Original article

Evaluation of the safety of ancient strains of wheat in coeliac disease reveals heterogeneous small intestinal T cell responses suggestive of coeliac toxicity

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Summary

Background & aims: Coeliac disease is a chronic small intestinal immune-mediated enteropathy triggered by dietary gluten in genetically predisposed individuals. Since it is unknown if all wheat varieties are equally toxic to coeliac patients seven Triticum accessions showing different origin (ancient/modern) and ploidy (di-, tetra- hexaploid) were studied.

Materials and methods: Selected strains of wheat were ancient Triticum monococcum precoce (AA genome) and Triticum speltoides (BB genome), accessions of Triticum turgidum durum (AABB genome) including two ancient (Graziella Ra and Kamut) and two modern (Senatore Cappelli and Svevo) durum strains of wheat and Triticum aestivum compactum (AABBDD genome). Small intestinal gluten-specific T-cell lines generated from 13 coeliac patients were tested with wheat accessions by proliferation assays.

Results: All strains of wheat independent of ploidy or ancient/modern origin triggered heterogeneous responses covering wide ranges of stimulation indices.

Conclusion: Ancient strains of wheat, although previously suggested to be low or devoid of coeliac toxicity, should be tested for immunogenicity using gluten-specific T-cell lines from multiple coeliac patients rather than gluten-specific clones to assess their potential toxicity. Our findings provide further evidence for the need for a strict gluten-free diet in coeliac patients, including avoidance of ancient strains of wheat.

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1. Introduction

Coeliac disease (CD) is a chronic small intestinal immune-mediated enteropathy precipitated by exposure to dietary gluten in genetically predisposed individuals.1 Treatment involves a long-life gluten-free diet (GFD) which excludes wheat, rye, barley and possibly oats. Gluten is however difficult to avoid because of its widespread use in food processing. It is used in the production of soups, sauces, meat products, potato chips, candies, ice cream and as excipients in medicines and vitamins supplements, etc.2 Therefore the GFD is restricted, unpalatable and causes social disadvantage for the individuals resulting in poor compliance amongst CD patients. The symptoms include diarrhea, weight loss and fatigue, although a broad spectrum of clinical presentations occur. The condition is diagnosed by the presence of some degree of small intestinal villous atrophy with raised number of intra-epithelial
lymphocytes which normalise when gluten is removed from the
diet.

In the submucosa of the small intestine the enzyme tissue
transglutaminase (tTG), generally involved in tissue repair, dea-
midates gluten peptides, that allows high-affinity binding to the
human leucocyte antigen class molecules HLA-DQ2 and HLA-DQ8,
subsequently triggering an inflammatory reaction in patients with
CD.2 The activation of gluten-sensitive CD4+ T-cells is central to the
inflammatory reaction, although innate immune mechanisms are
also thought to be involved.4

Gluten is a term that describes storage proteins in wheat
endosperm and related cereals. Wheat storage proteins are divided
into two major groups of proteins: gliadins and glutenins. Gliadins
are further divided into α-, γ- and ω-gliadins and glutenins into
high- and low-molecular weight glutenins. The α-gliadins are
proteins encoded by a gene family at the Gli-2 loci, which may
contain from 25 to 35 to even 150
proteins encoded by a gene family at the Gli-2 loci, which may
ploidy. Wheat accessions were as follows: the diploid
(ploidy. Wheat accessions were as follows: the diploid
T. monococcum (considered the progenitor or otherwise very close
to the ancestor of the BB genome). In particular, some ancient, that is
ancient, strains of wheat have been suggested to be less toxic for
coeliac patients or even lack CD toxicity. For example gliadins of
T. monococcum.

