Proceedings of the 29th Meeting

WORKING GROUP on PROLAMIN ANALYSIS and TOXICITY

Edited by
Peter Koehler
German Research Centre for Food Chemistry

8 - 10 October 2015
Tulln, Austria

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on PROLAMIN ANALYSIS and TOXICITY

Edited by
Peter Koehler
Deutsche Forschungsanstalt für Lebensmittelchemie /
German Research Centre for Food Chemistry
Freising

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Cover picture* and picture of participants
Thomas Mothes

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* Bronze sculpture of Michael Nogin: "Encounter of Kriemhild, Queen of Burgundy, and Etzel, King of the Huns, in Tulln", on the mirrored steeples of the St. Stephan church of Tulln. Kriemhild is the third and Etzel is the fourth from left side.

Preface

The Austrian City of Tulln was the venue of the 29th meeting of the Working Group on Prolamin Analysis and Toxicity from 8 to 10 October 2015. The meeting was hosted by Romer Labs Division Holding GmbH, and the Austrian Coeliac Society assisted in the registration of the participants. Simone Schreiter, the local organiser, was present during the entire meeting. More than 60 persons participated in the meeting. This shows the ongoing scientific significance of the topic gluten and gluten hypersensitivities. From the 12 current members of the PWG, nine participated in the meeting. Peter Koehler, chairman of the PWG, welcomed the group, one invited speaker, participants from industry, research institutes as well as delegates from European coeliac associations. Industry delegates came from starch producers, manufacturers of gluten-free foods, and producers of analytical test kits for gluten quantitation.

Analytical and clinical work in the field of coeliac disease and gluten done in the labs of the PWG members as well as results of guest speakers were presented in 15 talks and intensely discussed at the meeting. In addition, two presentations addressed regulatory aspects of gluten analysis and labelling. A symposium on “Innate Immunity and Coeliac Disease” with three presentations of internationally recognized experts highlighted the latest advances in the field of the early steps of CD pathogenesis and non-celiac gluten/wheat sensitivity.

At this occasion, I would like to express my thanks to all participants for their active contributions and to all persons that made the meeting possible. I would like to thank the local organizing team, in particular Simone Schreiter of Romer Labs and also Christian Petz and Hertha Deutsch of the Austrian Coeliac Society, who perfectly organized the hotel, the venue, and the registration. Also, very special thanks go to Katharina Scherf for her help in proofreading. Finally, I would like to express my appreciation to all friends, colleagues and sponsors for their ongoing support of the PWG and the meeting.

Freising, March 2016 Peter Koehler

Table of Contents

1 Executive summary

Among the topics of the meeting were analytical issues of gluten, novel approaches for coeliac disease diagnosis, aspects of the innate and adaptive immune response, as well as legal issues and standardization activities.

Analytical session

Six presentations were given in this session. The opening lecture and an additional talk within the session discussed the requirements and current activities in the production of suitable reference materials for gluten quantitation. Another analytical topic was the specificity of different immunochemical kits for gluten quantitation towards gluten fractions and gluten from different plant species. Two presentations were on the detection and quantitation of gluten by mass spectrometry and, finally, breeding approaches on reducing or elimination of coeliac disease activity of wheat and barley were presented.

Clinical session

This session also included six presentations. The first talk was on the gluten-free diet of the Spanish coeliac population and the nutrient intake compared to the gluten-containing diet. Another presentation was on signalling in the innate immune response in coeliac disease. A novel approach for endomysium antibody testing by using liver tissue instead of oesophagus tissue was also presented. A talk on the use of mass cytometry to image cell populations in different coeliac disease conditions was followed by a presentation on the activity of avenin from oats in coeliac disease.

Symposium: Innate Immunity and Coeliac Disease

Three recognized experts in innate immunity presented results from their research. Viral infections as well as gliadin peptides were discussed as potential triggers of the innate response of the immune system in coeliac disease pathogenesis. Finally, research on amylase-trypsin-inhibitors (ATI) was presented, which are thought to be related to the innate response of the immune system. They have been postulated to act as triggers of non-celiac gluten sensitivity but also as “second” and “third hits” in the pathogenesis of a number of chronic inflammatory diseases.

29th Meeting of the Working Group on Prolamin Analysis and Toxicity (PWG), Tulln, Austria, 8 - 10 October 2015

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3 Programme

THURSDAY, 8 October 2015

20:00 Arrival of Prolamin Working Group and all participants
Informal get-together with dinner
Welcome by Romer Labs
Location: Hotel Römerhof, Tulln

FRIDAY, 9 October 2015

08:00 Bus transfer to UFT Tulln

08:30 Opening of the meeting (Peter Koehler)

08:45 Towards reference materials for food allergen and gluten-free analysis
Prof. Dr. Roland Poms, Neutal, Austria

09:25 THE PROLAMIN WORKING GROUP RESEARCH REPORTS

- Analytical research reports
Chirdo, Ciclitira, Feighery, Gilissen, Koehler, Koning, Lundin, Mothes, Schuppan; guests

10:30 Coffee break

11:00
- Analytical research reports (continuation)

12:30 Lunch

13:30 THE PROLAMIN WORKING GROUP RESEARCH REPORTS

- Clinical reports
Catassi, Chirdo, Ciclitira, Feighery, Koning, Lundin, Mothes, Schuppan, Troncone; guests

16:00 Coffee break

16:30 Tour through the facilities of Romer Labs

16:30 THE PROLAMIN WORKING GROUP EXECUTIVE MEETING
(members only)

17:30 Bus transfer to the hotel

18:30 Bus departure from the hotel for all participants for the joint dinner close to Vienna
Location: Heuriger "Zum Martin Sepp"

23:00 Bus departure to the hotel

SATURDAY, 10 October 2015

	SYMPOSIUM
09:00	Innate Immunity and Coeliac Disease *Chair: Prof. Dr. Fernando Chirdo, La Plata, Argentina*
09:05	Viral infections in coeliac disease pathogenesis *Dr. Valentina Discepolo, Chicago, U.S.A.*
09:50	Gliadin peptides as trigger of the stress/innate immune response of the coeliac small intestine *Prof. Dr. Riccardo Troncone, Naples, Italy*
10:30	Coffee break
11:00	The role of ATIs: A redefinition of NCGS? *Prof. Dr. Detlef Schuppan, Mainz, Germany*

11:45 Discussion of current developments concerning gluten analysis, clinical and legal aspects

Statements by participating organisations, representatives from industry and guests

- Outline: Action plan 2016 of the Prolamin Working Group

13:00 Farewell, bus transfer to train station in Tulln and Vienna airport with packed lunch

Afternoon

- Extra time for informal meeting and additional PWG executive meeting concerning action plan

- Departure of the Prolamin Working Group

4 Analytical research reports

4.1 Comparative analysis of prolamin and glutelin fractions with ELISA test kits

Barbara Lexhaller, Christine Tompos, Peter Koehler, Katharina A. Scherf

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Introduction

Immunochemical methods such as enzyme-linked immunosorbent assays (ELISAs) are specific and sensitive analytical tools for gluten quantitation. They do not require specialised equipment, are comparatively fast and easy to use also in routine applications and recommended by legislation [1]. Due to these advantages and lack of a fully validated alternative method to quantitate gluten proteins, ELISAs are most commonly used to monitor the safety of gluten-free foods for coeliac disease (CD) patients. More than 20 ELISA test kits for gluten analysis are currently on the market. Each of these test kits has its specific features regarding the procedure to extract gluten proteins from the food matrix, the test format (sandwich or competitive), the reference material used for calibration (PWG-gliadin [2], gluten or wheat protein), the type of antibody (monoclonal or polyclonal), and the specificity and sensitivity of the antibody against different epitopes depending on the antigen that was originally used for immunisation (Tab. 1). In addition to various polyclonal antibodies (pAbs), current sandwich ELISA test kits are based on the R5 [3], G12 [4], and 401.21 (Skerritt) [5] monoclonal antibodies (mAbs). Gluten is composed of the alcohol-soluble prolamin fraction and the alcohol-insoluble glutelin fraction that is only soluble after addition of disaggregating and reducing agents. Analytical methods should be capable of detecting the total gluten content, because both prolamins and glutelins harbour CD-immunogenic epitopes [6]. Most ELISA test kits are assumed to only recognize the prolamin fraction and the gluten content is calculated by multiplying the prolamin content by a factor of two. However, detailed comparative studies on antibody sensitivities and specificities against the prolamin and glutelin fractions from wheat, rye, and barley are missing. Therefore, the aim of this study was to compare these different gluten fractions within one ELISA test kit as well as to compare the same gluten fraction between test kits.

Materials and methods

Modified Osborne fractionation and quantitation by RP-HPLC

Flours of wheat (cultivar Akteur), rye (cultivar Visello), and barley (cultivar Marthe) were sequentially extracted with dilute salt solution (albumin/globulin fraction), 60%

(v+v) aqueous ethanol (prolamin fraction) and 50% (v+v) 1-propanol/0.1 mol/L Tris-HCl, pH 7.5 containing 0.06 mol/L (w+v) dithiothreitol at 60 °C under nitrogen (glutelin fraction) [7]. After centrifugation, the respective extracts were made up to volume (2 mL), filtered (0.45 µm) and the gluten protein concentrations were quantitated by RP-HPLC using PWG-gliadin [2] as calibration reference.

Analysis with ELISA test kits

The fresh prolamin and glutelin extracts were diluted appropriately to fit within the respective calibration range of the five sandwich ELISA test kits (Tab. 1).

Table 1. *Overview of the differences in commercially available sandwich ELISA test kits used for the comparative analysis of prolamin and glutelin fractions.*

Anti-body	Extraction	Reference material	Antigen used for immunization	Main epitope
R5 mAb	Cocktail solution/ 50 °C/40 min/ addition of 80% ethanol to yield 60% ethanol	PWG-Gliadin	ω-Secalin	QQPFP
G12 mAb	Extraction solution/ 50 °C/40 min/ addition of 80% ethanol to yield 60% ethanol	Gluten	33-mer peptide of α2-gliadin	QPQLPY
401.21 mAb[1]	40% ethanol with extraction solution mix/45 °C/5 min	Gluten	ω-Gliadin	PQPQPFPQE/ PQQPPFPEE
pAb1	40% ethanol/ 22 °C/5 min	Gliadin	Gliadin	several epitopes
pAb2	Extraction buffer with SDS/β-mercaptoethanol/ 22 °C/12 h	Wheat protein	Gliadin	several epitopes

mAb, monoclonal antibody; pAb, polyclonal antibody; [1]also known as Skerritt mAb

Five serial dilutions of each prolamin and glutelin extract were measured in duplicate with each test kit. The last dilution step was always performed with the sample dilution buffer included in the test kit and the ELISA procedures were carried out strictly according to the manufacturers' instructions.

Data analysis

The prolamin and glutelin concentrations quantitated by RP-HPLC were plotted against the absorbance which was measured by the ELISA test kit. Then the ELISA protein concentrations were calculated using the respective reference material

provided as calibrator in the test kit. This allowed creating plots of the prolamin and glutelin concentrations quantitated by ELISA against those quantitated by RP-HPLC (Fig. 1). Ideally, both concentrations would be the same.

Results and discussion

Overall, the comparative analysis of prolamin and glutelin fractions from wheat, rye and barley with five commercial ELISA test kits for gluten quantitation covered three different mAbs (R5, G12 and Skerritt) as well as two pAbs. Taking the respective dilutions into account, the prolamin and glutelin concentrations quantitated by RP-HPLC were always used as independent base values for all calculations and comparisons of ELISA results.

The specificities and sensitivities of each antibody against the different gluten fractions were variable within one test kit (Fig. 1). The R5 mAb had the highest affinity towards rye and barley prolamins as well as rye glutelins. Wheat prolamins were detected with about equal sensitivity as the kit standard, which was according to expectations, because PWG-gliadin is used for calibration. The glutelins of wheat and barley only showed very limited reactivity with the R5 mAb. Wheat, rye and barley prolamins were detected by the G12 mAb with almost equal sensitivity that was also comparable to the kit standard. However, the affinity of the G12 mAb towards wheat and rye glutelins was very low and it was unable to detect barley glutelins, as has been reported before [8]. In contrast to the R5 and G12 mAbs, the 401.21 (Skerritt) mAb reacted with wheat, rye and barley glutelins with even higher sensitivity than with the kit standard. Wheat and rye prolamins and the kit standard were detected with comparable sensitivity, but the affinity towards barley prolamins was very low. Both pAbs differed considerably in their abilities to accurately detect wheat, rye and barley prolamins and glutelins. Wheat and rye prolamins were recognized by the pAb 1, but all other fractions showed very limited reactivity. The pAb 2 detected all fractions with high sensitivity except for barley glutelins.

When comparing the analyses of the same gluten fraction with the five different test kits, the results also showed a high degree of variability. The only exception were wheat prolamins (gliadins), which were only slightly overestimated by the R5, Skerritt and pAb 2 assays and slightly underestimated by the G12 and pAb 1 assays. This result was according to expectations, because most test kits are calibrated against gliadins. However, wheat glutelins were overestimated about 9-fold by the Skerritt mAb and underestimated by a factor of about 10 by the R5 and G12 mAbs and the pAb 1, whereas the pAb 2 yielded results that agreed with the RP-HPLC quantitation. In the case of rye prolamins, the R5 and pAb 2 assays overdetermined the concentration, the pAb 1 assay slightly underestimated the concentration and the G12 and Skerritt mAb assays showed a good correlation to the RP-HPLC results. The immunochemical determination of rye glutelins revealed that this fraction was overestimated by the R5, Skerritt and pAb 2 assays, but underestimated by up to 10-fold by the G12 and pAb 1 assays. The analyses of barley gluten fractions showed the

highest overall degree of variability. Barley prolamins were overestimated by up to 6-fold using the R5 and pAb 2 assays, but the pAb 1 and Skerritt assays hardly showed any detection. The G12 assay resulted in quite accurate values. In contrast, the Skerritt assay was the only one that was able to detect barley glutelins. All other mAbs and pAbs showed virtually no affinity towards barley glutelins.

