Celiac Disease—Multidisciplinary Approaches

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Celiac disease (CD), also known as gluten-sensitive enteropathy, is a permanent intestinal intolerance to proteins found in wheat, rye, barley, and possibly oats that causes mucosal lesions and nutrient malabsorption in genetically predisposed individuals. The precipitating factors are the storage proteins contained in these cereals, widely termed “gluten” in the field of CD. The current essential treatment for CD is strict lifelong adherence to a gluten-free diet. The frequency of CD has long been underestimated, but with the development of sensitive serological tests it is becoming clear that CD is one of the most prevalent food intolerances in many parts of the world. Intense multidisciplinary studies have contributed to substantial progress in understanding and treatment of CD during the last two decades. Due to the complexity of the disease, many experts in the fields of medicine, chemistry, food technology, and law have been involved in the research on CD. This article provides an overview of the different approaches to studying CD.

History
Aretaeus of Cappadocia, a Greek physician practicing in Rome and Alexandria during the second century A.D., was the first to describe a disease feature similar to the current description of CD. He called his patients “koiliakos” based on the Greek term “koilia” (abdomen). Many centuries later, in 1888, English physician and pediatrician Samuel Gee published the first modern description of CD and recommended a dietary treatment without knowing the precipitating factor(s) (21). Based on his recommendations, extreme dietary therapies were used for many years. For example, all sources of carbohydrates (e.g., bread, cereals, and potatoes) were excluded or a strict banana diet was recommended. The discovery by Dutch pediatrician W. K. Dicke in 1950 that the ingestion of wheat, and later the ingestion of rye and barley, is responsible for CD provided the breakthrough required for development of an effective therapy (12).

Fractionation of wheat flour and testing led to the conclusion that gluten (the rubber-like protein mass that remains when wheat dough is washed to remove starch and soluble constituents) is toxic to individuals with CD, whereas starch and water-soluble albumins are not (67). Since then, all proteins that trigger CD have been integrated into the collective term “gluten” in the field of CD, and a “gluten-free diet” has been introduced as the conventional treatment for CD.

Epidemiology and Genetics
In the past, CD was considered a rare childhood disorder, with a frequency of 1 in 1,000–2,000 individuals. However, modern serological screening followed by small intestinal biopsy, has revealed that CD is one of the most prevalent food intolerances worldwide and can occur at any age. CD is most prevalent in Europe and regions to which Europeans have emigrated, including Australia and North and South America. Serological data suggest an overall high prevalence ranging from 0.2 to 1% in many geographic regions. Recently, CD has increasingly been found in areas of the developing world such as north Africa, the Middle East, and India. The incidence among first-degree relatives has been reported to be strongly elevated (≈10%), and the rate in monozygotic twins has been reported to be ≈75%.

Human leukocyte antigen (HLA) class II alleles HLA-DQ2 and HLA-DQ8 at the major histocompatibility complex have the strongest genetic association with CD. The large majority of CD patients are DQ2 positive (95%); the remainder are DQ8 positive. The absence of the genes is a reliable negative predictor of the disease. However, these genes are also common in portions of the non-CD population (>25%). Therefore, further genome research to identify risk factors has been performed that implies the involvement of non-HLA genes (55). Unfortunately, the results indicate little consensus and show that each of the non-HLA genes has a relatively modest effect (39). Thus, identification of non-HLA genes related to disease susceptibility is an ongoing challenge.

Clinical Features
Numerous symptoms are associated with CD and can be divided into intestinal features and extra-intestinal features caused by the malabsorption of essential nutrients. The clinical appearance of CD is highly variable and can range from asymptomatic to full-blown CD symptoms. In infants classic symptoms such as diarrhea, abdominal distension, vomiting, failure to thrive, and apathy appear after weaning and introduction of cereals into the diet. In older children and adolescents the clinical presentation is usually less obvious, with diarrhea, loss of appetite, fatigue, anemia, short stature, and delayed puberty predominating. In addition to classic symptoms, adults show increased effects of in mineral and vitamin deficiencies, such as anemia, bone pain and fractures, osteoporosis, dental defects, skin

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lesions, night blindness, and infertility. Untreated CD may lead to a higher risk of malignancies such as T-cell lymphoma. A minor number of patients show psychological or psychiatric symptoms. It is worth noting that there is a large number of undiagnosed subjects with no or negligible symptoms but who have villous atrophy (silent CD). CD is frequently associated with other disease processes such as type I diabetes, thyroid disease, and dermatitis herpetiformis.