It is unknown if all wheat varieties are equally toxic to in-
dividuals with CD. Since large variations exist in the amount of
T-cell stimulatory peptides present in different wheat cultivars,10
numerous accessions have been studied to identify those with a
lower coeliac toxic profile.2,10–12 It has been suggested that a diet
based on wheat varieties reduced in T-cell stimulatory epitopes
may help in prevention of CD since the amount and duration of
gluten consumption are associated with the initiation of CD. The
argument then goes that this would especially benefit children in
which the onset of CD may be delayed or even prevented and in
undiagnosed coeliac patients (which are the vast majority of all
coeliac sufferers) might strongly reduce their symptoms.7 Never-
theless this assumption is controversial and still subject to much
debate and investigation.

In an attempt to identify grains which were less toxic to patients
suffering from coeliac disease, several scientists strongly focused on
the analysis of grains considered forerunners of modern grains
including Triticum monococcum (bearing the AA genome) and Tri-
ticum speltoides (considered the progenitor or otherwise very close
to the ancestor of the BB genome). In particular, some ancient, that is
ancestral, strains of wheat have been suggested to be less toxic for
coeliac patients or even lack CD toxicity. For example gliadins of
T. monococcum were reported to lack coeliac toxicity in an in vitro
organ culture system suggesting new dietary opportunities for CD
patients.13 Some ancient wheat varieties including T. monococcum
and T. speltoides were shown to be low in α-gliadin T-cell epi-
topes10,13 or other gliadin and glutenin epitopes involved in the
pathogenesis of CD.10,11 More recently it has been suggested that
some monococcum lines (Monlis and ID331) are to be considered
toxic for coeliac patients.14 Misinterpretations of the existing liter-
ature and contradictory evidence on the safety of T. monococcum
accessions could potentially influence CD sufferers to consume
ancient cereals that are unsafe for them.

In the last decades, a huge number of durum wheat cultivars
have been obtained by artificial selection, generally based on high
yield, disease resistance and technological qualities. On the other
hand, to preserve genetic variability and reduce genetic erosion it
is extremely important to develop and maintain local crops, including
old cultivars and landraces, which were not subjected to massive
selective breeding or genetic modifications. Examples of this are
Graziella Ra, an accession (not a cultivar) of durum wheat that
recently appeared on the market as Graziella Ra8, and Kamut6. The
latter is considered ancient relative of modern durum wheat. In
their paper testing the hypothesis of a potentially reduced or absent
coeeliac toxicity of these strains of wheat, Gregorini et al.15 reported
that both Graziella Ra and Kamut, are not only putatively as CD
toxic as the modern durum accessions analysed, but also contain
greater amounts of α-gliadin.

In the present study we sought to test whether the low immu-
nogeneity hypothesis of ancient vs modern wheat varieties is
confirmed or not, since any cereal from the tribe Triticeae has to be
considered toxic unless thorough in vitro and in vivo evidence to the
contrary is produced. The analysis was carried out by using 13
polyclonal gluten-sensitive T-cell lines (TCL) to assess their overall
reactivity in relation to their (i) origin (ancient/modern) and (ii)
ploidy. Wheat accessions were as follows: the diploid T. monococcum
precoce and T. speltoides, representing potential ancient progenitors
that might have hybridised into tetraploid strains of wheat16; four
accessions of the tetraploid (AABB genome) Triticum turgidum durum
including two ancient (Graziella Ra and Kamut) and two modern
(Senatore Cappelli and Svevo) durum wheat varieties17,18; and the
hexaploid (AABBDD genome) Triticum aestivum compactum.

2. Materials and methods

2.1. Subjects

Small intestinal biopsies were obtained at endoscopy performed
diagnostic or clinical management purposes in subjects with
suspected or known CD. Thirteen volunteer subjects diagnosed
according to British Society of Gastroenterology guidelines19
included twelve females and one male, median age 35 years,
range 23–72 (Table 1). Biopsy specimens were taken from the
second part of the duodenum. All participating subjects provided
written informed consent. The study was approved by the St
Thomas’ Hospital Research Ethics Committee (reference number
05/Q0207/167).