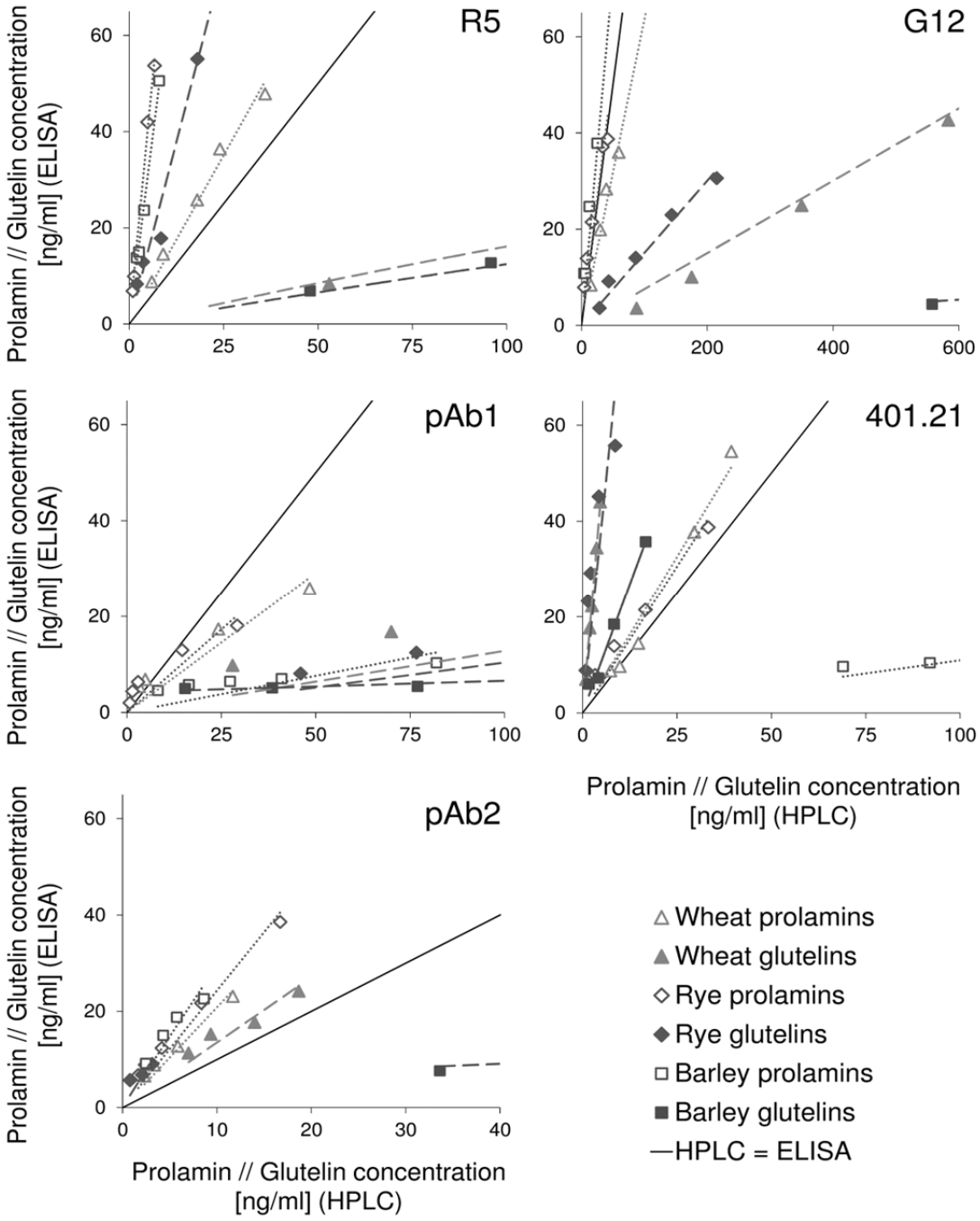

Figure 1. *Prolamin and glutelin concentrations [ng/mL] quantitated by RP-HPLC plotted against the prolamin and glutelin concentrations [ng/mL] quantitated by five sandwich ELISA test kits using the R5, G12 or 401.21 (Skerritt) monoclonal antibodies or two different polyclonal antibodies (pAb1 or pAb2)*

Conclusions

Different gluten fractions yielded variable results within one ELISA test kit just like different ELISA test kits yielded variable results within one gluten fraction. The mAbs and pAbs showed different sensitivities and specificities towards wheat, rye and barley and towards prolamin and glutelin fractions. Therefore, the gluten content was either over- (up to 6-fold) or, more seriously for CD patients, underestimated (up to 8-fold). A careful consideration of this variability of results between different ELISA test kits is important, especially when analysing samples where the gluten source is unknown.

Acknowledgement

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4.2 Progress towards the practical application of MS for gluten detection and quantification

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Introduction

Gluten is the collective name for a class of proteins found in wheat, rye, and barley. Coeliac disease (CD) is an immune-mediated inflammatory disease of the small intestine in a subset of genetically susceptible individuals that is triggered by the ingestion of gluten, resulting in intestinal inflammation and damage. The only current treatment for CD- and gluten-intolerants (≈70 million people globally) is lifelong avoidance of dietary gluten. Gluten-free (GF) foods are now commonplace, however, it is difficult to accurately determine the gluten content of GF products using current methodologies as the antibodies are non-specific and show cross-reactivity. In processed products measurement is further confounded by protein modifications and/or hydrolysis. The development of mass spectrometry- (MS) based methodology for absolute quantification of gluten is required for the accurate assessment of gluten, including hydrolysed forms, in food and beverages to support the industry, legislation and to protect consumers suffering from CD.

In this study, the hydrolysis of gluten was examined by proteomic profiling of beers subjected to size-fractionation. Selected proteolytic and hydrolysed peptide fragments were quantified by targeted mass spectrometry and the results were compared with ELISA data. Strikingly, those beers containing high levels of hydrolysed B-hordeins yielded potential false negatives by ELISA. The effect of the hydrolysed gluten fragments on the suppression of ELISA response was further investigated revealing interference that precludes the accurate quantification of beers using sandwich ELISA technology. Secondly, proteomic profiling of gluten-enriched fractions of 16 cereal grains including wheat, rye, barley, and oats focussing only on the grain (the edible seeds) provided the foundation for the selection of peptide markers unique to wheat. A rapid, robust, selective and sensitive method for detection of wheat contamination in a range of cereals using multiple reaction monitoring (MRM) MS was developed and applied to detect contamination in commercially available flour as well as in an intentionally contaminated soy flour.

Materials and methods

Suppression of ELISA by hydrolysed gluten

A selection of beers were collected (60 commercial beers and a lab-brewed beer made using barley cv. Sloop as listed in [1]) based on the stated ingredients or gluten content. Each beer was either: left whole or applied to a 30 k or 10 k molecular weight cut-off filter as described in [2]. The beers (whole and sub-30 k) were reduced by addition of 10 µL of 50 mmol/L dithiothreitol (DTT) under N_2 for 30 min at 60°C. To these solutions, 10 µL of 100 mmol/L iodoacetamide (IAM) was added and the samples were incubated for 30 min at room temperature. To each solution, with the exception of the 10 k fractions, 5 µL of 2 mg/mL trypsin was added and the samples incubated at 37 °C overnight. The digested peptide solution was acidified by addition of 50 µL of 1% formic acid and stored at 4 °C until analysis. All beer samples generated were analysed by liquid chromatography (LC-) MS/MS and proteins identified by automated database searching as described in [2]. Selected proteins (and their peptide fragments) were further analysed by targeted proteomics employing MRM MS [2].

Three beers were selected from the collection. Beers 6 and 31 gave a high and medium response by ELISA, respectively. Beer 13 gave near-zero ELISA response, but contained average levels of hordein by MS and high levels of hydrolysed gluten by MS. Beer 13, either undiluted or diluted 2-, 5- or 10-fold was added to sandwich ELISA wells containing beers 6 or 31 which had been diluted 1/50,000 or 1/5,000 respectively (so as to be in the middle of the Sloop hordein standard curve as described in [3]). In a second experiment, aliquots of beer 13, either unfiltered or filtered through 100, 30, 10 or 3 k molecular weight cut-off centrifugal filters were added to sandwich ELISA wells followed by aliquots of beers 6 or 31. All ELISA assays were processed according to manufacturer's instructions (ELISA Systems) and as described in [2].

Detection of wheat (gluten) contamination

Grains of wheat cv. Chara and 15 commercially relevant cereal grains were sourced as described in [4]. A gluten-enriched fraction was prepared by dissolving wholemeal flour (20 mg) in 200 µL 55% (v+v) propan-2-ol (IPA), 2% (w/v) dithiothreitol (DTT) with incubation at 60 °C for 30 min. The total protein extraction was performed by dissolving wholemeal flour (20 mg) in 200 µL of 8 M urea, 2% (w/v) DTT with incubation at RT for 30 min. The solutions were centrifuged for 15 min at 20,800 x *g* and the supernatant was kept. The proteins were reduced, alkylated and digested using trypsin [4] prior to LC-MS/MS analysis.

Results and discussion

Suppression of ELISA by hydrolysed gluten

We have previously demonstrated that barley-based beers that contain seemingly average levels of hordein by MS have very low or zero readings by ELISA [5]. We

have also demonstrated the existence of hordein peptide fragments in beers that have been filtered through 10 k molecular weight cut-off filters with no enzymatic digestion [1]. In this study, we sought to examine the relationship between the levels of hydrolysed hordein in beer with the effect on the ELISA response.

LC-MS/MS analysis of barley-derived beers revealed that certain classes of hordein were prone to hydrolysis (as judged by the percentage of each hordein present in the 30 k fraction relative to whole beer). Specifically, 57 ± 12% and 37 ± 7% hydrolysed hordein were detected for B1- and B3-hordein, 31 ± 13% for D-hordein compared to 18 ± 10% for γ3-hordein. The resulting peptide fragments shared significant homology with the immunotoxic epitopes determined for CD (Tab. 1). Strikingly, those beers that contained high levels of B-hordein fragments gave near zero values by ELISA.

Table 1. *Potential immunoreactive peptide sequences. Peptide sequences identified (≥95% confidence) in the sub-10 k fraction of 60 commercial beers. The sequences are aligned with the closest matching immunoreactive epitope(s). Glutamic acid residues shown in bold typefont (**E**) are produced by deamidation of glutamine (Q) by the enzyme tissue transglutaminase, but are present as Q in the native protein.*

Protein name	Uniprot accession	Peptide sequence	Immunoreactive epitope	#AA matching[a]
B1-hordein	I6TMW0	QPQPYPQQPQQPFPPQ	QQP**E**QPFPQ	8/9
B1-hordein	P06470	QPQPYPQQPFPPQ	QQP**E**QPFPQ	6/9
B1-hordein	I6SJ22	PQQPFPQQPPFG	QQPFP**E**QPQ	8/9
B3-hordein	I6SW30	QPQPYPQQPQPFPQ	PYP**E**Q**E**QPF	8/9
C-hordein	Q40055	PQPQQPNPQQPQQP	QQP**E**QPYPQ	6/9
HMW-GS	J3S7U9	GQGQSGYYPTSPQQP	QGYYPTSPQ	8/9
HMW-GS	C8CHI1	TSPQQGQQGQQGYYPTSPQ QSGQWQ	QGYYPTSPQ	9/9
75k γ-secalin	E5KZU9	PQQPQQSSPQPQQP	PQP**E**QPFPW	6/9
γ-prolamin	H8Y0J7	GQGIIQPQQPAQLEAIR	IQP**E**QPAQL	9/9
γ-prolamin	H8Y0J7	QPQQPQPFPQPSQP	PFPQP**E**QPF	7/9
γ-gliadin	J7HY97	IIQPQQPAQLEVLR	IQP**E**QPAQL	9/9
γ-gliadin	F6KV50	PQQPQQPAQLEGIR	IQP**E**QPAQL	8/9
LMW-GS	G0YLZ2	QQQPFPQQPP	QQPFP**E**QPQ	8/9
γ-gliadin	L7R5Y9	FPQPQQTIPHQPQ	PFPQP**E**QPF	6/9
γ-gliadin	B6DQC6	IIQPQQPAQLEVIR	IQP**E**QPAQL	9/9

[a] number of amino acids matching

Gluten fragments detected in the sub-10 k fraction contained epitopes that would likely be recognized by the antibodies used in currently accepted ELISA assays. The Skerritt antibody recognizes PQPQPFPQE & PQQPPFPEE, while the Mendez R5 antibody recognizes **QQPFP**, QQQFP, LQPFP & QLPFP. Typical examples of gluten

hydrolysis fragments detected include: B1-hordein (Uniprot: P06470 and I6SJ22) peptide fragments QPQPYP**QQPFP**PQ and P**QQPF**PQQPPFG; B3-hordein (Uniprot: I6SW30) peptide fragment QPQPYPQQPQPFPQ. QPQPY**PQQPFP**PQ shares 6/9 in the DQ8 T cell epitope (EQ**PQQPFP**Q) and P**QQPFPQQP**PFG shares 8/9 residues in the DQ2 T cell epitope (**QQPFPEQP**Q) rendering them likely to possess immunoreactivity.

The hydrolysed fragments that persist in beer show a dose-dependent suppression of ELISA measurement of gluten despite using a hordein standard for calibration of the assay. One drawback of sandwich ELISAs is they cannot adequately quantify gluten that has been highly hydrolysed [6]. Sandwich ELISAs require two epitopes or antibody binding sites. When a protein is hydrolysed, the various fragments may not contain two epitopes. Many of the hydrolysed gluten fragments present in beer 13 have one potential antigenic site. It was hypothesised that their binding to the capture antibody precludes the binding of intact gluten proteins resulting in suppression of the ELISA response. Beer 13 showed a concentration-dependent suppression of the ELISA response (Fig. 1A, B) in both a high-gluten containing wheat beer (beer 6) and

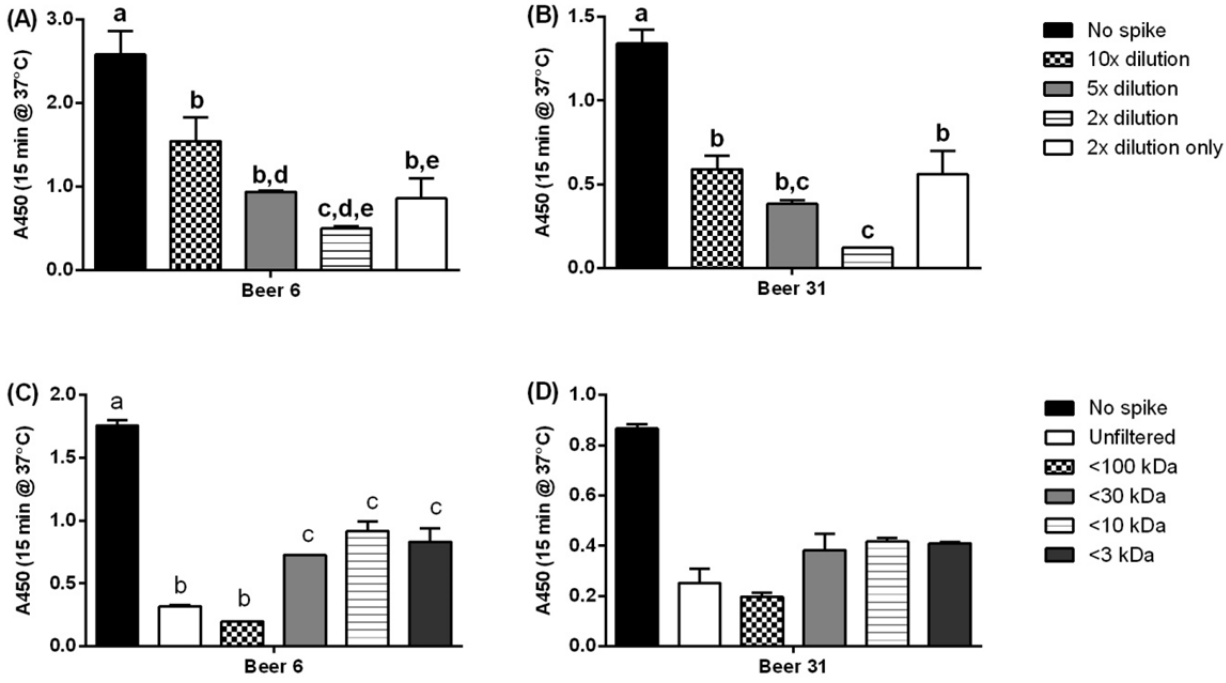

Figure 1. *Suppression of ELISA response by hydrolysed gluten present in whole beer. Beer 13 (that contained high levels of hydrolysed gluten) was spiked into samples of beers with differing levels of total gluten: (A) beer 6 (high); or (B) beer 31 (medium). The columns represent (from left to right) no spike, spike with diluted beers (at either 10-, 5- or 2-fold dilutions) or the 2-fold diluted spike only. The mean A450 ± SD are shown. Beer 13 was subsequently subjected to size-fractionation prior to addition in to the same two beers: (C) beer 6; and (D) beer 31. Analysis of variance (ANOVA) was carried out (2D: A and B; 1D, C and D). Within a group, columns with different letters were significantly different by Tukey's multiple comparison test ($p \leq 0.01$)*

a medium gluten-containing beer (beer 31). The suppression was demonstrated to be due primarily to the <3 k fraction, i.e. hydrolysed peptide fragments (Fig. 1C, D). Suppression was also caused by the high MW fraction (30 - 100 k) possibly the result of aggregation (Fig. 1C, D).

Detection of wheat (gluten) contamination

In this study, 16 cereal grains that may be subject to contamination have been comprehensively characterised using discovery proteomics to identify wheat-specific peptide markers. As the focus of our work is the detection of gluten proteins, an IPA/DTT extract that has been shown to preferentially solubilise the gluten fraction [3,7] was used. In order to accurately identify the specific gluten isoforms present in the extract, a combination of three proteolytic enzymes: trypsin, chymotrypsin and thermolysin; was used to improve the protein sequence coverage (Tab. 2).