Small intestinal biopsies of the jejunal intestine typically reveal a flat mucosa with a partial or complete absence of normal villi (Fig. 1). Histological examination further demonstrates a cellular infiltrate of the lamina propria, which consists of plasma cells and lymphocytes (43). Intestinal appearance can vary from a normal mucosa with an increase of intraepithelial lymphocytes (IEL) (latent CD) to a completely flat mucosa (Marsh [43] has proposed a four-stage disease progression). In untreated CD, specific serological antibodies to gliadin and transglutaminase-2 (TG2) are elevated, which can be used for the diagnosis of CD.

Testing CD Toxicity

Numerous in vivo and in vitro methods have been used to identify the CD toxicity of cereal proteins and peptides (reviewed by Troncone and Auricchio [64] and Shewry et al. [59]). Most investigators agree that in vivo testing is the gold standard. Early researchers established CD toxicity using a series of feeding tests based on the manifestation of symptoms such as steatorrhea and malabsorption of fat or xylose. One of the most important impediments, however, was that high amounts of gluten equivalents (10–100 g/patient) were necessary. Direct instillation into the small intestine followed by biopsy after several hours and histological judgement enabled use of smaller amounts of gluten (=1 g), and solid information on CD-specific toxicity was obtained (27). Because only a limited number of test patients and limited amounts of pure proteins and peptides were available, a series of in vitro tests was developed. Organ culture of intestinal tissue of CD patients challenged by a substance to be tested (milligram amounts) has been proposed as the most reliable in vitro model. More recently, T-cell lines and clones from the intestinal tissue of CD patients were used to measure the immunogenic effects of cereal proteins and peptides. However, T-cell tests frequently differ in their reactions to antigens, and immunogenicity does not always correspond to the toxicity demonstrated by in vivo or organ culture tests.

**CD-Toxic Cereals**

Soon after the CD toxicity of wheat flour was established (12), a series of investigations led to the conclusion that rye and barley are also harmful to those with CD, whereas corn, rice, and buckwheat are not (reviewed by Shewry et al. [59] and Kasarda [34,35]). There is still some disagreement concerning the CD-toxicity of oats. Other seeds used for food production have not been subjected to controlled CD-toxicity testing. According to Kasarda (34,35) the taxonomy of plants may provide useful guidance in categorizing plants as safe or unsafe for those with CD. All grains that are known to be toxic to those with CD (wheat, rye, and barley) are found in a single tribe, the Triticeae, within the Poaceae (grass) family. Due to this botanical relationship and their protein patterns (19,73), all wheat species (common and durum wheats, spelt, kamut, emmer, and einkorn), the wheat/rye hybrid triticale, and all botanical forms of rye and barley should be avoided by CD patients. Oats, over which there is continuing controversy regarding CD toxicity, belong to a separate tribe, the Aveneae. All common cereals that are not toxic to those with CD (rice, corn, sorghum, and millet) are more distant from the Triticeae tribe and show separate evolutionary lines within the Poaceae family. Therefore, rarely used cereals that are closely related to corn, millet, or sorghum, such as teff, ragi, and Job’s tears, are not likely to be toxic to CD patients. All seeds outside the Poaceae family, such as buckwheat, amaranth, and quinoa, are assumed to be safe for CD patients.

The results of early studies on the CD-toxicity of oats based on fat or xylose malabsorption were contradictory. Unfortunately, the oat samples used for the in vivo challenge had not been tested for contamination with wheat, rye, or barley. Most recent studies found that pure oats or purified avenins were well-tolerated clinically, did not cause histological changes, and did not induce immune response (28,31,32,36,37,61). A small number of patients, however, showed partial villous atrophy and CD-specific immune responses after challenge with oats (2,42). Thus, the toxicity of oats for CD patients is still being debated.

Approaches aimed at reducing or abolishing the CD toxicity of wheat are currently underway. In addition to the strategy to reduce the CD toxicity of wheat through conventional breeding (68,69), the recombinant production of completely safe gluten proteins is also a goal and is based on current knowledge of the elimination of CD-toxic fragments (40,47). The first approach is based on the assumption that the dose of exposure to gluten-derived CD-active epitopes would contribute to a general reduction of the prevalence of CD and symptom severity in the population.

**CD-Toxic Proteins and Peptides**

Cereal proteins are classically grouped into water-soluble albumins; salt-soluble globulins; prolamins, which are soluble in aqueous alcohols; and glutelins, which are soluble in aqueous alcohols only after reduction of disulfide bonds (52,74). All fractions are complex mixtures of numerous proteins that are related to some degree. Albumins and globulins are mainly metabolic proteins such as enzymes and enzyme inhibitors, and prolamins and glutelins are storage proteins that provide

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**Fig. 1.** Cross-sections of normal (left) and celiac disease-damaged (right) intestinal mucosa. (Source: German Celiac Society, http://dzg-online.de/pressebilder.2680.0.html)
the embryo with amino acids and nitrogen during germination. Trivial names have been given to the storage proteins from different cereals: gliadins (prolamins) and glutenins (glutelins) from wheat, secalins from rye, hordeins from barley, avenins (prolamins) from oats, zeins from corn, oryzins from rice, and kafirins from sorghum and millet. Prolamins occur mostly in grains and flours is in part monomeric and in part polymeric. The number of amino acid residues ranges from 203 (avenins) to 815 (HMW-GS). The amino acid compositions are characterized by high Gln (26–53 mol%) and Pro (11–29 mol%) contents. Proteins in the HMW group also have high Gly contents (16–20 mol%). Furthermore, the aromatic amino acids Phe + Tyr (5–10 mol%) are predominant.