2.2. Cereal sources of gliadins

Ancient and modern wheat accessions with their correspondent
ploidy, genomes and abbreviated names used in this manuscript

Table 1

<table>
<thead>
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<th>Subject</th>
<th>Sex</th>
<th>Age at time of biopsy</th>
<th>GFD (in years)</th>
<th>DQ status</th>
<th>Histology</th>
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<td>35</td>
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<td>F</td>
<td>72</td>
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<td>53</td>
<td>9</td>
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<td>3a</td>
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<td>0</td>
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<td>3a</td>
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<td>13</td>
<td>F</td>
<td>28</td>
<td>1.75</td>
<td>DQ2</td>
<td>3a</td>
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</table>

GFD, gluten-free diet; histology 0, normal mucosal architecture; 1, normal mucosal
architecture with an increased number of intraepithelial lymphocytes (IEL); 3a,
increased IEL with hyperplastic crypts and mild villous atrophy; 3b, increased IEL
with hyperplastic crypts and moderate villous atrophy. Subject number 1 had
positive CD serology 3 months prior to endoscopy and family history of CD.
are presented in Table 2. Ancient wheat varieties included T. monococcum precoce (ITA0340003), T. speltoides (CGN 10684), T. turgidum durum: Kamut and Graziella Ra. Modern wheat varieties included T. turgidum durum: Senator Cappelli and Svevo, and T. aestivum compactum (CGN 04210).

2.3. Preparation of antigens for T-cell assays

Kernels of wheat accessions were milled with an analytical grinder and sieved through a 0.5 μm mesh. The resultant flour was washed twice with phosphate buffer (0.067 M NaK phosphate buffer in 0.4 M NaCl, pH = 7.6) to remove concomitant albumins and globulins. Gliadins were extracted twice with 60% EtOH, and the extracts combined. They were then dried and sequentially digested with agarose-bound pepsin (P0609, Sigma-Aldrich) in PBS containing 1 mM CaCl2 and 370 m cell/ml guinea pig liver tTG (T5398, Sigma) according to the manufacturer’s instructions. Deamidation mixtures contained 100 μg/ml guinea pig liver tTG (T5398, Sigma–Aldrich) in PBS containing 1 mM CaCl2 and 370 μg/ml PTG digests of prolamins. Incubation was carried out for 4 h at 37 °C.

2.4. Establishment of small intestinal T-cell lines

The methods were as previously described. Plasmocin (ant-mpt, Invivogen, Cayla, Toulouse-Cedex, France) was used at all stages. Small intestinal biopsies obtained from coeliac patients were incubated overnight in the presence of 5 mg/ml peptic-tryptic digest of industrial gluten (PTG) bought from Roquette Ltd, Corby Northants, UK (418, batch NW552). This was in order to stimulate activation by the whole range of gluten proteins that are likely to be encountered by individuals consuming a modern gluten containing diet. Biopsies were then mechanically disrupted to release lymphocytes and passed through a 70 μm cell filter (BD Falcon). The collected cells were cultured in RPMI medium with 10% heat inactivated autologous plasma plus 1 × 105/ml irradiated (22 Gy) autologous peripheral blood mononuclear cells. Human recombinant interleukin 2 (IT5903, Sigma) was added on day 5 and subsequently twice a week at 10 U/ml. Cells were restimulated every seven days with PTG, pre-treated with tTG, at a final concentration of 200 μg/ml. Autologous irradiated (22 Gy) peripheral blood mononuclear cells acted as antigen presenting cells.

2.5. T-cell proliferation assays

T-cell proliferation assays were performed a minimum of seven days after antigenic stimulation. The antigens tested were tTG deamidated PTG and tTG deamidated gliadins from the seven strains of wheat. Antigens were incubated overnight with antigen presenting cells (5 × 105) at 100 μg/ml prior to addition of T-cells (5 × 104). Following incubation for 48 h, tritiated thymidine (NET355, Perkin Elmer, Boston, MA, USA) was added for 18 h prior to harvesting and measurement of thymidine incorporation. The stimulation index (SI) was calculated by dividing the mean counts per minute (cpm) in the presence of antigen by the mean cpm in the absence of antigen. An SI of 2 or more was considered positive.

