A small bowel mucosa biopsy will be performed at the start and the end of the treatment period to evaluate villous height-to crypt ratio and IELs density. Blood samples will be routinely taken to evaluate tTG2 antibody levels while the symptoms will be recorded using the celiac symptoms index (CSI) survey, Table 2.

(f) *Immunomodulation with Necator-hookworm*

Several clinical trials are evaluating the suppression of mucosal inflammation in CD by experimental infection with hookworm *Necator americanus*. A phase 2a randomized, double blinded, placebo controlled study tested the effect of *Necator americanus* larvae inoculation (at week 0 and 12) on the suppression of the immune response induced by gluten (16 g of gluten daily for 5 days at week 20-group) in 20 GFD-treated CD patients (NCT00671138). No clear protective effects were reported of this trial by Daveson et al. [58], although the basal production of IFNγ and IL17A from duodenal biopsy culture was suppressed in hookworm-infected compared to uninfected patients [59].

In another clinical trial, a 52-week phase1/2 study (NCT01661933), 12 CD adult patients were inoculated with 20 *Necator americanus* larvae and received escalating gluten challenges as pasta. The results shown the combination of infection and gluten micro-challenge promoted tolerance and stabilized or improved all evaluated indices of gluten toxicity (mucosal damage, symptoms and the percentage of inflammatory and regulatory T cells), [60]. A phase 1b multicentre clinical trial (NCT02754609) was completed in 2019, not followed by the publication of the data yet.

(g) *Evaluation of probiotic supplementation*

Dysbiosis could play a key role in the pathogenesis of CD influencing the intestinal permeability and the regulation of the immune system. The administration of probiotics might potentially represent a novel strategy to treat CD. The effects of several probiotic formulations have been investigated in mouse models, demonstrating a modulatory activity on innate and adaptive immune responses activated in CD [61, 62].

Several clinical trials assessed the safety and efficacy of different probiotic strains in the treatment of CD. A mixture of probiotics containing 8 different strains of bacteria (*Streptococcus thermophilus DSM 24731, bifidobacteria B. breve DSM 24732, B. longum DSM 24736, B. infantis DSM 24737*, lactobacilli *L. acidophilus DSM 24735, L. plantarum DSM 24730, L. paracasei DSM 24733, L. delbrueckii subsp. bulgaricus DSM 24734*) attenuated the inflammation and symptomatology of colitis in induced colitic mice models, [63, 64]. This preparation is currently under investigation in a phase 4 study (NCT04160767). The study started in 2019 involves enrolling 90 CD patients on GFD and will test the effect of this probiotic

mixture on vitamin B6, B12, 25'OH D, folic acid and omocystein levels, metabolic and inflammatory status, and gut microbiota metabolomics, in the CD patient group that received the drug compared to placebo CD group.

6 Conclusion

GFD is associated with high economic and societal burden, decreased quality of life and in some cases not satisfactory response. For these reasons the search for therapies alternative to GFD has become a priority. In fact, an increased comprehension of pathogenetic mechanisms has revealed new therapeutic targets and also new biomarkers useful to assess the efficacy of new treatments. Although we still lack an animal model recapitulating all the features of CD, progress in this area have contributed to test new strategies particularly those based on immunomodulation [50, 51, 59, 60].

One of the unsolved problems is how to evaluate the response to the new drugs and which outcomes to privilege: gluten-dependent symptoms, gluten-specific T cell response, CD-specific autoantibodies and gut histology after prolonged gluten challenge have been considered. Ideally, the protection of the intestinal mucosa from gluten-induced damage should represent the gold standard, but so far, only in one study investigating the effect of an enzymatic preparation [33] such a goal has been reached. Most approaches have been tested for their ability to attenuate symptoms in patients non responsive to GFD. In fact, the relationship between symptoms and objective endpoints such as gluten-specific T cell response, autoantibodies and gut histology after gluten challenge has not been fully understood. One other important limitation is the lack hitherto of paediatric studies, a part those based on the use of probiotics; the suitability of such approaches in children remains to be assessed.

In conclusion, at moment no drug has been licenced for the therapy of CD and GFD remains the cornerstone of the treatment. On the other hand, the most promising candidates have entered phase 3 trial and we may expect that advances in the comprehension of CD pathogenesis will help to identify new targets and new strategies (Fig. 1). In such respect, CD remains a model for other autoimmune diseases, such as type 1 diabetes, and progress in this area will certainly impact on their management.

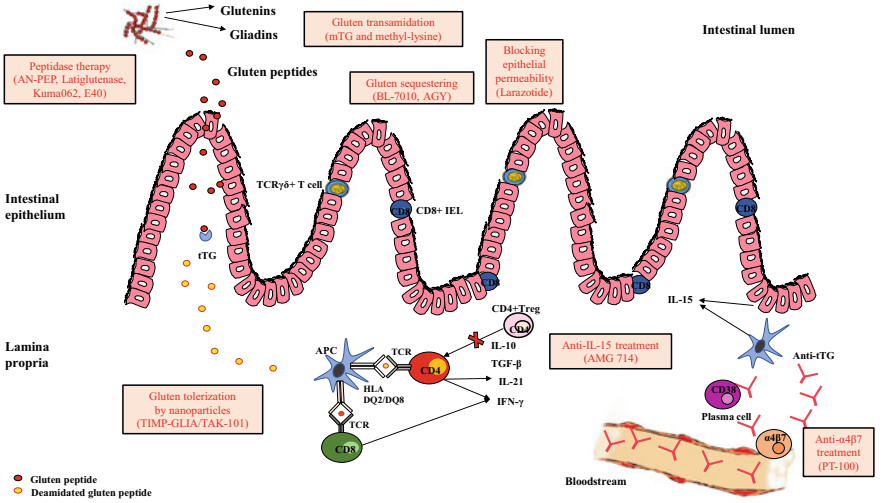

Fig. 1 Celiac Disease pathogenesis and main pathways targeted by drugs under clinical investigation. Several therapies currently in clinical trials are designed to reduce the load of gluten immunotoxic sequences thus inhibiting the contact with the gut-associated immune cells. Among these drugs, glutenases are efficient enzymes that degrade gluten proteins in non-immunogenic short peptides. Transamidation reaction by microbial transglutaminase operates by masking the immunogenic sequences to the recognition of cognate CD4+ T cells resident in lamina propria. Biocompatible polymers and polyclonal antibodies are designed to sequester gluten proteins hampering transport through the epithelial layers. Other experimental molecules act to suppress the proinflammatory CD4+ T cells reactive to gluten, inducing an anergic status

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