Based on numerous in vivo and in vitro tests on proteins and peptides (reviewed by Shewry et al. [59] and Wieser and Koehler [74]), most investigators involved in CD research agree that the entirety of storage proteins from wheat, rye, and barley (prolamins and glutelins) and possibly oats (prolamins) have the potential to activate the disease. CD-toxic storage proteins have a high degree of structural homology and can be classified into three groups according to a range of molecular weights and similarities in amino acid sequences. Each group contains closely related protein types (74). The high molecular weight (HMW) group contains HMW-glutenin subunits (GS) (wheat), HMW secalins (rye), and D-hordeins (barley). The medium molecular weight (MMW) group consists of α- and γ-gliadins (wheat), γ-75-secalins (rye), γ- and B-hordeins (barley), and avenins (oats). Some structural characteristics of representatives of the different cereal protein types are summarized in Table I. Their chemical state in grains and flours is in part monomeric and in part polymeric. The number of amino acid residues ranges from 203 (avenins) to 815 (HMW-GS). The amino acid compositions are characterized by high Gln (26–53 mol%) and Pro (11–29 mol%) contents. Proteins in the HMW group also have high Gly contents (16–20 mol%). Furthermore, the aromatic amino acids Phe + Tyr (5–10 mol%) are predominant.

Generally, the amino acid sequences can be subdivided into different structural domains, including the repetitive domains, which are predominant and unique for each type (72). The repetitive domains vary considerably in number, length, and composition of repetitive units, but the frequent occurrence of Gln and Pro is common (Table I). The repetitive domains are largely responsible for resistance to gastroenterological enzymes and CD toxicity. This is well supported by the numerous isolated and synthesized peptides identified as agents that are toxic for CD patients by in vivo and in vitro tests (summarized by Ciccocioppo et al. [7], Dewar et al. [11], Stern et al. [63], and Wieser and Koehler [74]). Table II presents a selection of CD-toxic peptides from different protein types. Certain peptides drive adaptive immune response, while others elicit an innate response.

Molberg et al. (48) discovered that TG2 selectively modifies CD-toxic peptides through deamidation of Gln before they are recognized by T cells. TG2 has a specificity only for select Gln residues that depends on the amino acids neighboring the target Gln (bold in Table II). The sequencess QXP, QXXXF, and QQQXF (X representing any amino acid and F representing hydrophobic amino acids), but not QP or QXXP, were identified as preferred substrates for TG2 (17,65). Some CD-toxic peptides, however, do not require deamidation to activate CD. Substitutions of single amino acids within the CD-toxic epitopes showed that certain amino acids, particularly Pro and Gln, take up an anchor position for binding to HLA-DQ2 and HLA-DQ8 molecules and T-cell receptors (TCR) (15,50).

Pathomechanism

During the last two decades great progress has been made in understanding the pathomechanism of CD (reviewed by Brandtzaeg [4], Hourigan [29], Jabri and Solid [30], Kagnoff [33], and Schuppan et

Table 1. Characterization of storage protein types from wheat, rye, barley, and oatsa