The use of internal negative controls in proliferation assays is not standard practice. Further, using another dietary coeliac non-toxic protein that has been treated in the same way as gluten proteins results in SI < 2. Hence, we employed medium only as a negative control.

2.6. Data analysis and statistics

Data are presented as arithmetic mean of cpm ± standard deviation of at least triplicates with corresponding SI. Statistical analysis was performed with GraphPad Prism version 5.03, using the Friedman test. Spearman non-parametric correlation analysis choosing a two-tailed P value was performed to investigate the correlation between SI values and the duration of the GFD in the subjects from which the TCLs were obtained.

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## Table 2

<table>
<thead>
<tr>
<th>Wheat species (accession)</th>
<th>Ploidy and genome</th>
<th>Name in the manuscript</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ancient wheat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. monococcum precoce</td>
<td>diploid, AA</td>
<td>Tmp_AA</td>
</tr>
<tr>
<td>T. speltoides</td>
<td>diploid, BB</td>
<td>Ts_BB</td>
</tr>
<tr>
<td>T. turgidum durum</td>
<td>tetraploid, AABB</td>
<td>GR_AABB</td>
</tr>
<tr>
<td>(Kamut)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Graziella Ra)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Modern wheat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. turgidum durum</td>
<td>tetraploid, AABB</td>
<td>SC_AABB</td>
</tr>
<tr>
<td>(Senatore Cappelli)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T. turgidum durum</td>
<td>tetraploid, AABB</td>
<td>S_AABB</td>
</tr>
<tr>
<td>(Svevo)</td>
<td></td>
<td></td>
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<tr>
<td>T. aestivum compactum</td>
<td>hexaploid, AABBD</td>
<td>Tac_AABBD</td>
</tr>
</tbody>
</table>

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Results of proliferation assays showing individual TCL tested with gluten (positive control) and gliadins from four ancient (Tmp_AA, Ts_BB, GR_AABB, K_AABB) and three modern wheat accessions (SC_AABB, S_AABB, Tac_AABBDD).