Table 2. *Identification of HMW-glutenin isoforms was achieved using a combination of three proteolytic enzymes by obtaining maximum sequence coverage.*

	ProteinPilot Sequence Coverage (95% confidence)					
Spots	**Accession**	**Family**	**Trypsin**	**Chymo**	**Thermo**	**Combined**
1-4	Q599I0	Dx2	27.1	62.8	10.6	66.4
5-8	Q41553	Ax2	48.4	60.6	5.9	75.7
9-15	Q45R38	Bx7	35.5	65.0	16.5	84.3
16-19	Q6RX92	12Dy	41.5	66.7	9.3	73.3
20-24	Q03871/ Q0Q5D8	1By9/ 1By8	30.6	41.3	10.9	52.2

The tryptic peptide products identified in the global LC-MS/MS analyses were assessed to identify prototypic peptides that were unique to wheat and that were high responding in the MS analysis, that is, peptides that yielded good peak area/intensity. The peak area/intensity is dependent on the ionisation efficiency of the peptide, but also the abundance of the protein and, therefore, the peptide and the efficiency of proteolytic digestion under the experimental conditions. The top 20 highest responding peptides were selected and subjected to BLASTp analysis (NCBI BLASTp server) against all other cereals to ensure specificity to wheat based on protein sequences that exist in the public databases. As not all of the grains examined have been sequenced at the genome level, it was expected that the public databases would be incomplete. To ensure the selected peptide markers were wheat-specific, the 16 grain extracts were screened using the LC-MRM-MS method developed herein. Peptide MRM transitions that showed interference, broad peaks and/or low intensity peaks were excluded from further analysis. The 10 best wheat peptides were selected for subsequent MRM analyses.

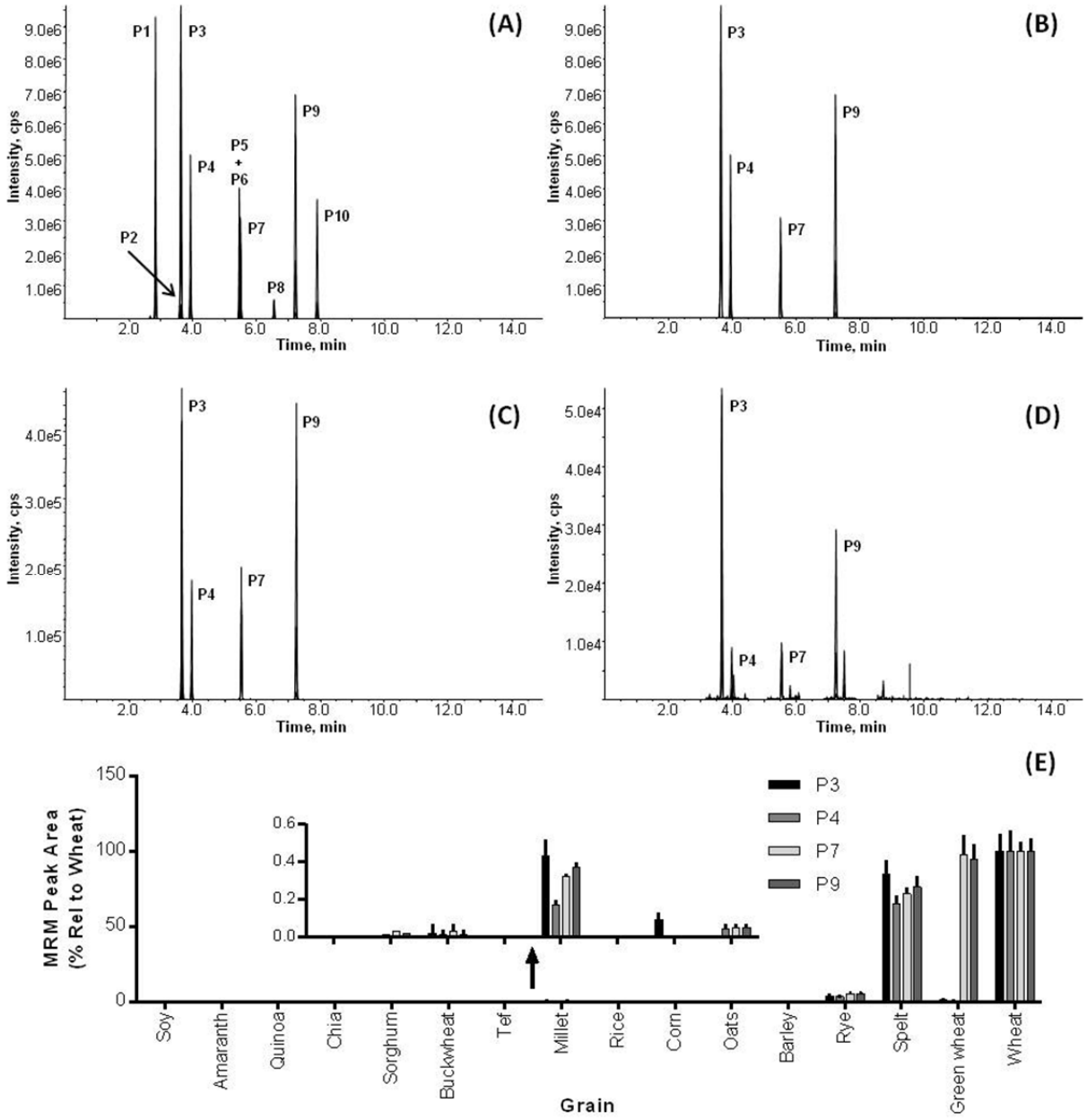

Figure 2. *From the top 10 wheat-specific peptide markers (A), the best 4 were selected (B) and allowed the detection of wheat contamination in rye (C) and millet (D) flour. Detection of contamination in commercial products: (E) Flour from rye, millet, sorghum, buckwheat, and oats were confirmed with low level wheat contamination*

Fig. 2 shows the LC-MRM-MS analysis targeting the wheat-specific peptide markers. The total ion chromatogram (TIC, Fig. 2A) shows ten peptide markers (labelled P1-P10) from which peptides P3, P4, P7 and P9 were selected and are shown in the extracted ion chromatogram (XIC, Fig. 2B). Fig. 2E shows the results of the LC-MRM-MS analysis of 16 grains using four wheat-specific peptide markers. As expected, the markers were present in high abundance in wheat, green wheat (Freekeh) and spelt. However, all four peptide markers were also detected in rye (average MRM peak area ≈4.2% relative to wheat), in millet (0.3%), and buckwheat (0.02%), whilst

three of the four peptide markers were detected in sorghum (0.03%) and oats (0.05%). The XIC for the analysis of pre-milled rye (Fig. 2C) and millet (Fig. 2D) flour revealed peaks matching to all four peptide markers (P3 at 3.6 min; P4 at 4.0 min; P7 at 5.5 min; and P9 at 7.3 min) indicating that the commercial flour samples of both rye and millet were contaminated by wheat (≈4.2% in rye and ≈0.3% in millet). Uncontaminated whole grain of rye and millet were obtained, visually inspected, milled to fine flour after careful cleaning of the mill, analysed and shown to be devoid of wheat confirming the detection of the peptide markers was due to wheat contamination and not the presence of endogenous proteins. Trace levels of the wheat peptide markers were also observed in commercial flours from oats, sorghum, and buckwheat, but the levels detected were <0.05% relative to wheat.

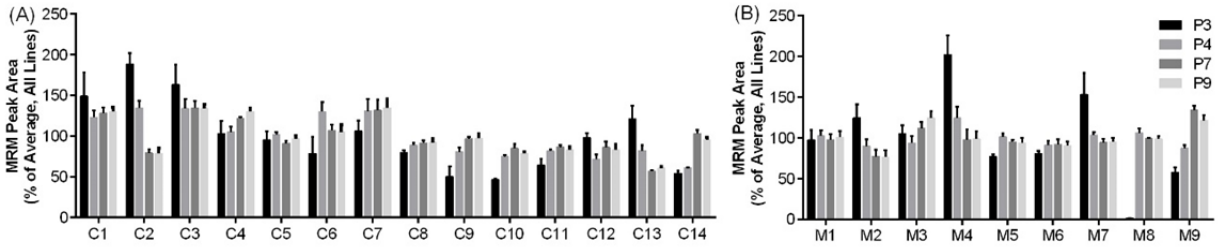

Figure 3. *Peptide markers are useful for diverse wheat varieties. The wheat peptide markers were assessed across 14 commercial wheat lines (A) and nine MAGIC parent lines (B)*

The wheat peptide markers were selected from analysis of a single wheat cultivar Chara. In order to assess the utility of the wheat peptide markers in broader applications, we investigated their presence and level in 14 commercially available wheat cultivars and in nine parent lines of the 4 and 8 way MAGIC populations developed by CSIRO [8], that are thought to represent ≈80% of the genetic diversity within the bread wheat genome (Fig. 3). Variation in the peptide levels of 22-26% for the 14 commercial lines and 11-16% for the nine MAGIC parent lines for P4, P7 and P9 was observed. Only one peptide (P3) was absent in the wheat cultivar Xioayn, a Chinese winter cultivar used for noodles, and showed higher variation between cultivars (≈43-57%). Overall, these data demonstrate that the selected peptide markers are suitable for detection of wheat across a wide variety of wheat representative of those used commercially.

Conclusions

Analysis of barley-derived beers revealed that high levels of gluten (B-hordein) hydrolysis correlated with low values by ELISA. The hydrolysed fragments that persist in beer caused a dose-dependent suppression of ELISA gluten measurement. Global proteomic analysis of 16 economically important cereals utilising SDS-PAGE, Western blotting and LC-MS/MS was used to characterise the “gluteome” and select

peptide markers specific to gluten and/or wheat for targeted quantitative MS assays. Wheat-specific peptide markers were detected in 14 wheat varieties that together constitute 80% of modern wheat genetic diversity and facilitated the detection of wheat contamination in commercial flours.

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4.3 Studies on the analysis of gluten-containing cereals

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Introduction

Coeliac disease (CD) is an inflammatory disorder of the upper small intestine caused by the ingestion of gluten proteins from wheat (gliadins, glutenins), rye (secalins), barley (hordeins), and possibly oats (avenins). The only effective therapy for CD patients is a strict gluten-free diet by consuming gluten-free foods, which contain less than 20 mg gluten/kg [1]. To ensure the safety of gluten-free products for CD patients, it is essential that appropriate analytical methods with high specificity and sensitivity are available. The most commonly used method for gluten analysis is an enzyme-linked immunosorbent assay (ELISA). Currently, the ELISA based on the R5 monoclonal antibody is endorsed for gluten analysis in maize-matrices [2] and is defined as type I method [3]. In general, with ELISA it is only possible to determine the prolamin content in foods, and not the whole gluten content. The gluten content is then calculated by multiplying the prolamin content by a factor of 2. This calculation is based on the assumption that the ratio of prolamins to glutelins is 1. The problem is that different types of grains contain different proportions of prolamins and glutelins. As a result, the gluten content may be either over- or underestimated. In particular the latter is problematic for CD patients [4].

Therefore, a new independent non-immunochemical method for the quantitation of prolamins and glutelins (= total gluten) is urgently needed to verify the results determined by ELISA. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) appears to be a suitable method for the quantitation of gluten.

Therefore, the aim of this study was to identify marker peptides from wheat, barley, rye, and oats to develop a stable isotope dilution assay for the quantitation of intact and partially hydrolysed gluten.

Materials and methods

Defatted flours from wheat, barley and oats (a mixture of four cultivars of each grain, harvest 2013) were extracted by a modified Osborne procedure [5]. First, albumins/globulins were extracted with 0.067 mol/L K_2HPO_4/KH_2PO_4-buffer with 0.4 mol/L NaCl (pH = 7.6) at 20 °C. Second, the residue was extracted with 60% (v+v) ethanol at 20 °C to obtain the prolamin fraction. Finally, the glutelin fraction was extracted with 50% (v+v) 1-propanol/urea/Tris-HCl/dithiothreitol (pH = 7.5) at 60 °C. Albumins/globulins were discarded because they were not relevant for gluten analysis.

Different protein types were isolated using preparative reversed-phase high-performance liquid chromatography (RP-HPLC). Gliadins (wheat) were separated into ω5-, ω1,2-, α-, and γ-gliadins, and glutenins (wheat) were separated into low-molecular-weight- (LMW-GS) and high-molecular-weight glutenin subunits (HMW-GS). Barley prolamins were divided into γ- and C-hordeins, and barley glutelins into B- and D-hordeins. Furthermore, all isolated protein types and fractions were characterized by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to verify the molecular weights and the purity of the preparations.

In order to be able to develop a LC-MS/MS method for gluten proteins, partially hydrolysed reference proteins were necessary. For this purpose, the isolated reference proteins (protein types, protein fractions) as well as the flours were incubated with chymotrypsin for 24 h at 37 °C and pH 7.8 (ratio enzyme:protein; 1:200). After incubation, the peptide mixtures were purified by solid phase extraction on DSC-18 cartridges. Then, the hydrolysates were analysed by LC-MS/MS.

Results and discussion

Prolamin and glutelin fractions from wheat (gliadins/glutenins) and barley (hordeins) were separated into protein types by preparative RP-HPLC. Avenins were not further separated. As an example, Fig. 1 shows the chromatogram of the glutenins, which were separated into HMW- and LMW-GS. All isolated protein types were characterrized by RP-HPLC and SDS-PAGE to verify the range of the molecular weights.

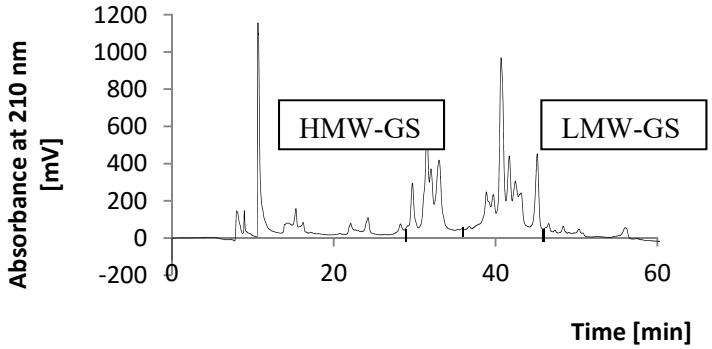

Figure 1. *Preparative RP-HPLC of isolated wheat glutelins (glutenins; 25 mg/mL, injection volume: 300 µL). HMW-GS, high-molecular-weight glutenin subunits; LMW-GS, low-molecular-weight glutenin subunits*

All isolated protein types from wheat, barley, and oats were hydrolysed and analysed by LC-MS/MS to identify gluten marker peptides which will be used for gluten quantitation. These marker peptides must fulfil specific requirements to be suitable for gluten quantitation. Firstly, the amino acid sequences must be characteristic for each protein type. Ideally, the peptide sequences should not occur in other protein types of gluten or other proteins. Secondly, the marker peptides should have a peptide length of 8 to 20 amino acids, because too small peptides are not specific enough and peptides longer than 20 amino acids are unsuitable for quantitation due to high complexity and

the large number of fragments. Thirdly, the marker peptides should not contain cysteine residues because of their tendency to oxidation.

To define gluten marker peptides, all isolated protein types from wheat and barley were hydrolysed and analysed by LC-MS/MS in the first step. By using the NCBI-Database (National Center for Biotechnology Information) and the MASCOT-software (Matrix Science, London, UK), gluten peptides were identified. In the second step, the criteria for gluten marker peptides were applied to get candidate peptides. To verify this selection, all protein fractions from wheat, barley, and oats as well as the flours were hydrolysed and analysed by LC-MS/MS. Finally, peptides which were identified in all hydrolysates (protein type, protein fraction and flour) and which fulfilled the requirements were suitable for gluten quantitation. For each protein type, two or three gluten marker peptides were defined (Tab. 1 and Tab. 2). For ω5-gliadin, only one marker peptide was defined because of the low amount in flour.