<table>
<thead>
<tr>
<th>Group Typeb</th>
<th>Codec</th>
<th>Residues</th>
<th>Statad</th>
<th>Repetitive Unitc (frequency)</th>
<th>Partial Amino Acid Composition (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMW-GS x</td>
<td>Q6R2V1</td>
<td>815</td>
<td>p</td>
<td>QQPGGQ (72×)</td>
<td>Q 36 P 13 F + Y 5.8 G 20</td>
</tr>
<tr>
<td>HMW-GS y</td>
<td>Q52JL3</td>
<td>637</td>
<td>p</td>
<td>QQPGGQ (50×)</td>
<td>Q 32 P 11 F + Y 5.5 G 18</td>
</tr>
<tr>
<td>HMW secalin x</td>
<td>Q94IK6</td>
<td>760</td>
<td>p</td>
<td>QQPGGQ (66×)</td>
<td>Q 34 P 15 F + Y 6.7 G 20</td>
</tr>
<tr>
<td>HMW secalin y</td>
<td>Q94IL4</td>
<td>716</td>
<td>p</td>
<td>QQPGGQ (60×)</td>
<td>Q 34 P 12 F + Y 5.0 G 18</td>
</tr>
<tr>
<td>D-Hordein</td>
<td>Q40554</td>
<td>686</td>
<td>p</td>
<td>QQPGGQ (26×)</td>
<td>Q 26 P 11 F + Y 5.5 G 16</td>
</tr>
<tr>
<td>MMW group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ω5-Gliadin</td>
<td>Q402J5</td>
<td>420</td>
<td>m</td>
<td>(Q)QQQFP (65×)</td>
<td>Q 53 P 20 F + Y 10.0 G 0.7</td>
</tr>
<tr>
<td>ω1,2-Gliadin</td>
<td>Q6DLCL3</td>
<td>373</td>
<td>m</td>
<td>(Q)QQQFP (42×)</td>
<td>Q 42 P 29 F + Y 9.9 G 0.8</td>
</tr>
<tr>
<td>ω-Sealin</td>
<td>O04365</td>
<td>338</td>
<td>m</td>
<td>(Q)QQQFP (32×)</td>
<td>Q 40 P 29 F + Y 8.6 G 0.6</td>
</tr>
<tr>
<td>C-Hordein</td>
<td>Q40555</td>
<td>327</td>
<td>m</td>
<td>(Q)QQQFP (36×)</td>
<td>Q 37 P 29 F + Y 9.4 G 0.6</td>
</tr>
<tr>
<td>LMW group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Gliadin</td>
<td>Q9M4M5</td>
<td>273</td>
<td>m</td>
<td>QPQFPPQQYP (5×)</td>
<td>Q 36 P 15 F + Y 7.4 G 2.6</td>
</tr>
<tr>
<td>γ-Gliadin</td>
<td>Q9G491</td>
<td>284</td>
<td>m</td>
<td>(Q)QQQFP (15×)</td>
<td>Q 36 P 18 F + Y 5.2 G 2.9</td>
</tr>
<tr>
<td>LMW-GS</td>
<td>Q52NZ4</td>
<td>282</td>
<td>p</td>
<td>(Q)QQQFP (11×)</td>
<td>Q 32 P 13 F + Y 5.7 G 3.2</td>
</tr>
<tr>
<td>γ-40K-Sealinf</td>
<td>Q4132O</td>
<td>–</td>
<td>m</td>
<td>QPQFPF</td>
<td>– – – – – – –</td>
</tr>
<tr>
<td>γ-75K-Sealin</td>
<td>Q9RF41</td>
<td>436</td>
<td>p</td>
<td>QPQFPFF (32×)</td>
<td>Q 38 P 22 F + Y 6.1 G 1.6</td>
</tr>
<tr>
<td>γ-Hordein</td>
<td>P17990</td>
<td>286</td>
<td>m</td>
<td>QPQFPFF (15×)</td>
<td>Q 28 P 17 F + Y 7.7 G 3.1</td>
</tr>
<tr>
<td>B-Hordein</td>
<td>P06470</td>
<td>274</td>
<td>p</td>
<td>QPQFPQ (13×)</td>
<td>Q 30 P 19 F + Y 7.3 G 2.9</td>
</tr>
<tr>
<td>Avenin</td>
<td>Q09072</td>
<td>203</td>
<td>m</td>
<td>PFVQQQ (3×)</td>
<td>Q 33 P 11 F + Y 8.4 G 2.0</td>
</tr>
</tbody>
</table>

a Wieser and Koehler (74).

b HMW, MMW, and LMW = high, medium, and low molecular weight, respectively; GS = glutenin subunits.

c Databank Uni Prot KB/TREMBL (http://pir.georgetown.edu).

d p = polymeric; m = monomeric.

e Basic unit frequently modified by substitution, insertion, and deletion of single amino acid residues.

f Fragment.
al. [55]). The first step in gluten processing after intake is characteristic for all humans. Their high proline content renders CD-toxic proteins resistant to complete proteolytic digestion (58). Thus, relatively large fragments (peptides) accumulate in the small intestine, in particular those derived from the Gln- and Pro-rich repetitive domains of the proteins (Table I). These pass through the enterocyte layer by means of exo- and endocytosis (78) and arrive at the lamina propria, where they trigger two CD-specific immunological pathways: adaptive and innate immune responses.

The mechanism of the adaptive immune system is well understood. Gln- and Pro-rich peptides containing more than eight amino acid residues are bound to the histocompatibility antigens HLA-DQ2 and -DQ8 expressed on the surface of antigen-presenting cells (e.g. dentritic cells, macrophages, and B cells). Here, the peptides are processed by TG2 deamidating specific Gln residues to Glu residues (17,65) and then presented to the TCR of gluten-sensitive CD4 helper cells. Models of the interactions of CD-toxic epitopes with DQ2 molecules and TCR have been presented by Ellis et al. (15), Dewar et al. (11), and Jabri and Sollid (30).