<table>
<thead>
<tr>
<th>Medium only</th>
<th>Gluten</th>
<th>Tmp_AA</th>
<th>Ts_BB</th>
<th>K_AABB</th>
<th>GR_AABB</th>
<th>SC_AABB</th>
<th>S_AABB</th>
<th>Tac_AABBDD</th>
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<tbody>
<tr>
<td>TCL1</td>
<td>4751 ± 870 (1)</td>
<td>24,469 ± 2932 (5.2)</td>
<td>12,915 ± 2412 (2.7)</td>
<td>10,457 ± 1138 (2.2)</td>
<td>14,252 ± 1915 (3.0)</td>
<td>12,736 ± 958 (2.7)</td>
<td>11,084 ± 1118 (2.3)</td>
<td>10,319 ± 1185 (2.2)</td>
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<td>TCL2</td>
<td>152 ± 17 (1)</td>
<td>9077 ± 975 (59.8)</td>
<td>1599 ± 277 (10.5)</td>
<td>4137 ± 483 (27.3)</td>
<td>3292 ± 559 (21.7)</td>
<td>2538 ± 393 (16.7)</td>
<td>2274 ± 175 (15.0)</td>
<td>2620 ± 255 (17.3)</td>
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<td>TCL3</td>
<td>1018 ± 187 (1)</td>
<td>33,858 ± 4790 (33.2)</td>
<td>1064 ± 162 (1.0)</td>
<td>1192 ± 224 (1.2)</td>
<td>3785 ± 653 (3.7)</td>
<td>1098 ± 167 (1.1)</td>
<td>3244 ± 370 (3.2)</td>
<td>434 ± 64 (0.4)</td>
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<td>TCL4</td>
<td>66 ± 11 (1)</td>
<td>29,613 ± 2001 (488.7)</td>
<td>3224 ± 511 (48.9)</td>
<td>5400 ± 340 (81.8)</td>
<td>6701 ± 543 (101.5)</td>
<td>7350 ± 160 (111.4)</td>
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<td>1034 ± 56 (1)</td>
<td>9409 ± 1171 (9.1)</td>
<td>10,701 ± 971 (10.4)</td>
<td>9095 ± 1079 (8.8)</td>
<td>5907 ± 755 (5.7)</td>
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<td>5674 ± 1039 (5.5)</td>
<td>4487 ± 612 (4.3)</td>
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<tr>
<td>TCL6</td>
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<td>28,744 ± 1504 (194.6)</td>
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<td>8177 ± 1448 (55.4)</td>
<td>14,120 ± 1152 (95.6)</td>
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<td>1792 ± 247 (2.8)</td>
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<td>TCL11</td>
<td>464 ± 84 (1)</td>
<td>1473 ± 224 (3.2)</td>
<td>986 ± 171 (2.1)</td>
<td>1492 ± 274 (3.2)</td>
<td>1818 ± 563 (3.9)</td>
<td>2169 ± 400 (4.7)</td>
<td>1499 ± 120 (3.2)</td>
<td>1708 ± 264 (3.7)</td>
</tr>
<tr>
<td>TCL12</td>
<td>364 ± 63 (1)</td>
<td>3129 ± 184 (8.6)</td>
<td>1452 ± 106 (4.0)</td>
<td>312 ± 72 (0.9)</td>
<td>610 ± 112 (1.7)</td>
<td>358 ± 101 (1.0)</td>
<td>272 ± 49 (0.7)</td>
<td>405 ± 81 (1.1)</td>
</tr>
<tr>
<td>TCL13</td>
<td>1001 ± 139 (1)</td>
<td>57,985 ± 4633 (57.9)</td>
<td>18,547 ± 1834 (18.5)</td>
<td>16,314 ± 1626 (16.3)</td>
<td>23,328 ± 1602 (23.3)</td>
<td>27,530 ± 2053 (27.5)</td>
<td>28,621 ± 2092 (28.6)</td>
<td>21,779 ± 2022 (21.7)</td>
</tr>
</tbody>
</table>

Results (expressed as cpm) are presented as arithmetic mean ± standard deviation and in bracket corresponding stimulation index. TCL: T-cell line; Tmp_AA (Triticum monococcum precoce); Ts_BB (Triticum speltoides); K_AABB (Triticum turgidum durum Kamut); GR_AABB (Graziella Ra); SC_AABB (T. turgidum durum Senatore Cappelli); S_AABB (T. turgidum durum Svevo); Tac_AABBDD (Triticum aestivum compactum).
The Dunn's multiple pair-wise comparison post test determined that, with reference to SI, only Tmp_AA, Ts_BB and S_AABB were significantly different from Tac_AABBDD ($P < 0.05$, $P < 0.01$, $P < 0.01$, respectively) (data analysis not shown but available on request). Therefore, small intestinal T-cell responses to hexaploid wheat Tac_AABBDD were significantly higher compared to both diploid strains of wheat (Tmp_AA and Ts_BB) and one tetraploid wheat (S_AABB).

The correlation between SI values and the duration of GFD in the subjects from which the TCLs were obtained was investigated. Non-parametric analysis showed no significant correlation (for $P < 0.05$) between SI of any of the antigens and duration of GFD (data not shown but available on request).

4. Discussion

The only effective treatment available for CD patients is a strict exclusion of gluten from the diet. Nevertheless, the existence of thousands Triticum accessions and hundreds of alleles for gliadins and glutenins has raised the question of whether they are equally toxic to CD patients. Moreover, it has been speculated that ancient strains of wheat that have not been subjected to major genetic improvements may show potentially reduced or absent toxicity and therefore potentially better suited to be introduced into the diet of people suffering from wheat intolerances or allergies, including coeliac disease.