Table 1. *Selection of marker peptides for wheat gluten, their mass-to-charge-ratios, and the peptide scores, which were detected in flour.*

Wheat marker peptides			
Sequence	***m/z* (charge state)**	**Species (protein type)**	**Score[a]**
QQQPLPPQQTFPQQPL	938.4 (2^+)	Wheat (LMW-GS)	41
GQQPQQQQL	527.7 (2^+)	Wheat (LMW-GS, γ-gliadin)	33
VQQQIPVVQPSIL	725.3 (2^+)	Wheat (LMW-GS, γ-gliadin)	30
SIILQEQQQGF	645.9 (2^+)	Wheat (LMW-GS, γ-gliadin), rye, barley	71
LQPGQGQQGY	538.2 (2^+)	Wheat (HMW-GS), barley	49
TASLQQPGQGQQGHYPASL	985.0 (2^+)	Wheat (HMW-GS), barley	42
HVSVEHQAASL	589.3 (2^+)	Wheat (HMW-GS), rye	36
ASIVAGIGGQ	872.2 (1^+)	Wheat (γ-gliadin)	28
NIQVDPSGQVQW	685.7 (2^+)	Wheat (γ-gliadin)	57
LQPQQPQQSFPQQQQPL	1011.3 (2^+)	Wheat (γ-gliadin), rye	63
LQLQPFPQPQLPYPQPQPF	755.2 (3^+)	Wheat (α-gliadin)	63
FQPSQQNPQAQGF	738.7 (2^+)	Wheat (α-gliadin)	64
RPQQPYPQPQPQY	813.7 (2^+)	Wheat (α-gliadin)	48
QQYPQQQPSGSDVISISGL	677.9 (3^+)	Wheat (ω5-gliadin)	53
GSSLTSIGGQ	453.5 (2^+)	Wheat (ω1,2-gliadin)	43
FPHQSQQPF	558.1 (2^+)	Wheat (ω1,2-gliadin)	26

[a] Scores of peptides >40 indicate identity, scores in the range of 15-40 were additionally verified

In general, it was also an important criterion to select marker peptides with a high score to increase the chance to detect them in different food matrices. Although the oat peptide DPSEQYQPYPEQQEPF was detected with a low protein score of 15, it was defined as marker peptide because it contains a deamidated T cell epitope (PYPEQEQPF) (Tab. 2) [6].

Table 2. *Selection of marker peptides for gluten from barley and oats, their mass-to-charge-ratios, and the peptide scores, which were detected in flour.*

	Barley marker peptides		
Sequence	***m/z* (charge state)**	**Species (protein type)**	**Score[a]**
AIDTRVGV	415.6 (2^+)	Barley (B-hordein)	32
QQPQPQQGQQQQVPQSVF	1040.3 (2^+)	Barley (B-hordein)	39
AQQQPSIEEQHQL	768.3 (2^+)	Barley (γ-hordein)	52
GGGLTTEQPQGGKQPF	801.2 (2^+)	Barley (D-hordein)	40
TQQKPGQGYNPGGTSPL	863.3 (2^+)	Barley (D-hordein)	38
IIPQQPQQPFPLQPHQPY	1078.4 (2^+)	Barley (C-hordein)	38
RQLNPSSQEL	586.2 (2^+)	Barley (C-hordein)	35
	Oats marker peptides		
VQQQPPFVQQEQPF	850.4 (2^+)	Oats (Avenins)	62
DPSEQYQPYPEQQEPF	990.7 (2^+)	Oats (Avenins)	15
LQPQLQQQL	548.2 (2^+)	Oats (Avenins)	38

[a] Scores of peptides >40 indicate identity, scores in the range of 15-40 were additionally verified

Conclusions

The LC-MS/MS analysis of hydrolysed proteins from wheat, barley and oats yielded a number of peptides which can be used as gluten marker peptides. Peptides, which were identified in hydrolysed protein types, protein fractions as well as the flour, were selected as marker peptides. For each protein type, two to three marker peptides were defined, which provide the basis to develop a non-immunochemical independent method for gluten quantitation.

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4.4 Strategies to reduce or prevent wheat coeliac-immunogenicity and wheat sensitivity through food

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Abstract

Cereals are among the oldest foods of humans. Wheat is one of these. In present times, several syndromes are, whether true or false, increasingly attributed to the consumption of wheat, with increasing costs for medical care and decreasing turnover for the food industry, especially the bakery sector. Many western societies show remarkable annual increases in their health care costs, often surmounting their economic growth rates. Governmental health policies should urgently revert towards the stimulation of disease prevention practices instead of maintaining the stimulation of expensive medical care.

Here we review and discuss possible strategies to prevent or reduce the incidence of wheat-related conditions through application of breeding and food-related technologies. Breeding includes selection and crossing for low-immunogenic wheat varieties using varieties, accessions, and wild relatives, silencing the expression of gluten genes, and advanced genome editing techniques to eliminate gluten genes, such as CRISPR/Cas9 technology. Food-related approaches include the reduced application of vital gluten, exclusion of gliadin from isolated gluten by separation, increased use of sourdough fermentation and malting, utilisation of patient-specific gluten epitope-sensitivity profiles, introduction of the gluten contamination elimination diet (GCED) especially in individuals that are non-responsive to the gluten-free diet, to acquire more fundamental knowledge on immune modulating factors, and the design of an intervention study to learn about the medical and mental motives of people to switch towards a 'gluten-free' diet. Finally, we discuss the development, testing and promoting of efficient disease prevention measures within the societal context.

Introduction

Agriculture started some 10,000 years ago. It is a myth, however, that the consumption of cereal grains by humans begun only at that time. The divergence of early humanoids from man-apes occurred about 6 My ago with leaving the African forest and moving into the savannah areas. Concomitantly, the humanoids increased their feeding on small and hard grass (cereal) seeds. This has been concluded from the shape of the molars and the increased thickness and the molecular composition of the enamel [1-3]. From 1.5 My ago onwards, *Homo* species increased the fraction of C4-based plant resources (i.e. cereals) in their diet as was also concluded from enamel

analysis [4]. As early as 120,000 years ago, during an interglacial period, the Levant, where barley and wheat are endemic species, was a fruitful settling and meeting place where West-European Neanderthals exchanged genes with early modern humans [5]. Neanderthals from the Iraq region are known to have consumed cooked *Triticeae* grains, especially barley, about 50,000 years ago [6]. Excavations from 32,600 years ago in Italy revealed the occurrence of thermal pre-treatment and grinding of cereal seeds (oats) by humans [7] indicating that cereal food technology must have been developed much earlier. From more recent times, some 15,000 years ago, remainders from barley groat meals and porridge have been found, as well as remains from unleavened bread from barley flour [8]. Further, data from 11,000 years ago show ground collection of wild barley and wild emmer wheat seeds as an intermediate step between seed collection by hunter-gatherers and cereal harvesting by early farmers [9]. Agriculture and cereal (barley and wheat) consumption, therefore, did not appear suddenly 10,000 years ago, but had a long and gradual origin.

Remarkable in this apparently long tradition of grain consumption by humans is the development of bread wheat and spelt wheat (both with the AABBDD genome). These wheats are considered as the natural hybrids between (early cultivated) emmer wheat (with AABB genome) and wild *Triticum tauschii* (with the DD genome). The hybridization is suggested to have occurred some 8,000-9,000 years ago, maybe as a side-result of early agricultural activity [10]. More recently, 2,000 years ago, coeliac disease (CD) was identified (but not yet directly related to the consumption of wheat, barley and rye) and received its name from Arathaeus of Cappadocia [11]. Undoubtedly, this disease must have existed much longer and most probably at significant frequency.

In the 20th century cereal breeding and cereal food processing have become highly advanced. Wheat and barley can be grown now also at high latitudes and large acreages. Millers are able to refine cereal meal and separate many fractions that can be used in innumerable applications. Simultaneously, the prevalence of and mortality due to undiagnosed CD increased four times [12]. During the last years, non-celiac wheat/gluten sensitivity (NCWGS) seems a new condition, although some case reports were already known from the seventies of last century [13,14]. A recently estimated prevalence in the general population of NCWGS, obtained from indirect evidence, is slightly more than 1% (which is similar to the prevalence of CD in the general population [15]). NCWGS is mostly reported to occur in females in the age group of 30-50, although also paediatric cases are known. In spite of the generally low estimated prevalence of both CD and NCGWS, more than 10% of the adults in the USA and the UK changed their diet towards gluten-free, mainly on the basis of self-diagnosis [15]. The situation may be even more extreme in the USA, where 30% of restaurant visitors demand gluten-free food [16]. Nevertheless, in NCWGS a direct relationship with gluten itself or with other wheat-related compounds like amylase trypsin inhibitors (ATI), lipid transfer proteins (LTP), fermentable sugar compounds (FODMAPS), or other compounds or factors as causing agents remains unclear [15]. This situation is different for CD. While the relationship with gluten consumption has

been established clearly and is well-understood, 80-90% of the CD patients has not yet been diagnosed or has been diagnosed wrongly. This large part of the CD population maintains the daily consumption of gluten-containing foods, unaware of the risk of worsening their health status. These figures together show an *underestimated* population of people with CD, and a *self-overestimated* population of NCWGS people, both following a (strict) gluten-free diet.

In this short review we propose a broad array of strategies to meet the desire of CD and NCWGS populations and individuals for safe(r) cereal food (see also [17-19]). These strategies fall into two categories: 'plant-related strategies' that use various advanced breeding technologies for the reduction of the occurrence of CD epitopes in especially gliadins, and 'food-related strategies' that focus on targeted processing to enable gluten avoidance.

Plant-related strategies

Selection

Selection of low CD-toxic wheat varieties and accessions has been performed using epitope-specific monoclonal antibodies (mAbs): out of hundreds of such lines only a few gave a relatively low mAb response [20,21]. The utility of screening with epitope-specific mAbs is limited as they do not recognize the complete epitope (mAbs recognize a maximum linear sequence of six amino acid residues, whereas the T cell epitope is nine amino acids long) and the signal may be ambiguous and not quantitative (i.e., also responding to non-intact epitope sequences). A direct approach combining deep sequencing of the N-terminal region of gliadin transcripts as a pre-screening of developing seeds of a single variety, followed by quantitative proteomics of ripe seeds [22] enabling simultaneous quantification of several CD epitopes is much more straightforward and conclusive.

Traditional breeding

In barley, a reduction of the gluten (hordein) content to below 5 mg/kg (the threshold for gluten content in 'gluten-free' products in the Codex Alimentarius is 20 mg/kg) was achieved using traditional breeding strategies by combining three recessive alleles, which act independently of each other to lower the hordein content in the parental varieties. Further breeding was applied to increase the grain size to near wild-type levels. The plants were agronomically acceptable and the produced grains showed good malting characteristics and brewed successfully. The advantage in traditional breeding of barley over bread wheat is its diploid genome level and the occurrence of only four hordein protein families [23].

Synthetic hexaploids

The D-genome of bread wheat contributes most to CD-immunogenicity with the highest number and diversity of epitopes [24], but shows the lowest degree of variation compared with the A- and the B-genome. Newly created synthetic hexaploids carrying

a diversity of D-genomes are now in a screening program which aims to identify hexaploid wheat lines with reduced CD-immunogenicity [18].

Deletion of loci

A ‘deletion line’ lacking a large part of the short arm of chromosome 6D, which eliminated the 6D alpha-gliadin locus, showed strongly decreased mAb responses against the Glia-alpha1 and Glia-alpha3 epitopes. Changes in dough mixing properties of this line could be compensated by the addition of oat prolamins (avenins), which are not CD-immunogenic. Such deletion lines are especially useful as model systems but lack economic value as they grow poorly. This is probably an effect of the loss of hundreds of other genes on this chromosome arm [25].

RNAi

Two different approaches have been applied to reduce the amount of gliadins in wheat through silencing of gliadin gene expression using RNA interference (RNAi). In the first approach, the gliadin genes themselves were targeted directly and successfully [26-31]. Flour made from grains in which gliadins were silenced showed no T cell elicitation, but retained baking quality [30]. The other approach used RNAi to suppress homologs of the DEMETER (DME) gene. This gene encodes a 5-methylcytosine DNA glycosidase which demethylates the promoter region of gliadin and LMW glutenin genes in wheat endosperm, a step that is necessary to switch on the expression of these genes during endosperm development. Suppression of expression of DME resulted in >75% reduction in the amount of immunogenic prolamins [32].

Mutation breeding and genome editing

Mutation breeding is a relatively old technology using chemical (especially ethyl methane sulfonate, EMS) or ionizing irradiation (e.g. gamma-radiation, fast neutrons) to introduce random mutations in the DNA. Mutation selection on populations of mutant progenies can be carried out through Targeting Induced Local Lesions in Genomes (TILLING), a technology based on molecular DNA sequence analysis followed by bioinformatics analysis. It is expected that no single line from such an experiment would exist in which all gliadins have been mutated simultaneously, but this strategy may be used in combination with other strategies mentioned above.

Precise genome editing recently sees a stormily increasing interest as the CRISPR/Cas9 technology enables inducing mutations and deletions at specific, targeted locations within the genomes of animals as well as plants. Several groups currently apply CRISPR/Cas9 in wheat, but no results have been published yet.

Food-related strategies

Reduction of vital gluten

Vital wheat gluten is a major side-product in the production of wheat starch which is the raw material for further production of native and modified starch, glucose syrup, liquid and crystalline polyols, maltodextrins, all with high-impact applications in the food industry. Wheat starch is also used for the production of alcohol, including bioethanol. Since wheat starch is economically the most important product from wheat (much more important than flour for bakery purposes), the production of its side-product, vital gluten, is enormous. Vital gluten is increasingly applied as protein additive in food products for technological improvement. The large-scale addition of vital gluten to a wide range of processed food products has contributed to the increase in total gluten consumption: between 1977 and 2007, gluten intake in the western world has tripled [33], and most of this increase took place after 2000. The increase relates only partly to bread and bakery products, where vital gluten is applied to maintain high loaf volumes, but is mainly the result of its use in the form of 'hidden' ingredient, often not labelled, in a large number of food products. Currently, a movement is going on in the modern bakery in The Netherlands towards 'back to basics'. This includes 'clean label' strategies and avoidance or strong reduction of the application of bread quality-improving ingredients such as enzymes and vital gluten in artisanal and industrial whole grain breads. There are, however, no indications of reduced application of vital gluten in other sectors of food production. On the contrary, more and more vital gluten application in foods is occurring. In addition, industrial conversion of wheat starch into other food-grade carbohydrates will not eliminate all stable and potentially immunogenic gluten. Therefore, foods containing these modified carbohydrates may still contain low levels of gluten. Together these processes have led to a steady increase of gluten accumulation in the general daily diet.

Elimination of gliadins from gluten

HMW-glutenins give dough its elasticity, while the gliadins can be replaced by other proteins, e.g. by the coeliac-safe avenins, for maintaining good baking quality [25]. The HMW-glutenins also have a relatively low coeliac-immunogenicity. This means that separation of glutenins from total gluten is a realistic option that may contribute to new applications in foods aiming at a strongly reduced immunogenic gluten load. Nearly complete separation can be achieved at lab scale [34]. Industrial upscaling to economically and technologically viable levels seems to be more recalcitrant [35], and would need further research.

Sourdough fermentation

In Germany, the prevalence of CD (measured using a single antibody test) was found to be remarkably low: 0.4% and 0.2% in women and men, respectively [36], which confirmed earlier data showing 0.1-0.4% prevalence on the basis of anti-tTG and EMA positivity [37]. Recent estimations of the CD prevalence in Germany go up to 0.8%,

especially in children up to 17 years old [38]. The authors suggest that this may reflect underdiagnosis in earlier studies, but another explanation is also possible.

Germany has a longstanding tradition of sourdough bread consumption. Sourdough fermentation has been suggested to enable the manufacture of 'gluten-free' or 'low-in-gluten' wheat-based products through proteolytic gluten degradation [39]. More recently, a two-step hydrolysis including fungal peptidases and *Lactobacillus* endo-peptidase activity, respectively, resulted in a wheat flour-derived product with a gluten content below 10 mg/kg. This product appeared not harmful to individuals with CD [40]. These observations are promising, but need further research on the potential of sourdough products in a safe 'gluten-free' diet for individual cases. For this, first a strict definition of the type of sourdough is required and the process of gluten degradation needs to be analysed in detail. Further, the question is whether large-scale consumption by the general population of well-defined sourdough products can indeed contribute to the reduction of the incidence and symptom severity of CD and NCWGS. The answer requires large-scale epidemiologic research. If sourdough indeed reduces the exposure to intact gluten epitopes, then the recent increase in prevalence of CD in young people may in fact reflect a change in the diet of German children and adolescents towards fast food consumption (white and yeast-based bread).