Within the CD-toxic epitope Pro 62–Pro 71 of α2-gliadin (15) residues, Pro 62, Glu 65, Pro 67, Tyr 68, and Gln 70 are bound to the binding groove of the DQ2 molecule, while residues Gln 63, Pro 64, Leu 66, Pro 69, and Pro 71 may interact with TCR (Fig. 2). T-cell stimulation leads to the secretion of pro-inflammatory cytokines, in particular γ-interferon and interleukin2, and tumor necrosis factor, metalloproteinases, and nitric oxide (Th-1 response). Activated T cells also elicit an anti-inflammatory Th-2 response that promotes B-cell maturation and expansion of plasma cells that produce IgA and IgG serum antibodies to gliadin and autoantibodies to TG2 (13,18). These antibodies can be used as specific indicators for non-invasive screening tests to diagnose CD, but they are unlikely to cause CD symptoms (39).

In contrast, the rapid innate immune system is characterized by a massive increase in IEL, one of the hallmarks of CD, which can be observed very early in the disease, before the onset of villous atrophy. Two subsets of IEL bearing the αβTCR or the γδTCR are linked to CD innate immune response. IL-15 has been considered a central player in this part of the gluten-induced immune response. IL-15 is produced by epithelial and lamina propria cells in active CD, but not by T and B cells involved in the adaptive immune response. Recently, it has been suggested that amylase inhibitors present in cereal flours are coactivators of the innate immune system (56).

**Diagnosis of CD**

Classic symptoms of CD such as diarrhea, failure to thrive, iron deficiency anemia, and weight loss are the most common indications of CD incidence. In these cases, the physician should initiate a serological screening test, e.g., determine anti-gliadin and anti-TG2 antibodies, which guarantees sensitivity and specificity at nearly 100%. For confirmation of positive results, it is mandatory to perform a jejunal biopsy according to the European Society for Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) procedure. Previously, three steps were implemented: 1) biopsy → flat mucosa; 2) gluten-free diet, biopsy → mucosa remission; and 3) gluten challenge, biopsy → flat mucosa (45). The question of whether three biopsies and supervised gluten rechallenge are necessary led to the suggestion by ESPGHAN in 1990 that it is not mandatory to proceed to a gluten challenge if a gluten-free diet has resulted in good improvement in the symptoms and morphology of the biopsy specimen (71).

**CD Therapy**

After a diagnosis of CD has been established, permanent lifelong adherence to a gluten-free diet is the current essential treatment. The daily intake of gluten should be <20 mg. CD patients may consume gluten-free foods from two different categories. First, they may eat a wide range of common products such as meat, fish, fruits, and vegetables. However, they should be aware of numerous composite foods that contain hidden sources of gluten, such as thickened sauces and soups, puddings, and sausages. Second, CD patients may consume gluten-free dietetic

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**Table II. Amino acid sequences of select celiac disease-toxic peptides from different protein types**

<table>
<thead>
<tr>
<th>Origin</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Gliadins</td>
<td>PQPQLPYQPQQLPY</td>
</tr>
<tr>
<td>α-Gliadins</td>
<td>LGQQQPFPQQPQY*</td>
</tr>
<tr>
<td>γ-Gliadins</td>
<td>FPQPQPYPQPQP</td>
</tr>
<tr>
<td>γ-Gliadins</td>
<td>FSQPQPQFPQPQ</td>
</tr>
<tr>
<td>LMW glutenins</td>
<td>QQQQPPSQQQSPFS</td>
</tr>
<tr>
<td>LMW glutenins</td>
<td>QQPPQQQPQPLPQ</td>
</tr>
<tr>
<td>HMW glutenins</td>
<td>QQGYPSPSQSQ*</td>
</tr>
<tr>
<td>HMW glutenins</td>
<td>PGQGQQGYPSTPSQSQGQ*</td>
</tr>
<tr>
<td>γ-Secalins</td>
<td>QFPFPQQPFPFQ</td>
</tr>
<tr>
<td>γ-Hordeins</td>
<td>QFPFPQPPQFPFPQP</td>
</tr>
<tr>
<td>Avenins</td>
<td>QYQYPPEQQPFPVQ</td>
</tr>
</tbody>
</table>

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*a* Dewar et al. (11), Ciccocioppo et al. (7), and Wieser and Koehler (74).

*b* LMW and HMW = low and high molecular weight, respectively.

*c* One letter codes for amino acids; bold Q residues are modified to E residues by TG2; * indicates deamidation is not necessary.
foods. These are primarily alternatives to products containing wheat, rye, and barley, such as breads, other baked products, pastas, and beer.