To test this hypothesis, in the present study, we analysed how TCLs from 13 subjects with CD responded to seven wheat accessions selected according to their origin (ancient/modern) and ploidy (di-, tetra-, hexaploid). Our findings revealed that all the wheat varieties tested, irrespective of their ancient/modern origin or ploidy, evoked heterogeneous small intestinal T-cell responses. For example hexaploid wheat Tac_AABBDD evoked a wide range of intestinal T-cell responses which include negative (in 2 out of 13 gluten-sensitive TCLs), very high stimulation indices in 2 TCLs and a moderate SI in all other lines. A roughly similar trend was observed with all other modern and ancient wheat accessions. Our results are not surprising from a clinical point of view, since majority of wheat strains are likely to be toxic to coeliac patients. However, our findings highlight the occurrence of a heterogeneous response by different coeliac patients. Our results are in agreement with previous studies where Camarca et al. who showed that intestinal T-cell responses to gluten peptides are largely heterogeneous and Speajni-Dekking et al. who showed that wheat varieties either induced low, medium or high T-cell responses independent of the ploidy or genome background of the accession. Our results are in further agreement with Molberg et al. who showed that polyclonal T-cell line reactivity patterns to ancient wheat varieties are heterogeneous despite the ancient strains of wheat being shown to be low in certain T-cell epitopes.

In relation to assessing the safety of ancient and modern wheat varieties for consumption by individuals with CD, the present study further supports the necessity of testing them with TCLs from several different individuals. Due to the wide heterogeneity of T-cell responses there is a realistic chance they would test negative if small numbers of TCLs were employed and results extrapolated to the safety of ancient wheat varieties for all coeliac subjects. This is particularly the case if clones as opposed to TCLs were used. Gluten-specific clones cover only a limited number of specificities and thus do not represent the whole repertoire of gluten-reactive T-cells within coeliac lesions. This is well demonstrated by the results obtained in the present paper. For example, the ancient diploid accession Ts_BB and modern hexaploid accession Tac_AABBDD have been tested with selected clones and shown to be low in some gluten epitopes, including the immunodominant peptide from $\alpha$-gliadins and two other $\gamma$-gliadin peptides. In our experiments, testing gliadin extracts from these two strains of wheat with polyclonal T-cell lines showed that the majority of TCLs (10 out of 13 for Ts_BB and 11 out of 13 for Tac_AABBDD) responded with a positive SI, with no overall trend in reduction of polyclonal T-cell responses. In fact, responses to diploid Ts_BB were observed to be in a comparable range to those of all four durum strains of wheat (ancient K_AABB and GR_AABB; modern SC_AABB and S_AABB); T-cell responses to Tac_AABBDD, which was shown to be low in the immunodominant epitope were significantly higher than to both diploid strains of wheat (Tmp_AA and Ts_BB, $P < 0.05$ and $P < 0.01$, respectively) and one tetraploid wheat (S_AABB). This might indicate that other gliadin peptides from Ts_BB and Tac_AABBDD which were not tested in Speajni-Dekking’s study may also trigger T-cell responses, perhaps peptides from $\omega$-gliadins or other $\alpha$- and $\gamma$-gliadins showing different amino-acid sequences. Additionally, we cannot exclude that other unknown CD-triggering epitopes in gliadins might have triggered T-cell responses in our polyclonal TCLs.

The literature suggests that, compared to diploid and tetraploid wheat, hexaploid wheat, might be increased in T-cell stimulatory epitopes that exacerbate CD because of the presence of the D genome. Indeed, with respect to T-cell toxicity as far as induced by $\alpha$-gliadin epitopes, the D genome should be considered as the most relevant as it codes for several $\alpha$-gliadin toxic epitopes. This might explain higher stimulation indices obtained for our modern wheat accession Tac_AABBDD. On the other hand, this particular accession has been shown to be low in immunodominant and other CD toxic epitopes. Such contrasting results obtained by the two groups working on the same wheat accession further supports the need to use polyclonal T-cell lines to assess the CD toxicity of wheat varieties as opposed to gluten-sensitive T-cell clones. This is because a lack of certain T-cell stimulatory sequences does not imply that their gluten proteins may not have any T-cell stimulatory properties.