Malting

This process is not only relevant for brewing, but has also been applied since ages in the bakery sector. Endogenous proteolytic enzymes become active during malting, i.e. the first steps in seed germination, to produce amino acids from the storage proteins in the endosperm as building blocks for new structural and house-keeping proteins for the young growing seedling. During this germination process, which lasts several days until the seed has been transformed into a seedling, gliadins may become degraded first. This means that (specific) gliadinases will show early activity. Application of enzyme supernatants from a small stock of germinated seeds (especially barley seeds) have recently been suggested to be applicable in large-scale production of flour reduced in prolamin content [41,42]. In addition, it has been shown that germinated and fermented rye and wheat sourdough effectively degraded 99.5% and 95% of the prolamins, respectively [43,44].

Patient-specific epitope-sensitivity profile

Genomic data of plant species and cultivars are rapidly increasing in number and detail; this also includes the wheat genome (with the sequences of dozens of gluten (gliadin) genes and epitopes and their expression profiles). Similarly, genomic data of humans, also at the individual level, are laying the basis for personalized food and for personal health and medical strategies. Individuals with CD appear to have different and epitope-specific sensitivities to gluten [45,46]. Combining both these sets of gliadin (gluten) and human genomics data may reveal possibilities to further investigate wheat varieties with epitope profiles that may fit to the specific sensitivity profile of an individual patient or specific groups of patients.

Gluten contamination elimination diet (GCED)

Some coeliac patients have persistent symptoms and villous atrophy despite strict adherence to a gluten-free diet. They are referred to as having 'non-responsive CD', a subset of which may have true 'refractory CD'. Some non-responding CD patients simply react to minor amounts of gluten, which may be present as contamination in regular, processed gluten-free foods. As mentioned above (in the paragraph on 'Reduction of vital gluten') foods labelled as gluten-free may contain minor amounts of gluten or stable gluten fragments due to their introduction into these food products through complex food processing. This may even happen in increasing numbers. Most (over 80%) of the non-responsive patients, however, improved on a so-called 'gluten contamination elimination diet' (GCED) that included only fresh and unprocessed foods. These patients appeared not to suffer from 'refractory CD' and were able to return to a traditional gluten-free diet without return of symptoms [47].

Immune-modulating factors

The high frequency of individuals sensitised to a broad range of cereal (i.e. wheat) proteins led to the suggestion that wheat (and related cereal) foods may have high immunogenic potential, perhaps greater than other foods in general, although without any occurrence of clinical symptoms [48]. We hypothesised that the consumption of wheat positively influences the maturation of the immune system in early childhood in 'cross-talking' with the intestinal microbial community [49]. This may especially be relevant in populations not yet affected by the 'western lifestyle' with its increased hygiene [50], and not yet saddled with the related 'western lifestyle syndrome' characterised by disturbed immune and (intestinal) microbial functioning and increased susceptibility to chronic inflammatory diseases. Western urban environments often lack many of the microbes with which humans have co-evolved, and which may function as inducers of immune-regulatory circuits [51,52]. Integration of research on factors related to gut microbiota (including foetal programming by maternal microbial exposure, neonatal programming and hygiene), on diet and lifestyle factors, and on human genetics and epigenetics, will hopefully reveal some of the immune-regulatory environmental factor(s) to explain the recent increase in immune-related diseases. Many studies have been conducted to identify the features of a 'healthy microbiome/microbiota' and the alterations on the host-microbiota cross-talk, promoting the progress from health to disease in diverse disorders. These studies show the enormous complexity and challenges of this scientific arena. Advances could open ways to the development of microbiome-informed strategies for (personalised) preventive and therapeutic measures including dietary strategies that help optimise the partnership between the gut microbiota and host immunity, increasing microbiome homeostasis in, and health resilience of, the host [53]. The manipulation of the gut microflora is in its infancy but may have enormous potential for the future with regard to prevention of immune system-mediated diseases [51,52]. How intestinal microbiota may play a role in the development of the immune system was demonstrated during a helminth therapy (through experimental hookworm infection), showing a significant

increase in microbial species richness and, interestingly, a promoted tolerance in coeliac subjects to escalating gluten challenges [54,55]. Helminth infections are known to be correlated with a Th2 response [56]. In this regard, measurement of IgE or other Th2 markers might be of interest to demonstrate a possible reversion of the immune system away from autoimmunity (i.e. away from CD).

Health Grain Forum Intervention Study

The above refers to an increasing but still small percentage of the population. There are currently solid grounds to advise the general consumer, i.e., the majority of the population, to consume whole grain wheat products. Very large cohort studies clearly demonstrate that consumption of whole grain products, including wheat and especially the cereal bran fraction, significantly reduces the risk of several chronic diseases [57,58]. These diseases are common, and therefore reducing these risks has a major effect on life expectation and wellbeing. Nevertheless, worldwide, an anti-wheat and anti-gluten hype has developed over the last five years, with significant impact on all parts of the cereal supply chain and concomitantly significantly reduced sales of bread, breakfast cereals and pasta products in various markets. The question arises why so many people (≈30% in the USA, 15% in Australia, and increasing numbers in other Western countries) seemingly feel more comfortable on a gluten-free or wheat-free diet. Hard data about negative human health effects (beyond CD and wheat allergy) are lacking, although popular books such as ‘Wheat Belly’ [59] and ‘Grain Brain’ [60] suggest differently. In these books, the cereal supply chain is blamed for feeding the world with sick-making food, but several of the arguments used are objectively wrong [61,62]. The clinical picture of NCWGS is variable and usually includes IBS-like gastrointestinal manifestations and neurological symptoms, such as foggy mind and headache [15]. Expert criteria for the diagnosis of NCWGS have been recommended, but specific and sensitive biomarkers for NCWGS are lacking [63]. Health Grain Forum now proposes to perform a double-blind placebo-controlled human intervention study addressing the effects of different wheat species, their components, and various processing steps towards end products. This study aims at measuring effects on human metabolism and health parameters to obtain reliable data useful for future human dietary recommendations and appropriate food processing and product development. *In vivo* effects, to be measured in this study, are related to gut feeling and adverse effects, analysis of gut microbiota and colonic metabolism, and to gut permeability. The intervention will also include the measurement of nocebo effects on the consumer’s perception after consumption. The intervention will include a well-controlled cohort of IBS patients. A consortium of research organisations and stakeholder cereal food companies has recently been established. The project may start in 2016 [64].

The societal context

Health care costs in The Netherlands (with 17 million inhabitants) amounted to almost 100 B€ in 2015, with an estimated annual increase of 6%, which is much higher than the total Dutch economic growth. With this amount, the Dutch spend *per capita* almost as much as a citizen of the USA, which is the highest in the world. The spectacular rise of these costs is partly due to an increasing elderly population, to increasing prices of special medical treatments in health care, and last but not least to the enormous increases in the prices and in the applications of medicines. In this regard, the pharmaceutical industry plays a dubious role [65]. Remarkably, in Cuba, the health care budget *per capita* is 20 times lower compared to the USA, however, with an equal life expectation. People have argued that part of the health care budget should be bent towards the development, testing and promotion of efficient prevention measures. We, therefore, argue that also 'big food industries' should take a responsibility for the health status of the population.

Conclusions

The societal context demonstrating increasing costs for health care should strongly urge national governments to reconsider their health care policies and change these into disease prevention policies with a focus on the maintenance of the health of its individual people. In this paper, we give a variety of examples for plant and food-technological related strategies that may contribute as small steps towards the reduction of the incidence of remarkable food-induced syndromes, specifically CD and NCWGS, caused by the consumption of one of our oldest and most frequently consumed foodstuffs worldwide.

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4.5 Ultra-low gluten barley

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Introduction

Coeliac disease (CD) is a condition that is estimated to affect about 1% of most populations worldwide [1] and at present the only treatment is a lifelong gluten-free diet with the exclusion of gluten-like proteins in wheat (gliadin and glutenins), barley (hordeins), rye (secalins) and in some coeliacs, oats (avenins) [2]. Untreated coeliacs face adverse health outcomes including low bone mineral density and increased intestinal malignancy [3]. Gluten-free diets are traditionally more expensive, low in fibre, high in fat and sugar, which in turn can lead to adverse health outcomes [4-6].

In addition to CD, a second condition known as non-celiac gluten sensitivity (NCGS) is thought to affect up to 10% of the population in some countries [7,8]. Little is known about NCGS, but it has been shown that for those who suffer NCGS a gluten-free diet is beneficial.

Barley contains four classes of hordeins, the B-, C-, D-, and γ-hordeins. The B- and C-hordeins are the major classes representing over 90% of the hordeins [9,10]. The B-hordeins and C-hordeins are encoded by the *Hor2* and *Hor1* loci, respectively, with both loci located on the short arm of chromosome 1. In the 1970s, the search for high lysine barleys at the Carlsberg Institute identified two mutants, Risø 56 and Risø 1508, which had reduced hordein content. Risø 56 does not accumulate B-hordeins due to the deletion of the *Hor2* locus [9], while Risø 1508 does not accumulate C-hordeins and this is the result of a mutation in the *Lys3* locus on chromosome 5H [11]. The D-hordeins are encoded by the *Hor3* locus, which encodes a single 105 k protein [12], located about 9 cM from the centromere on the long arm of chromosome 1H [13]. An Ethiopian derived landrace, R118 [14], contains a single spontaneous mutation which prevents accumulation of the D-hordeins. The γ-hordein family comprises two genes, *γ-1* and *γ-3 hordein* at the *Hor5* locus on the short arm of 1H which encode three γ-hordein proteins (1, 2, and 3) [15,16]. The γ-hordein loci are very tightly linked to the B-hordein locus at 0.2 cM [17,18].

We report the isolation of the first ultra-low gluten barley with hordein levels below the Codex limit of 20 mg/kg for gluten in gluten-free food. In addition, the grain shape and size have been improved and are similar to current malting lines. These new grains have utility in the preparation of malt, food and beverages for those who suffer from CD, wheat allergy and gluten intolerance.

Materials and methods

Barley lines cv. Sloop and Bomi (wild-types) were obtained from the Australian Winter Cereals Collection (Tamworth, Australia). The single hordein-null lines, Risø 56 and Risø 1508 were obtained from the Nordic Germplasm Bank (Alnarp, Sweden). A line which was null for D-hordein, Ethiopian R118 [14], was obtained from The John Innes Centre Public Collections, Norwich. The Ethiopian R118 line was a land-race observed to segregate for 2-row and 6-row phenotypes and for black pigmented and green seeds. A two-rowed, green seeded line was selected and backcrossed to Sloop to produce a BC2 D-hordein null line with 87.5% Sloop background.

Analytical methods

Plant analysis: Kernel weight, dimensions and screenings, a measure of seed size, were determined using a SeedCount^{TM} SC4 (Seed Count Australasia, Condell Park, Australia). Total flour nitrogen was determined by the method of Dumas [19]. Starch, β-glucan, α-, and β-amylase were determined by the methods of McCleary [20-23]. Free sugar composition was by the anthrone method [24]. Fatty acids were extracted and analysed according to the method of Zhou [25]. Free amino acids were determined by the Australian Proteome Analysis Facility (Macquarie University, Sydney) according to the method of Cohen [26] with norvaline (Sigma) as an internal standard [27].

Protein Analysis: Protein extraction, one and two dimensional SDS PAGE and ELISAs were carried out as described previously [28]. Mass spectrometry was conducted as described previously [29-31].

Results and discussion

Creation and analysis of ULG 3.0

Risø 56 and Risø 1508 were intercrossed to produce a line that lacked B- and C-hordeins and was called ULG 2.0. The hordein concentration was lowered further by crossing ULG 2.0 with the Ethiopian R118 line in a Sloop BC2 background to produce ULG 3.0, which lacked B- C- and D-hordeins. Analysis of the hordein content in these lines by ELISA (Tab. 1) revealed that it had been reduced to approximately 4 mg/kg in ULG 3.0. 2D-PAGE, in-gel digestion and MS/MS analysis of ULG 3.0 confirmed that the only detectable hordein in an alcohol soluble protein fraction from ULG 3.0 was γ-3-hordein (Uniprot: P80198) (data not shown).

Seeds of both ULG 2.0 and 3.0 were shrunken due to the pleiotropic effect of the *lys3a* gene derived from Risø 1508 [32] and had high levels of screenings. When grown in the field at Ginninderra Experiment Station, ACT, seed weight of ULG 2.0 was 33.5 ± 0.40 mg/ kernel, while ULG 3.0 had slightly larger seeds 39.2 ± 0.31 probably due to the contribution of cv. Sloop germplasm. The screenings also decreased dramatically from ULG 2.0 (96.2 ± 1.2% of grains <2.8 mm) to ULG 3.0 (41.3 ± 3.7% <2.8 mm).

Table 1. *Hordein content of ULG lines as measured by ELISA, values are mean ± SE, n=3.*

Line	Hordein content [mg/kg]
Sloop	56,600 ± 3,300
R56	33,300 ± 1,100
R1508	4,900 ± 260
ULG 2.0	1,670 ± 70
ULG 3.0	3.9 ± 0.7

Improving seed size, development of ULG 3.2

ULG 2.0 was crossed to cv. Sloop, Yagan and Baudin. F2 hordein double-null lines were identified in the three backgrounds 2S (Sloop), 2Y (Yagan) and 2B (Baudin) respectively, each containing 50% of the respective parental germplasm. These hordein double-null lines were crossed with a ULG 3.0 line named T2 and F2 hordein tri-nulls identified in the three backgrounds T2S, T2Y and T2B respectively. The hordein tri-null lines were intercrossed in pairs in both directions e.g. T2S x T2Y and T2Y x T2S, to form F1 lines, SY and YS, respectively, to create biparental lines, all with hordein tri-null phenotypes. The F1 lines were either selfed to create F2 bi-parental families or intercrossed and selfed to create segregating quad-parental families all with a hordein tri-null phenotype.

Through a field selection process over two generations the best 20 lines were identified. Progeny of these lines were carried through a further three generations of field selection to identify agronomically acceptable lines that produced seeds that were larger, with short, well-filled heads. The best two lines, 43.2 and 124.1, were selected and fixed by three rounds of single-seed descent. These lines had the lowest hordein levels, and improved seed weight (>47.2 mg), and improved screenings near that expected for commercial malting barley (3-14%).

Proximal analysis of ULG 2.0, 3.0, and 3.2

Starch, monosaccharides, β-glucan, free amino acids, and fatty acid content were also determined to establish whether removal of seed hordeins, a significant seed sink, impacted other components of the grain (Tab. 2).

The starch content of ULG 3.0 was lower than the controls Sloop and Baudin, while the two ULG 3.2 lines 043.1 and 124.1 had starch contents similar to the controls. There was no consistent trend in monosaccharide content between the lines with 043.2 and 124.1 having similar levels to that seen in Baudin. The protein content of ULG 3.0 was higher than that seen in the control lines, while the 043.2 and 124.1 had a lower protein content than the controls. Interestingly, the β-glucan content of the ULG 3.0 lines was significantly lower than that of the controls, with 043.2 and 124.1 having a

lower β-glucan content than ULG 3.0. The cause of this is unknown at present. The total free amino acid content in the ULG 3.0 lines was up to 15-fold higher than the levels in the controls and is likely to be a direct result of the absence of the hordeins and the inability of the lines to incorporate proline and glutamine, which are present in high concentrations in the hordein proteins, into other proteins in the absence of the hordeins.

Amylase activity

The level of α-amylase of the ULG 3.2 lines was not significantly different from the malting lines cv. Sloop, Baudin, Commander or Hindmarsh. Conversely, the level of β-amylase in the ULG 3.2 lines was reduced by approximately 10-fold compared to the malting lines above (data not shown).