These dietary restrictions pose a considerable challenge for CD patients. Therefore, a series of studies has been performed to search for alternative therapies (reviewed by Sollid and Koshla [60], Gianfrani et al. [23], Schuppan et al. [55], and Wieser and Koehler [76]). Typically, CD-toxic peptides derived from digested gluten survive the normal digestive process (58). Supplementation with additional proteolytic enzymes may degrade CD-toxic epitopes and avert an immune response. Peptidases from bacteria, fungi, and germinating cereals have been proposed as an oral therapy. Some of these approaches are already in Phase I and II clinical trials. Moreover, the use of inhibitors of zonulin, a key molecule for intestinal permeability (14), and TG2, the autoimmunogen involved in CD (26), the inhibition of gluten peptide presentation by HLA-DQ2 antagonists (77); and modulation or inhibition of proinflammatory cytokines (3) have been proposed. In summary, a wide range of studies have been performed on alternative therapies for CD. However, the risks, benefits, and costs of alternatives have to be carefully weighed, and the conditions and indications under which such alternative therapies might be warranted have to be accurately defined (33).

Legislation

Gluten as it pertains to the field of CD is defined in the Codex Alimentarius (8). From a legal point of view, “gluten is defined as a protein fraction from wheat, rye, barley, oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant and that is insoluble in water and 0.5M NaCl. Prolamins are defined as the fraction from gluten that can be extracted by 40–70% of ethanol. The prolamin from wheat is gliadin, from rye is secalin, from barley hordein and from oats avenin. The prolamin content of gluten is generally taken as 50%.”

Studies have shown that celiac patients can tolerate a low amount of gluten without adverse effects (6). Therefore, thresholds for gluten concentration have been established, and foods falling below the threshold can be labeled “gluten-free.” According to the latest revision (step 8) of the Codex Alimentarius (8), “Gluten-free foods are dietary foods consisting of or made only from one or more ingredients which do not contain wheat (i.e., all Triticum species, such as durum wheat, spelt, and kamut), rye, barley, oats or their crossbred varieties, and the gluten level does not exceed 20 mg/kg in total, based on the food as sold or distributed to the consumer,” and/or “consisting of one or more ingredients from wheat (i.e., all Triticum species, such as durum wheat, spelt, and kamut), rye, barley, oats or their crossbred varieties, which have been specially processed to remove gluten, and the gluten level does not exceed 20 mg/kg in total, based on the food as sold or distributed to the consumer.” Labeling of foods specially processed to reduce gluten content to a level higher than 20 mg/kg, up to 100 mg/kg, may be determined at the national level.

Legislation in the European Union (EU) largely follows the Codex Alimentarius, and definitions, thresholds, and labeling specified in European Commission Regulation 41/2009 (16) are the same as those in the Codex Alimentarius. Foods with a

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gluten content between 20 and 100 mg/kg may be labeled with the term “very low gluten.” Oats are specially regulated in the EU, and oats consumed by celiac patients must not be contaminated by wheat, rye, barley, or their crossbred varieties, and the gluten content of such oats must not exceed 20 mg/kg.

Division 24 of the Canadian Food and Drug Regulations (FDR) sets out specific regulations that apply to “Foods for Special Dietary Use.” As of August 4, 2012, section B.24.018 of the FDR states that it is prohibited to label, package, sell, or advertise a food in a manner likely to create an impression that it is a gluten-free food if this food contains any gluten protein or modified gluten protein, including any gluten protein fraction, referred to in the definition of “gluten” in subsection B.01.010.1. While no specific threshold is mentioned in the regulations themselves, the best available scientific evidence indicates that levels of gluten lower than 20 mg/kg in gluten-free foods would protect the health of the vast majority of people with CD. This level is recognized internationally in the Codex Alimentarius (8).

Based on the available scientific evidence, Health Canada considers that gluten-free foods, prepared under Good Manufacturing Practices, which contain levels of gluten not exceeding 20 mg/kg as a result of cross-contamination, meet the health and safety intent of section B.24.018 when a gluten-free claim is made. However, based on enhanced labeling regulations for allergens and gluten sources, any intentionally added gluten sources, even at low levels, must be declared either in the list of ingredients or in a “Contains” statement.