With reference to TCL responses to tetraploid accessions, the gliadins from the two ancient (K_AABB and GR_AABB) and two modern accessions (SC_AABB and S_AABB) triggered similar ranges of polyclonal T-cell responses. This is interesting particularly in relation to the amount of $\alpha$-gliadins these four durum accessions...
contain and the relevance of α-gliadins in the pathogenesis of CD. As Gregorini et al.\textsuperscript{15} showed – by using a monoclonal antibody specific for the immunodominant α-gliadin peptide,\textsuperscript{24} a monoclonal antibody to another coeliac toxic α-gliadin peptide (A-gliadin 31–49)\textsuperscript{25} and a commercial gliadin kit – gliadins from the two ancient accessions K_AABB and GR_AABB occur in greater amount than in modern durum varieties. We have shown in the present set of experiments that our T-cell lines reacted similarly to the gliadins of the four tetraploid strains of wheat which might indicate that (i) the response is not only dependent on the α-gliadin amount; (ii) other gliadin peptides contribute to the intestinal T-cell response;\textsuperscript{2} and (iii) different specificities of T-cells tested overall resulted in comparable stimulation indices.

Most attention in relation to the safety of ancient diploid wheat varieties and potentially new dietary opportunities for coeliac patients has been focussed on \textit{T. monococcum}. Molberg et al.\textsuperscript{31} showed that fragments identical or equivalent to the genes from wheat chromosome 6D, and are thus absent from \textit{einkorn} has the full potential to induce CD30 and Gianfrani et al.\textsuperscript{31} who showed that the monococcum lines Monlis and ID331 acti-
vate the CD T cell responses.\textsuperscript{14} We conclude that the T-cell responses to ancient and modern wheat varieties are indeed heterogeneous. Further, we have shown that any wheat variety that is suggested to be low in CD toxicity needs to be tested in multiple individuals with CD. Tmp_AA triggered positive T-cell responses of most TCLs tested overall resulted in comparable stimulation indices.

Most attention in relation to the safety of ancient diploid wheat varieties and potentially new dietary opportunities for coeliac patients has been focussed on \textit{T. monococcum}. Molberg et al.\textsuperscript{31} showed that fragments identical or equivalent to the genes from wheat chromosome 6D, and are thus absent from gluten of diploid einkorn (including \textit{T. monococcum}). Pizzuti et al.\textsuperscript{31} showed lack of toxicity of \textit{T. monococcum} gliadin in an in vitro coeliac small intestinal organ culture system. Vincentini et al.\textsuperscript{31} and De Vincenzi et al.\textsuperscript{25} showed that \textit{T. monococcum} did not exhibit any negative effect on Caco-2/TC7 and K562(S) cells. \textit{T. monococcum} was also suggested to be well tolerated for con-
sumption by coeliac sufferers.\textsuperscript{28} Our results underline strongly the need for all cereals from the tribe Triticaceae to be considered coeliac toxic.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contribution

The work presented here was carried out in collaboration amongst all authors who co-worked in experimental design and writing the paper. TS performed the experiments, data analysis and interpretation and drafted the paper. AG and MC performed strains of wheat selection, data analysis and interpretation, manuscript writing. HJE designed the experiments, performed data interpretation and manuscript writing. PJC undertook the clinical work, supervised the project and provided intellectual input. All authors read and approved the final manuscript.

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References

findings provide additional evidence for the necessity of a strict lifelong gluten-free diet in CD patients, without exception. Our results underline strongly the need for all cereals from the tribe Triticaceae to be considered coeliac toxic.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contribution

The work presented here was carried out in collaboration amongst all authors who co-worked in experimental design and writing the paper. TS performed the experiments, data analysis and interpretation and drafted the paper. AG and MC performed strains of wheat selection, data analysis and interpretation, manuscript writing. HJE designed the experiments, performed data