Table 2. *Proximal analysis of grain components from the ULG lines.*

Line	Starch [% flour]	Mono-saccharides [% flour]	Protein [% flour]	β-Glucan [% flour]	Free amino acids [mg/g]
Sloop	69.5 ± 0.5	2.70 ± 0.19	12.7 ± 2.7	2.43 ± 0.02	3.63 ± 0.01
Baudin	67.8 ± 1.1	3.58 ± 0.16	11.6 ± 0.3	2.85 ± 0.15	1.60 ± 0.02
Hindmarsh	59.9 ± 0.6	2.69 ± 0.06	12.3 ± 0.5	4.24 ± 0.07	1.70 ± 0.03
Risø 56	75.5 ± 1.3	4.19 ± 0.19	10.0 ± 0.7	2.17 ± 0.01	5.67 ± 0.08
Risø 1508	65.9 ± 1.5	4.34 ± 0.54	11.5 ± 1.2	2.29 ± 0.03	5.22 ± 0.09
ULG 3.0	64.1 ± 0.6	2.65 ± 0.01	14.1 ± 0.2	1.10 ± 0.04	18.10 ± 0.63
043.2	71.1 ± 1.9	3.38 ± 0.10	10.9 ± 0.8	0.51 ± 0.03	18.56 ± 0.08
124.1	68.4 ± 1.5	3.60 ± 0.25	11.0 ± 0.7	0.76 ± 0.02	19.82 ± 0.25

Values are means plus or minus standard deviation, n = 3

Conclusion

We report the selection and breeding of the first ultra-low gluten barley. The hordein level was extremely low, to the point where it was difficult to measure even with sensitive mass spectrometry or ELISA. The grain had a normal appearance and malting and brewing properties sufficient to make a useful malting grain. This grain has application in the preparation of food and beverages for coeliacs and gluten intolerants.

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4.6 Developing a hordein standard for barley-containing products

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Introduction

The use of a gliadin reference material for barley-containing products in the prolamin quantification by R5 ELISA causes overestimation [1,2]. The difference in prolamin composition between wheat and barley, and the different reactivity of each prolamin group with the antibody are the main reasons. To obtain an accurate value, a more specific reference material is needed when measuring 1) prolamin content in gluten-free barley starch; 2) prolamin content in gluten-free beer; 3) barley contamination in oat products. In this study, we investigated barley cultivars from several regions of origin, and analysed their behaviour in R5 ELISA quantification and their prolamin composition.

Materials and methods

A collection of common barley cultivars from Nordic countries, central Europe and North America was kindly provided by Boreal Plant Breeding Ltd., Finland. After water and 0.5 mol/L NaCl extraction of milled grains, the prolamins were extracted by 40% (v+v) 1-propanol with 1% (w+v) dithiothreitol (DTT), 30 min at 50 °C. To study the prolamin composition, prolamins of barley cultivars were separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% Bis-Tris, NuPage, Invitrogen). The gel was stained by Coomassie Brilliant Blue R-250 for 1 h, and destained for 2 h with 7.5% (v+v) acetic acid and 10% (v+v) ethanol. The gel was visualised by AlphaImager HP system (ProteinSimple, California, USA), prolamin bands were detected and the density was calculated.

The milled grains were also extracted by RIDA Extraction Solution (colourless) (R-Biopharm, Darmstadt, Germany). After optimised dilution, the prolamins were measured by sandwich R5 ELISA (R7001 RIDASCREEN Gliadin, R-Biopharm). In order to get the actual protein content, the RIDA extraction solutes were dialysed against water to remove the reagents prior to analysis, then dried and analysed by the Dumas method (nitrogen content × 6.25).

Results and discussion

From SDS-PAGE, the hordein pattern of cultivars can be divided into three groups by size, D-hordein, C-hordein, and B + γ-hordein (Fig. 1). When protein bands were taken for gel densitometry, we could compare the proportion of each hordein group among cultivars. The D-hordein size and proportion was very similar among different cultivars, around 10% of total hordein. However, C-hordein composition was different. For example, cultivar 79, 80, and Vilde had more bands than cultivar 88, 89, and Harbinger. The reported C-hordein composition was 10-20% of total hordein [3]. However we found that some cultivars had a C-hordein content around 30% of total hordein (Tab. 1, cultivar 51 and Scarlett). The R5 antibody recognized ω-type of prolamin; in the case of barley, the R5 antibody recognized C-hordein better than other hordein groups.

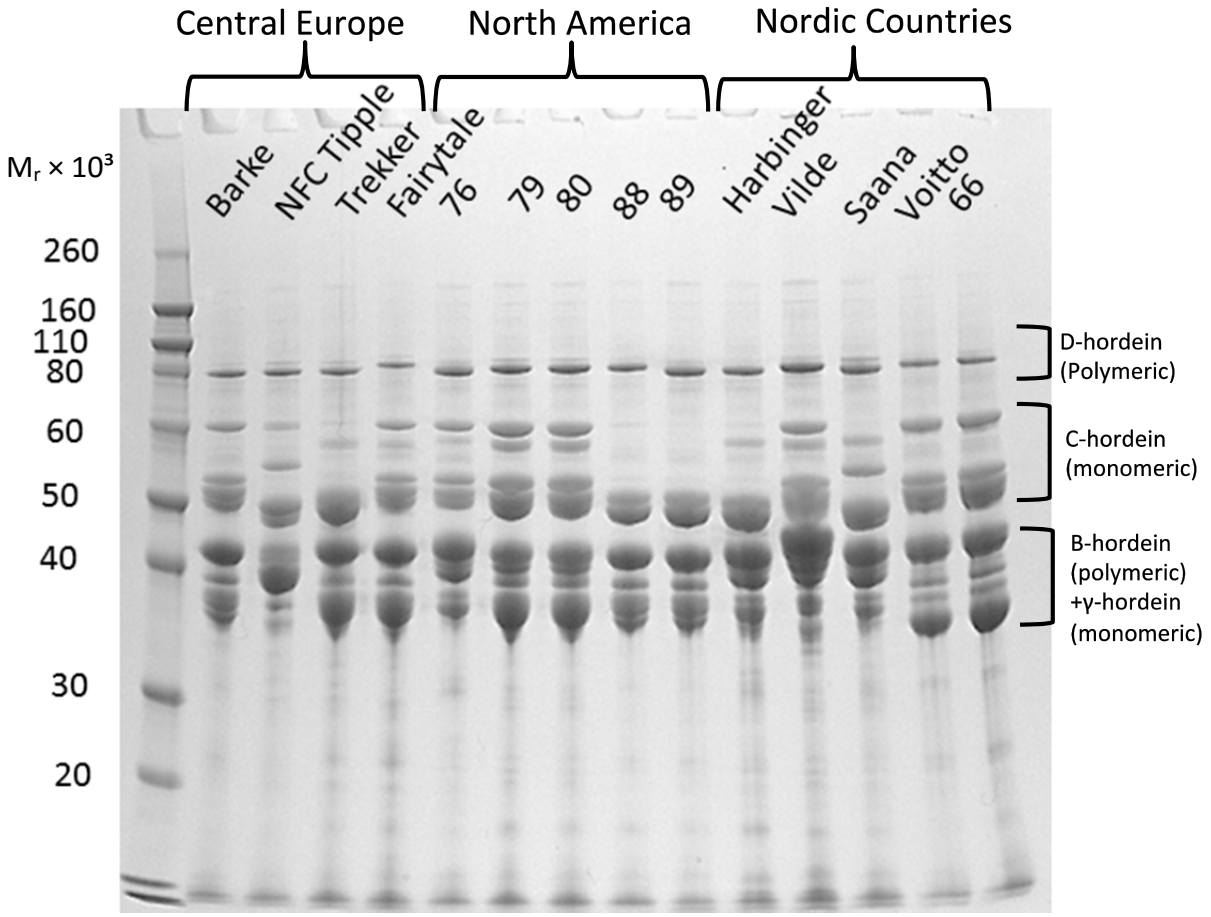

Figure 1. *SDS-PAGE of barley hordein of selected barley cultivars originating from Central Europe, North America, and Nordic countries*

Our hypothesis was that the high proportion of C-hordein was the reason for overestimation of barley prolamin quantification by the R5 antibody. When the C-hordein proportion was around 15% of total hordein, like ω-gliadin 10-20% of total gliadin in wheat (Shewry and Tatham, 1990), the response matched with the gliadin

standard (cultivar KWS Asta and Overture in Fig. 2). However, when looking at the distance between actual prolamin concentration and gliadin standard curve, the distance was greater at higher C-hordein proportion, which means more overestimation. The primary structure of C-hordein, composed of repetitive sequences of PQQPFPQQ, is one plausible cause of this overestimation. The R5 epitope QQPFP appeared 13 times in C-hordein, but only 5 times in B1-hordein and 3 times in B3-hordein. Other epitopes QQPYP and QLPFP, which are also recognised by the R5 antibody, appear once in C-hordein. When measuring pure C-hordein with R5 ELISA, the concentration was 15-20 times overestimated in the sandwich ELISA with gliadin standard, and 23-43 times overestimated in the competitive ELISA with hydrolysed prolamin standard (Huang et al., 2016). ω-Gliadin also contains a high number of epitope repeats (QQPFP×14, QLPFP×1, QQPYP×4, QQSFP×3), but the ω-gliadin proportion of whole gliadin does not vary that much among cultivars.

Table 1. *Hordein composition of selected barley cultivars.*

Origin	Cultivar	C-Hordein [%]	D-Hordein [%]	B+γ-Hordein [%]
Europe	KWS Asta	15.2	10.7	74.1
Europe	Overture	16.5	7.4	76.1
Nordic	Minttu	22.8	10.8	66.4
Nordic	51	28.2	7.9	63.9
Europe	Scarlett	31.8	11.6	56.6

We propose a hordein reference material from only one barley cultivar, instead of a combination of many cultivars. This cultivar should have an average C-hordein proportion, based on cultivars cultivated in the region. For example, the barley cultivars in Finland seem to have a higher C-hordein proportion than the ones in Central Europe, probably due to the climate influence. The one cultivar-based hordein reference material can be used to measure the prolamin content in barley starch with sandwich ELISA. The hydrolysed hordein can serve as a reference material in the competitive ELISA for measuring the prolamin content of barley-based beer. Another application of the hordein standard is to measure the barley contamination in oat products. However, this situation is more complicated and needs to be combined with other techniques, like mass spectrometry, to identify whether the contamination comes from wheat or barley beforehand.

Conclusions

The C-hordein proportion in barley cultivars varies much more than ω-gliadin in wheat. In general, the relative proportion of ω-type prolamin is higher in barley than in wheat. It is one plausible reason for overestimation of gluten in barley-containing products if a (wheat) gliadin standard is used in the gluten assays. We propose a

hordein reference material from one cultivar, which contains an average C-hordein proportion of total hordein in cultivars used in one region. Each region may have their own reference material, based on their most common cultivars.

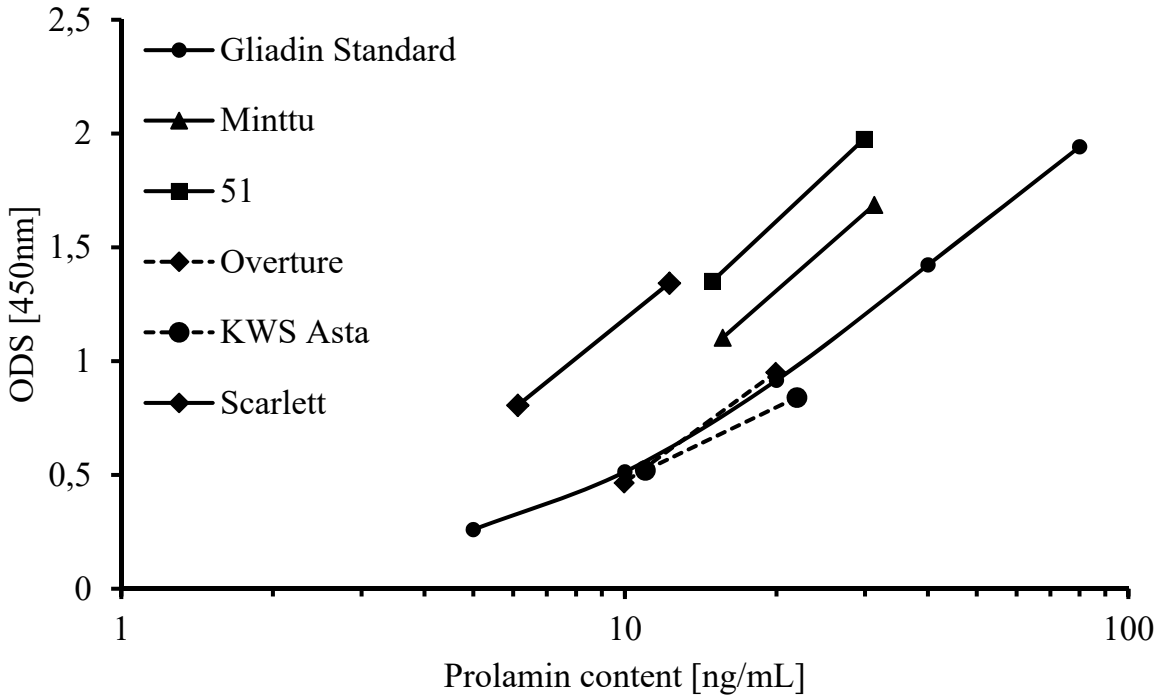

Figure 2. *The RIDA extraction solutes response in the R5 sandwich ELISA. The prolamins were extracted by RIDA extraction solution, the actual prolamin content was measured by Dumas nitrogen content × 6.25*

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5 Clinical research reports

5.1 Evaluation of diet composition and food habits in the Spanish coeliac population

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Introduction

The only treatment for coeliac disease (CD) consists of following a gluten-free (GF) diet and the ingestion of small amounts of gluten can cause major disruptions in gluten intolerants. Thus, an increasing interest in GF foodstuffs has arisen in recent years and the GF product market has become one of the most profitable markets in the field of food and beverages.

Although the target of this market has focused on the absence of gluten in GF foods, the nutritional quality of GF foodstuffs is an important aspect to consider [1]. Some authors have demonstrated that 20-38% of coeliac patients [2,3] show some nutritional deficiencies due to an inadequate intake of calories/protein [4], dietary fibre [5-7] or various micronutrients [7-9].

The correct GF diet should fulfil all energy and nutrient requirements and prevent individuals from any nutritional deficiency. Sometimes, following a GF diet implies an excessive restriction of cereal consumption in order to avoid gluten intake. This circumstance may lead to a low carbohydrate intake with an excess of fat and protein. Indeed, some studies highlight excessive saturated fats in GF products [5,10]. With regard to micronutrient intake, it has been shown that following a GF diet could also result in low intake of fibre and some micronutrients such as iron, folate, niacin, and zinc. Other authors also indicate that consumption of refined grains, which are used in the manufacture of processed GF products, entails a lower intake of B group vitamins, D vitamin and calcium [11].

In view of the above, the aim of the present work was to evaluate the possible consequences of following a GF diet in the nutritional requirements of coeliac patients. For this purpose, we evaluated the diet composition of Spanish coeliacs and measured their compliance with a balanced and healthy diet. Furthermore, we studied the nutritional composition of GF-rendered foodstuffs to highlight any difference with equivalent foods containing gluten.

Material and methods

Nutritional composition of GF-rendered foodstuffs and comparison to equivalent products with gluten

Nutritional information available on labels was analysed from GF-rendered foods (n = 206) as well as that of equivalent products containing gluten (n = 289). Foodstuffs were classified into different groups using the reference given by Gibert *et al.* [12].

Information about energy content, total and simple carbohydrates, proteins, total and saturated lipids, fibre, sodium, salt and cholesterol was collected. These data were included in a spreadsheet, and the nutritional composition of GF-rendered foods was compared to that of products containing gluten.

Dietary Assessment

Data from a female coeliac cohort recruited in 2011 from 3 regions in the north of Spain (Alava, Gipuzkoa, and Bizkaia) were used in the present study (n = 54, mean age ± SD: 34 ± 13). All participants had been compliant with the GF diet for at least one year, as previously described [13].

24-hour food recall for 3 days (two weekdays and one weekend day) and a food frequency questionnaire were collected for each woman. Food servings and amounts were determined using photographs and nutrient intake was calculated by a computerized nutrition program system (AyS, Software, Tandem Innova, Inc.).

The nutrient content of the specific GF products destined for coeliac people was collected from the manufacturers and added into the food composition database before calculations. As GF product labels did not indicate micronutrient contents (vitamin and minerals), an estimation with homologous products containing gluten was made [14].

Energy, nutrient and food intake of coeliac women was compared to that of a reference Spanish women population (data from ENIDE study) [15].