The situation in the United States is considerably different. Since the passage of the Food Allergen Labeling and Consumer Protection Act (FALCPA) on August 2, 2004, which amended the Federal Food, Drug and Cosmetic Act, the status of gluten-free labeling has not been finally regulated in the United States. Although FALCPA mandates the proposal of a rule to define and permit the use of the term “gluten-free” on food labels, it does not require the U.S. Food and Drug Administration (FDA) to establish a threshold level for gluten. To date a final rule has not yet been issued, leaving consumers confused. The proposed rule has also created issues for other agencies, such as the U.S. Alcohol and Tobacco Tax and Trade Bureau, as it relates to malt beverages, because the FDA believes that for some food matrices (e.g., fermented or hydrolyzed foods and beverages) there are no validated methods currently available that can be used to accurately determine gluten concentrations <20 mg/kg. In such cases, the FDA is considering whether to require manufacturers of such foods to use a scientifically valid method that will reliably and consistently detect gluten at ≤20 mg/kg before including a gluten-free claim in their food labels.

Legally, Australia and New Zealand are bound by the Food Standards Australia and New Zealand (FSANZ) Food Standards Code (FSC), Standard 1.2.8. In this code, “A claim to the effect that a food is gluten free must not be made in relation to a food unless the food contains (i) no detectable gluten; and no (ii) oats or their products; or (iii) cereals containing gluten that have been malted, or their products.” Foods with a “low gluten” content may have a gluten concentration of no more than 20 mg of gluten per 100 g of food. Such claims are only permitted provided certain specified conditions are met. Thus, in Australia and New Zealand gluten free means that gluten is not detected by the most appropriate currently available techniques. This situation is supported by the Australian Competition and Consumer Commission, a consumer advocacy group, which interprets the presence of a component claimed to be gluten free or absent as “false advertising.” At the moment it seems unlikely that the Australian Competition and Consumer Commission would ever accept gluten free as containing any detectable gluten.

Gluten Analysis

The detection and quantitation of gluten in foods is essential for CD patients, the food industry, and food control. Reliable methods should include sufficient sensitivity, selectivity, precision, and a suitable protein reference. Moreover, they should be applicable not only to raw but also to processed (heated, fermented) materials. Many laboratories have been searching for methods that are able to accurately quantitated gluten for the last 25 years (reviewed by Wieser and Koehler [74]). Most methods are based on the determination of wheat, rye, and barley prolams. For prolamin extraction from the material, aqueous ethanol (e.g., 60%) and propanol (50%) are generally applied. For material that has been heated, the use of a reducing agent (e.g., 2-mercaptoethanol or tris(2-carboxyethyl)phosphine), a dis-aggregating agent (e.g., guanidine or urea), and increased temperature (50 or 60°C), which allows coextraction of both prolams and glutelins, are also recommended (20,22).

Most techniques used for gluten quantitation are based on immunochemical methods, predominantly on enzyme-linked immunosorbent assays (ELISA) (reviewed by Denery-Papini et al. [10] and Wieser and Koehler [74]). Different commercial ELISA kits are available that use either monoclonal or polyclonal antibodies raised against wheat and rye prolamins. Two principles of ELISA have been applied: the sandwich test for intact gluten proteins and the competitive test for par-

![Fig. 3. Proportions of Osborne fractions (albumins/globulins, prolams, and glutelins) in commercially available protein references.](Image)
tially hydrolyzed gluten. Comparative studies of different kits, however, demonstrated that results for gluten quantitation can vary considerably. The R5-ELISA developed by the Méndez group in Madrid (66) was the most successful test. This sandwich test is based on a monoclonal R5 antibody raised against α-secalins and directed against CD-toxic sequences within the repetitive domains of prolamin. The assay has a detection limit of ≈3 mg of gluten per kilogram and is equally sensitive to wheat, rye, and barley prolamins; however, oat prolamins are not detected. R5-ELISA has been validated by collaborative studies and is established in the market. In 2005, R5-ELISA was endorsed as a type 1 method by the Codex Committee of Methods of Analysis and Sampling (CCMAS) and recommended by the recent Draft Revised Codex Standard (8). In the meantime, a competitive ELISA and ELISA stick are on the market. In addition to ELISA, other immunochemical methods, e.g., the magneto immunosensor technique (41), and non-immunochemical methods, such as mass spectrometry (46) and polymerase chain reaction (9,54), have been developed for gluten analysis. In particular, methods using mass spectrometry for gluten quantitation are being developed as independent methods to verify ELISA results for problematic samples. However, mass spectrometric methods providing reliable quantitative data are still not available, and therefore, routine application and common acceptance have not been achieved.

Various reference proteins that are essential for establishing a calibration curve have been produced by different laboratories and companies. Comparative investigations of commercially available references (gliadins, wheat gluten), however, indicate that they differ significantly in protein content and composition (Fig. 3) and lead to differing results when applied in ELISA (57). The European Working Group (PWG) on Prolamin Analysis and Toxicity produced a gliadin reference isolated from kernels of 28 representative European wheat cultivars (70). This reference material has been well characterized chemically and by ELISA tests and is now distributed by the PWG for collective use.

According to the most recent Codex Draft Revised Standard, prolamins should be extracted from the material and quantified by an immunochromatographic method (8). The gluten content must be calculated by multiplying the prolamin content by a factor of 2 assuming that the ratio of prolamins to glutenins is generally 1:0. However, this calculation was shown to be invalid through comparative analyses of prolamins and glutenins from cultivars of different wheat species, rye, barley, oats, and industrial wheat starches (75). The ratios ranged from 1.4 to 13.9 within the cereal flours and from 0.2 to 4.9 within the starches. Thus, gluten content is either over- or underestimated in many cases.

**Dietetic Products**

Strict lifelong adherence to a gluten-free diet is currently the only effective treatment for CD. This means that gluten-free dietetic products must be substituted for foods from wheat, rye, barley, and oats, such as breads, other baked products, pasta, and beer. The raw materials used for these alternative products are mainly non–CD-toxic cereals (e.g., corn and rice) and pseudocereals (e.g., amaranth and buckwheat). However, many of the gluten-free dietetic foods available on the market are of low quality and exhibit poor texture, mouthfeel, and flavor compared with conventional products.

The replacement of wheat bread and barley beer is one of the most critical aspects of a gluten-free diet and a challenge for food technologists, bakers, and brewers.

The unique quality of wheat bread is a result of the special properties of gluten proteins (gliadins and glutenins). They provide the flour with a high water absorption capacity; the dough with cohesivity, viscosity, elasticity, and gas holding ability; and the bread with high volume and a porous crumb (1,5). It is extremely difficult to mimic all of these desired properties. Usually, starches or starch-containing flours from non–CD-toxic plants (e.g., corn, rice, or potatoes) are the base materials used in production of gluten-free breads. The use of wheat starch is allowed provided that the gluten content is lower than 100 mg/kg. To imitate the water absorption capacity and dough viscosity of gluten proteins, several hydrocolloids are recommended. Hydrocolloids are hydrophilic polymers that act as water binders, improve the rheological properties of dough and bread texture, and slow down the retrogradation of starch (1). Hydroxypropylmethylcellulose, carrageenan, xanthan gum, and sodium alginate are examples of hydrocolloids used in gluten-free bread production. The replacement of gluten proteins with other protein sources is another approach used to improve bread quality. Dairy ingredients such as caseinates, skim milk powder or whey protein concentrate (62), soy products (49), or egg proteins (49) have been recommended. In addition to texture, these proteins improve the nutritional properties of gluten-free breads. However, lactose intolerance (milk) and allergic potential (soya) are limiting factors for the use of these gluten substitutes.

The use of lactic acid bacteria and gluten-free sourdough is another possibility for improving gluten-free bread quality (24).

Because beer based on barley malt is not included in gluten-free diets, the search for gluten-free brewing materials has been intensified during the last decade. Non–CD-toxic cereals and pseudocereals have been tested for application in beer production, and currently, gluten-free sorghum, millet, and buckwheat ingredients (51) are available on the market. However, the flavor of products made with these alternative ingredients may not be acceptable to CD patients.

Recent research has shown that gluten proteins and peptides can be degraded into non–CD-toxic fragments using specific peptidases, the so-called prolyl endopeptidases (reviewed by Wieser and Koehler [76]). Bacteria, fungi, and germinated cereal grains are sources of effective peptidases. Potential applications can be divided into therapeutic treatment of CD patients and treatment of gluten-containing raw materials and foods. The latter is of particular interest for foods that do not need gluten functionality for product quality. For example, sourdough lactobacilli produce specific peptidases that hydrolyze Pro-rich peptides (25). Together with fungal peptidases they are capable of degrading gluten in sourdough, which can then be used as an ingredient in gluten-free baked goods. Peptidase preparations using germinating cereals are also promising candidates for the degradation of gluten in foods, as was recently shown by its application in beverages such as kwas and malt beer (38). Similarly, the enzyme transglutaminase can be used to degrade gluten proteins and peptides into non–CD-toxic fragments. Patents have been filed concerning the effective treatment of wheat flours used in baked products (53) or cereal-based beers (44).

**Conclusions**

What remains to be done? This overview clearly shows that more action is needed in the field of CD. Combined interdisciplinary approaches are required to successfully address the problem of CD, including...
• Research on the pathomechanism of CD and other gluten intolerances to provide and improve the bases for new therapies
• In addition to wheat, rye and barley also require attention and further efforts are necessary to elucidate the CD toxicity of oats
• Research on the use of gluten-degrading enzymes for CD therapy and the production of gluten-free foods
• Improvement in gluten quantitation, in particular the development of reference methods and materials
• Harmonization of legal regulations
• Efforts by the food industry to further improve the safety and quality of gluten-free foods
• Research on the reduction or elimination of the CD toxicity of cereals through breeding or genetic modification

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