Statistical analysis

Statistical analyses were performed by using the IBM SPSS statistical program 19 (IBM Inc., Armonk, NY, USA). The results for continuous variables are given as the arithmetic mean ± SD and the range, whereas results for non-continuous variables are given as the frequency and the percentage. Statistical analyses were performed with Student's *t* test. P values <0.05 were accepted as significant.

Results and discussion

Nutritional composition of GF-rendered foodstuffs and comparison to equivalent products with gluten

When the composition of GF-rendered foodstuffs was compared to that of gluten-containing products significant differences were found (Fig. 1).

The group of GF breads and pasta had almost a third less protein than the equivalent gluten-containing foods ($p<0.001$). This fact has also been recently reported by others [16]. Besides, and in good accordance with Segura *et al.* [1], GF breads provided twice as much fat ($p=0.001$), mainly in the form of saturated fat. Additionally, the pasta group in GF products had declared more sodium and less fibre, as observed previously by our group in the case of cereal bars or flour [14].

Some of these nutritional differences could be based on the list of ingredients used. It is common for starches, hydrocolloids, gums, enzymes and other protein sources (soybean, amaranth, quinoa, etc.) to be used to produce GF products in order to improve their viscoelasticity and some other nutritional aspects [17,18]. The ingredients added for the optimization of texture and/or palatability could explain the higher fat intake, whereas the lower fibre intake might be due to qualitative or quantitative differences among bread and cereals in a GF diet.

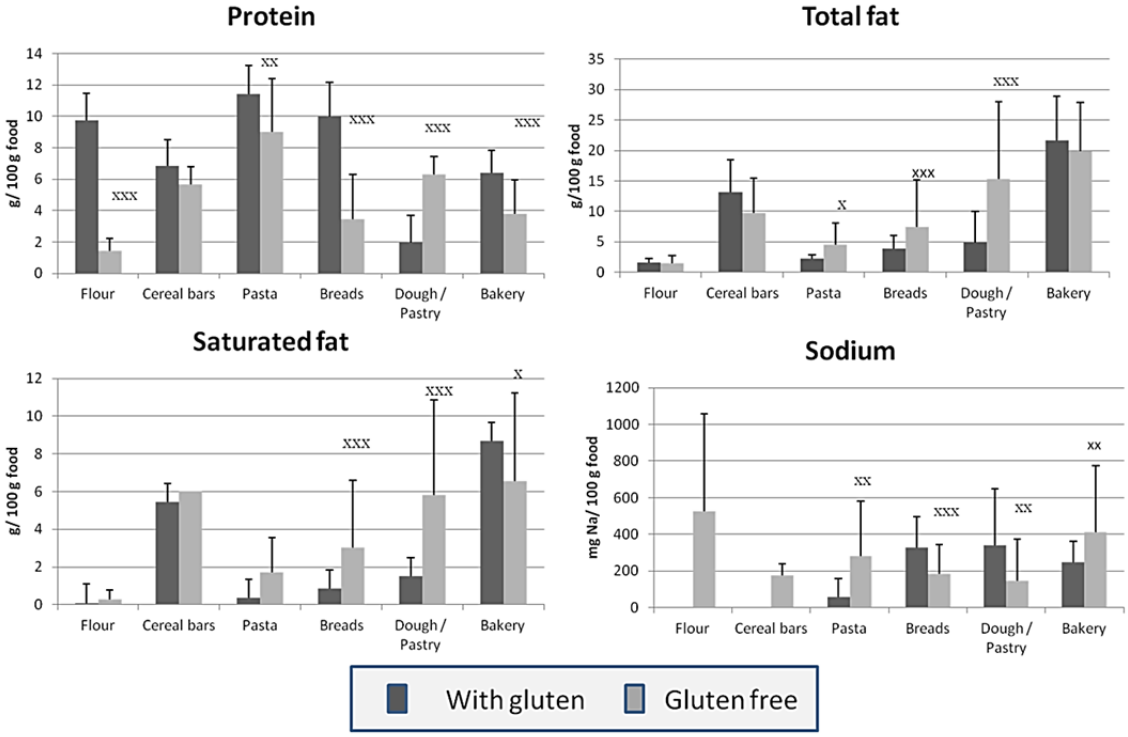

Figure 1. *Protein, total and saturated fat, and sodium label content per 100 g of product in gluten-containing and GF-rendered foodstuffs classified by food groups. Results are given as means with the positive error, shown by vertical bars. (x $p<0.05$, xx $p<0.01$; xxx $p<0.001$)*

Dietary Assessment

The average energy intake of coeliac women was in good accordance with their estimated energy expenditure, but was slightly lower than the dietary intake recommendations [19], which is similar to that observed in the Spanish female population in ENIDE studies [15]. Furthermore, when energy distribution was analysed, no differences were found between both groups (Tab. 1).

Excessive protein intake (17.3% of total energy intake) in coeliac women was due to a high consumption of animal protein (69% of total protein). With reference to this, an excessive consumption of meat was seen in more than three quarters of participants.

Carbohydrate (CHO) consumption (41.9% of total energy intake) was enough to cover the minimum established as Recommended Dietary Allowance (RDA) (130 g/day) by the Institute of Medicine of The National Academies (IoM) [20], but it was far from being the 45 to 60% of total energy proposed as the dietary reference value (DRV). Nevertheless, it should be pointed out that other research conducted with coeliac women members of the German Coeliac Society showed more adherence to the carbohydrate consumption (45.9% of total energy) [21].

With regard to lipid intake, the percentage of this macronutrient markedly exceeded the recommendations (Tab. 1), established as 30 to 35% of total energy. When fibre consumption was analysed, the coeliac group consumed a significantly lower daily amount than Spanish women from the ENIDE survey (16.4 ± 5.6 *vs* 18.9 ± 10.1; $p<0.001$). According to our data, coeliac women older than 45 years (n = 13) demonstrated better adherence to the Mediterranean diet and this was reflected in fibre consumption. This group consumed on average 19.0 ± 5.1 g/day while the registered intake in young adult coeliac women (younger than 45 years, n = 41) was 15.6 ± 5.5 g/day ($p=0.005$).

Table 1. *Energy and nutrient distribution in coeliac women and Spanish women (control women, ENIDE study).*

	Coeliac women (n=54)	**Spanish women -control women- (n=1734)**	***p***
Energy (kcal)	1847 ± 362	2038 ± 655	*0.003*
Protein (g)	79.2 ± 16.6	88.0 ± 37.8	*<0.001*
% total energy as protein	17.3	17.7	
Carbohydrate (g)	192.3 ± 40.7	199.7 ± 75.9	*0.002*
% total energy as CHO	41.9	40.1	
Fat (g)	84.6 ± 23.0	93.2 ± 35.6	*0.022*
% total energy as fat	40.8	42.2	

Notes: Values are means ± SD. Spanish adult women data were taken from the Spanish dietary nutritional assessment (ENIDE study, representative at national level of the adult population). CHO, carbohydrate; SD, standard deviation

As we previously reported [13], food consumption frequency data (Fig. 2) showed that cereal intake was very low in nearly all women. In fact, 48% consumed fewer than two servings per day and only 7% of coeliac women fulfilled cereal recommendations (4-6 servings per day). With regard to the source of cereals in the diet, most of the cereal

derivatives were naturally GF grains and not specifically GF-rendered products (3% of total energy intake).

The majority of participants, 70%, did not reach vegetable consumption recommendations (10 portions/week) [22], and one third of coeliac women did not consume the minimum recommended fruit quantity, two servings daily.

In connection with animal source foodstuffs, it is important to mention that consumption of meat and meat products was excessive in a large population of coeliacs (>75%), and dairy servings were also fulfilled in a large percentage (87%).

Regarding the consumption of legumes, 65% of coeliac women usually included two or more portions per week. When comparing these data with the Spanish general population [23], a great difference between the groups was observed (19.3 g/d *vs* 11.9 g/d). The strategy followed by coeliac people consisted of avoiding gluten in the diet, diminishing cereal intake and promoting the consumption of legumes instead of cereals [24].

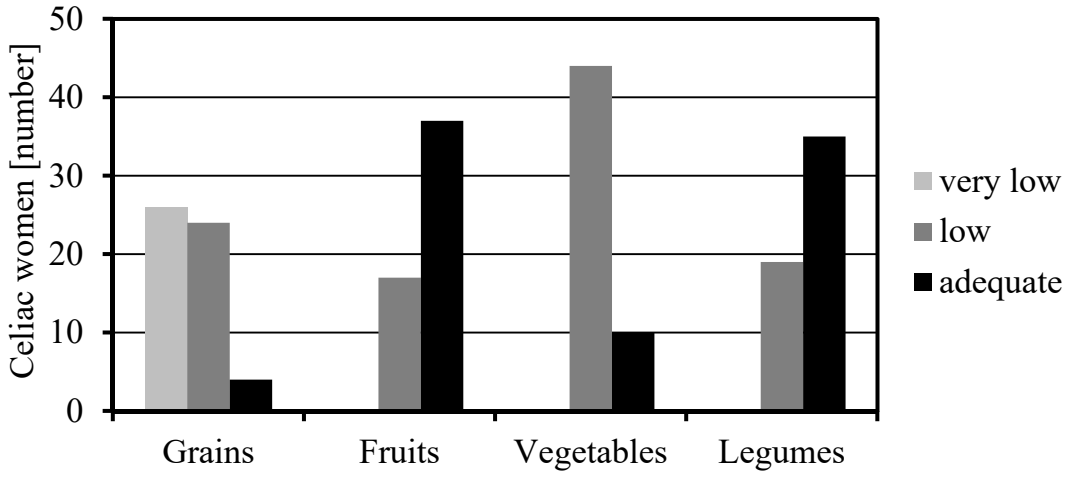

Figure 2. *Food frequency consumption (classified as very low, low or adequate) of coeliac women, expressed as number of women, according to Spanish Society of Community Nutrition (SENC) recommendations (grains: very low: <2 servings/day; low: 2-4 servings/day; adequate: >4 servings/day; fruits and vegetables: low: <2 servings of each per day; adequate: >2 servings of each per day; legumes: low: <2 servings/week; adequate: >2 servings/week)*

Conclusions

Considering the dietary assessment of coeliac adult women, it could be postulated that coeliac women and Spanish women -control women- follow similar trends as a whole, with an imbalanced distribution of macronutrients and an inadequate consumption of certain dietary components. Food frequency analysis suggests that some specific

nutritional education might be provided to coeliac people in order to improve eating habits and the nutritional status among adult coeliac women.

Furthermore, according to our results, there are marked differences between following a diet with GF products and a diet with gluten-containing products.

Taking into account the composition of GF-rendered foods, it might be thought that new ingredients based on vegetable sources would be helpful in improving these nutritional values. Additionally it is possible to draw the conclusion that in order to avoid wrong nutritional estimations, the different nutritional composition of products rendered GF demands specific diet design software containing a database of GF product compositions. As stated in the material and methods section, at the time when the present research was carried out, no software contained such a database.

In order to fill this gap, our group has recently developed appropriate software for the nutritional evaluation of a GF diet. This tool is available on www.ehu.eus/es/web/laboratorio_gluten/softwareak.

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5.2 Innate immune signalling pathways induced by gliadin peptide p31-43

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Introduction

Several studies have shown that a set of gluten-derived peptides are immunogenic and induce a potent gluten-specific $CD4^+$ T-cell response in the small intestinal mucosa in CD patients [1]. Particularly, transglutaminase 2 converts native into deamidated gluten peptides, which bind to HLA-DQ2 and -DQ8 molecules with higher affinity, thus enhancing T-cell activation [2]. On the other hand, some gluten peptides may mediate non-HLA related effects, which could lead to innate immune activation [3]. However the signalling pathways of innate immune activation by gluten peptides remain unclear.

The α-gliadin peptide, p31-43, which does not bind to HLA-DQ2/DQ8, has been used as a peptide model to study the innate responses that may be relevant in CD [3]. The described effects of p31-43 include apoptosis induction [4], upregulation of stress-inducible MHC class I polypeptide-related molecules (MICA) [5], and inhibition of epidermal growth factor receptor (EGFR) endocytic pathways [6]. P31-43 also delays traffic of EGF-carrying vesicles and late endosomes maturation in epithelial cells [7].

Most of the knowledge on the effects of p31-43 arises from *in vitro* studies using cell lines, duodenal biopsies or cells isolated from human samples [3]. To date, there is no evidence that p31-43 exerts any innate immune activation *in vivo*. Previous studies developed by our group have shown that p31-43 triggers enteropathy in a murine model that develops reduced villus height/crypt depth (V/C) ratio, infiltration of intraepithelial lymphocytes (IELs), increased cell death in the *lamina propria* and proliferative activity on the crypts [10].

Aims

Thus, the aim of this study was to evaluate the *in vivo* signalling pathways involved in innate immune activation by p31-43.

Materials and methods

Six to eight week old C57BL/6, MyD88$^{-/-}$, IFNαR$^{-/-}$ and C3H-HeJ mice received an intraluminal injection, by a microsurgical procedure, of p31-43 (100 μL, 100 μg/mL, 10 μg per mouse) or phosphate-buffered saline (PBS), following a procedure previously described [10, 11]. Mice were sacrificed 16 h post-surgery. Small intestinal samples were collected and fixed with formalin. 5 μm Paraffin-embedded sections were obtained, re-hydrated and stained with H&E.

Histological changes were evaluated using the V/C ratio and the number of IELs. Villus and crypt length ratio were measured on H&E stained intestinal sections. Pictures were obtained with a Nikon Eclipse Microscope and morphometric analysis was performed using a properly calibrated, image processing program (ImageJ). IELs were counted on 10 randomly chosen villus tips and results were expressed as IEL/100 enterocytes. For the statistical analysis an unpaired t-test and ANOVA were used.

Results and discussion

Inflammatory changes induced by p31-43 are myeloid differentiation primary response gene 88- (MyD88) dependent

In vitro studies have demonstrated that induction of proinflammatory cytokines and increased intestinal permeability driven by p31-43 are MyD88-dependent [12]. After intraluminal administration of p31-43 in C57BL6 mice, we observed intestinal damage, characterised by reduction in V/C ratio and increment in the numbers of IELs (Fig. 1A). We then tested whether MyD88, a key adaptor molecule in the TLR/IL-1R signalling pathways, is also involved in the effect induced by p31-43 *in vivo*. V/C ratio and number of IELs in MyD88 KO mice treated with p31-43 were similar compared with PBS-controls (Fig. 1B). Thus, MyD88 is critical for the *in vivo* development of small bowel inflammation in this model.

p31-43 effects are toll-like receptor 4- (TLR4) independent

TLR4-defective C3H/HeJ mice were used to evaluate whether TLR4 signalling pathway is involved in p31-43 induced effects. The morphometric analysis demonstrated reduction of V/C ratio after intraluminal injection of p31-43 in C3H-HeJ mice (Fig. 2) excluding a role for TLR4 signalling pathway.

Inflammation by p31-43 is type I interferone- (IFN) dependent

Type I IFNs may be implicated in the pathogenesis of CD [1]. To evaluate whether type I IFNs are involved in the mechanism of damage in our model, type I IFN receptor (IFNαR) KO mice were intraluminally injected with p31-43 and morphometric parameters assessed in intestinal sections. No changes in V/C ratio or IELs were observed in IFNαR KO mice treated with p31-43 compared with PBS-treated mice (Fig. 3) suggesting that type I IFN signalling pathway may play a key role in the inflammatory changes induced by p31-43.

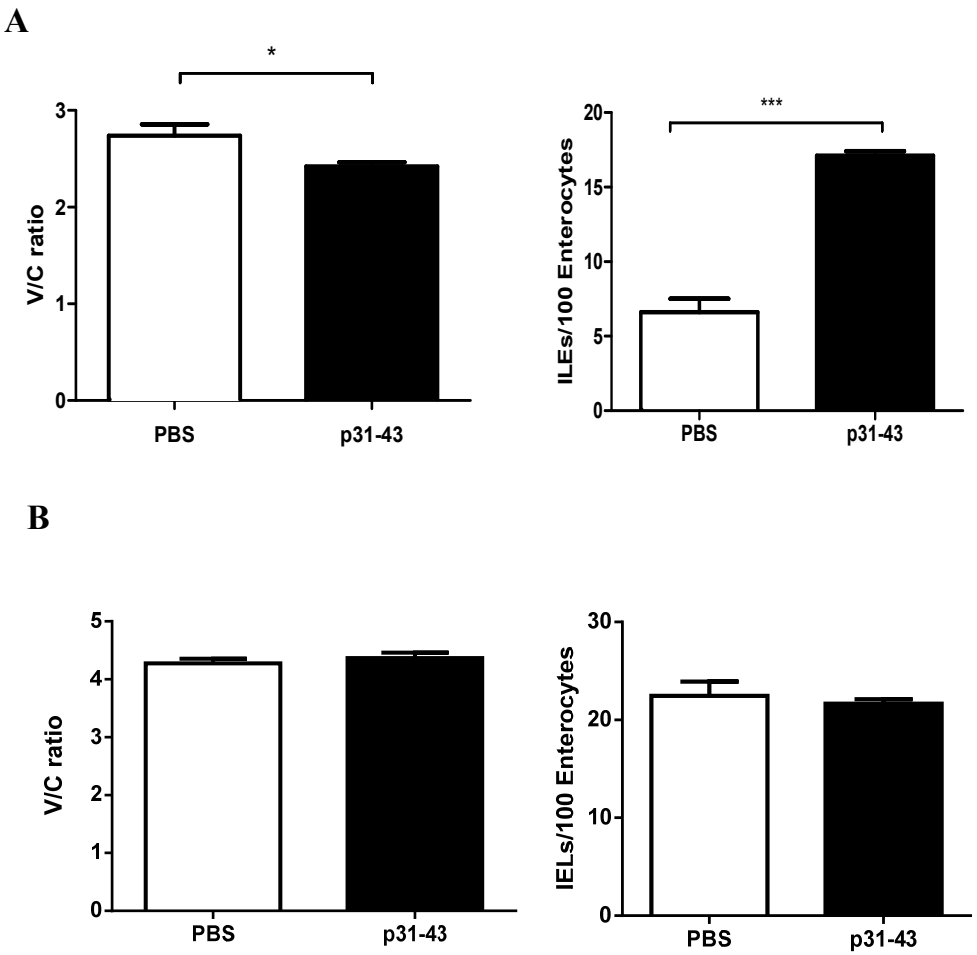

Figure 1. *Mucosal changes induced by p31-43 are MyD88-dependent.*
*Evaluation of V/C ratio and number of IELs in small intestine sections obtained from C57BL/6 wild type mice (A) and MyD88KO mice (B), 16 h post intraluminal injection of PBS or p31-43. N = 5 mice per group, ANOVA and unpaired t-test *p<0.05, ***p<0.001 were performed*

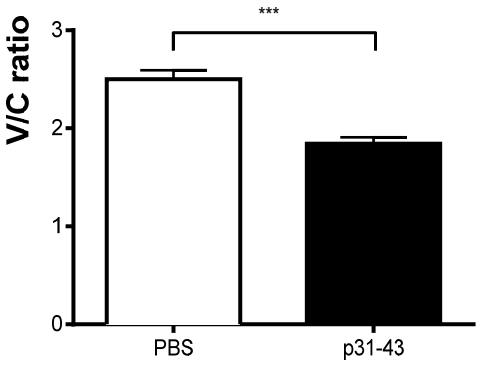

Figure 2. *Mucosal changes induced by p31-43 are TLR4-independent.*
*Evaluation of V/C ratio in small intestine sections obtained from C3H-HeJ mice 12 h after intraluminal administration of PBS or p31-43. N = 4 mice per group, unpaired t-test ***p≤0.001*

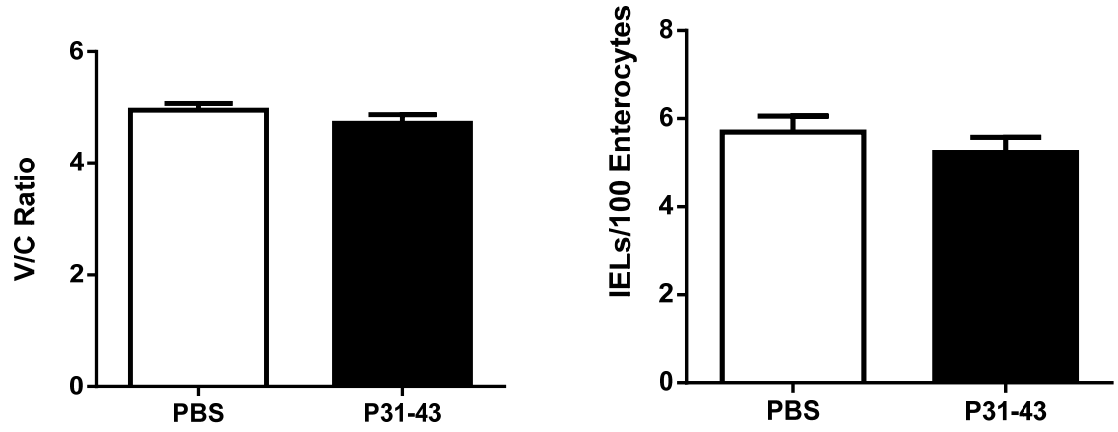

Figure 3. *Mucosal changes induced by p31-43 are type I IFN dependent. Evaluation of V/C ratio and IELs number in small intestine sections obtained from IFNαR KO mice, 16 h post intraluminal injection of PBS or p31-43, n = 5 mice per group. ANOVA was performed*

Conclusions

Our results confirm the previous observation that intraluminally delivered p31-43 causes inflammation in an experimental murine model and expands those observations showing that MyD88 and type I IFN signalling are required for the *in vivo* effects observed. Though a receptor for the p31-43 has not been identified yet, our results indicate that MyD88 and type I IFN participate in innate immune activation *in vivo* leading to inflammation and epithelial damage. It is possible that activation of these signalling cascades contributes to intestinal inflammation, tissue remodelling and cell death which could impact on the development of enteropathy in a genetically susceptible host.

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5.3 The prospective multicentre trial of antibody diagnostics in paediatric coeliac disease (AbCD): Status update after registration of 835 children

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Introduction

Already in 2012, the ESPGHAN proposed a biopsy-sparing guideline [1] for diagnosis of coeliac disease (CD) and stressed that the performance of these guidelines in clinical practice should be evaluated prospectively. Since then, a number of papers have been published on this topic, however, all of them describe retrospective studies [2-8]. Retrospective studies have serious limitations. Most of them were performed in coeliac-enriched populations with a high prevalence of CD and controls are sometimes missing completely. The patients included in these studies were preselected by previous antibody assays. In most cases, only the classical IgA antibodies against tissue transglutaminase (IgA-anti-TTG), were evaluated, but the performance of IgG antibodies against deamidated gliadin peptides (IgG-anti-DGP) were not considered.

In October 2012, the prospective multicentre trial of antibody diagnostics in paediatric coeliac disease (AbCD) was launched [9]. The primary objective of this trial is to validate a previously published [8] diagnostic cascade in patients with clinical suspicion of CD in which many biopsies can be avoided if antibody results are sufficiently clear. The basic trial flow and a blinded status report were already presented one year ago [10]. Here, after registration of 835 children, an update is given.

This is an ongoing trial and antibody results, clinical symptoms, duodenal histology and diagnosis can only be correlated after the last patient has finished the trial. Thus, after description of the current status of patient registration we will confine our report to the comparison of antibody test results from test kits of different manufacturers and on the course of antibody concentrations during follow-up.

Current status

From the 835 patients registered (key date September 14, 2015), an endoscopy was already documented from 778 children. Of them, 630 children have already been diagnosed. Currently, there are 393 CD patients, 228 controls, and 9 “unclear”

children, in whom a decision "CD: yes or no?" could not be made. The prevalence of CD was 62.4%.

Comparison of antibody test results from test kits of different manufacturers

The problem of differences between the antibody test kits was already addressed by the ESPGHAN [1]. The authors concluded in the appendix of their paper that "these results are regarded as an example for the characteristics of different tests and final conclusions could be drawn only from more systematic studies or from a longer survey".

In our trial, 7 different test kits for measurement of IgA-anti-TTG were applied in the local trial centres at the time of endoscopy and after follow-up. We compared the results obtained by the five most commonly used local tests with the results of the test of EUROIMMUN performed centrally (Tab. 1). We asked in how many cases the centrally performed trial test was above and the local test below the company cut-off and vice versa. The second question was in how many cases the centrally performed trial test was above and the local test below a higher cut-off. As higher cut-off, 10 times above the upper limit of normal (10 x ULN) was used.

Table 1. *Comparison of results of different test kits measuring IgA-antibodies towards tissue transglutaminase.*

	Local tests[a]				
Number of cases with results of	**Ph**	**GA**	**EI-L**	**ES**	**QL**
trial test below and local test above 1 x ULN	0	0	2	0	1
trial test above and local test below 1 x ULN	18	14	3	6	3
trial test below and local test above 10 x ULN	1	0	2	0	0
trial test above and local test below 10 x ULN	21	22	4	15	2
Total number of cases	242	75	57	39	41

Local tests performed in blood samples withdrawn less than three days before taking the blood for the trial test. [a] Abbreviations used: EC-C = EUROIMMUN test centrally applied as standard trial assay; EI-L = EUROIMMUN test locally applied in the trial centres; ES = Eurospital; GA = Generic Assays; Ph = Phadia (Celikey); QL = Quanta Lite; ULN = upper limit of normal

Our data confirm the view that comparison of results obtained by different test kits is problematic [11]. Therefore, each test kit has to be calibrated individually for its performance at different cut-offs.

Course of antibody concentrations during follow-up

There were 362 children with complete data sets for IgA-anti-TTG and IgG-anti-DGP after follow-up (key date September 14, 2015). Of these, 334, 266, or 257 children had initial concentrations of IgA-anti-TTG, IgG-anti-DGP, or of both antibodies, respectively, above the cut-off. During follow-up (17 to 507 days, median 109 days),

the antibody concentration decreased below the cut-off in 35.0% of the children for IgG-anti-DGP, but in only 15.3% for IgA-anti-TTG (Tab. 2).

Alternatively one can calculate how often the concentration was reduced by more than half of the initial value. The proportion of these patients was much larger (87.7% for IgA-anti-TTG and 70.0% for IgG-anti-DGP).

Table 2. *Decrease in antibody concentration during follow-up.*

	Children[a] with antibody concentration				
	Initially	**After follow-up**			
	Above cut-off	**Below cut-off**		**Reduced by more than half**	
	n	n	%	n	%
IgG-anti-DGP	266	93	35.0	186	70.0
IgA-anti-TTG	334	51	15.3	293	87.7
IgG-anti-DGP + IgA-anti-TTG	257	17	6.6	177	68.9

[a] Antibody data of all children with follow-up considered, some of these may not be coeliac patients

Conclusions

The final aim to include more than 900 children in the trial by the end of 2015 is achievable. Antibody data will be correlated with clinical symptoms, duodenal histology, and diagnoses only after the last patient has passed through the trial. Therefore, no results on the diagnostic performance of antibody assays can be presented now. Nevertheless, our data confirm the view that comparison of results of antibody assays obtained by different test kits is problematic. Special efforts for standardization of antibody assays are necessary. Thus, the data expected in the trial can only be valid for the trial test kits.

For detection of serological remission, the ESPGHAN suggests waiting “until the antibody titres fall below the cutoff” [1]. Our follow-up results show that it may be better to consider the relative instead of absolute antibody concentrations. We should keep in mind that earlier detection of serological remission spares additional later blood withdrawals! The concentration ratio indicates remission earlier than waiting for “negativity”. For calculation of a concentration ratio, however, identical tests for initial and follow-up measurement have to be used. Additionally, high antibody concentrations have to be measured exactly and not expressed as concentrations above measuring range (or out of range). If no initial values with the same antibody test are available, IgG-anti-DGP may be a better indicator for remission than IgA-anti-TTG.

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5.4 Liver tissue as an alternative substrate for endomysium antibody testing in diagnosis of paediatric coeliac disease

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Introduction

Immunofluorescence assays of antibodies against endomysium (EmA) still represent the gold standard in the serological testing for coeliac disease (CD). EmA are traditionally detected on oesophagus sections of primates. EmA bind to the connective tissue layers around the smooth muscle fibres of the *Lamina muscularis mucosae* and *Tunica muscularis* of oesophagus sections [1]. Other tissues, for instance human umbilical cord, were proposed as alternative substrate but became not accepted [2]. Nevertheless, EmA were gradually replaced in the last decade by ELISA tests detecting IgA and/or IgG antibodies against tissue transglutaminase (IgA-tTG or IgG-tTG), which is the main autoantigen in the connective tissue of oesophagus [3]. Currently, IgA-EmA are enjoying a diagnostic renaissance due to the recommendation as a confirmatory test in the ESPGHAN guidelines for diagnosis of CD [4].

The widespread tissue transglutaminase is also abundant in the endothelia of nearly each tissue [5]. Therefore, liver seems to be good substrate due to the high number of blood vessels/capillaries, called sinusoids. It is notable that some laboratories already use liver instead of oesophagus for EmA detection. But there is currently no study, which examined systematically the application of liver for EmA detection. Therefore, we analysed a retrospective dataset, in which liver tissue was used in parallel to oesophagus as substrate for EmA.

Materials and methods

We analysed serum from 872 children (298 CD patients and 574 disease controls, 379 boys and 493 girls, mean age 8.9 years, range 0.3 to17.9 years). The patients were recruited consecutively from six centres in Germany and Austria between 2004 and 2013. All patients were biopsied under gluten-containing diet due to suspicion of CD or other gastrointestinal disorders. The diagnosis of CD was considered confirmed in patients with Marsh 2 lesions and higher. Serum was collected around the time of duodenal biopsy under gluten-containing diet and was stored until measurement at -20°C. The study was approved by the ethical committee of the University of Leipzig and of the local ethical committees of the participating centres.

Sera for detection of IgA-EmA and IgG-EmA were diluted as described by the manufacturer (1:10 to 1:10,000) and incubated in parallel on commercial slides with acetone-fixed cryostat sections of monkey oesophagus and liver tissue from EUROIMMUN Medizinische Labordiagnostika AG (Lübeck, Germany). The cut-off was ≥1:10, respectively. All further incubation and wash-steps were performed as described in the test manual. Immunofluorescence patterns were estimated by using an Eurostar Plus microscope with Bluelight LED (EUROIMMUN Medizinische Labordiagnostika AG, Lübeck, Germany). Assessment of EmA titre on oesophagus and liver was performed consecutively by experienced observers and blinded to diagnosis and histology. IgG-tTG was also tested in all sera as described by the manufacturer (EUROIMMUN Medizinische Labordiagnostika AG, Lübeck, Germany).

Table 1. *Performance of IgA-EmA on oesophagus and liver tissue sections.*

	IgA-EmA		***p***
	Oesophagus	**Liver**	
TP	282	284	
TN	563	558	
FP	11	16	
FN	16	14	
Sensitivity ($CI_{95\%}$)	0.946 (0.913-0.968)	0.953 (0.921-0.973)	*0.71*
Specificity ($CI_{95\%}$)	0.981 (0.965-0.989)	0.972 (0.954-0.983)	*0.32*
$PPV_{34\%}$ ($CI_{95\%}$)	0.962 (0.932-0.980)	0.947 (0.913-0.968)	*0.95*
$NPV_{34\%}$ ($CI_{95\%}$)	0.972 (0.955-0.984)	0.976 (0.958-0.986)	*0.74*

χ^2 test was used to describe the differences between liver and oesophagus. $CI_{95\%}$ = 95% Wilson confidence interval, FN = false negative, FP = false positive, $NPV_{34\%}$ = negative predictive value for a prevalence of 34%, $PPV_{34\%}$= positive predictive value for a prevalence of 34%, TN = true negative, TP = true positive

We used maximum likelihood estimates for sensitivity, specificity, positive (PPV) and negative predictive value (NPV). The 95% Wilson confidence intervals for sensitivity, specificity, PPV and NPV were determined based on a normal approximation to avoid being overly optimistic [6]. Calculations and graphics were produced with SPSS 20. χ^2 test was used to describe the differences between liver and oesophagus.

Results and discussion

The results of IgA- and IgG-EmA testing on liver in comparison with oesophagus are depicted in Tab. 1 and 2, respectively.

For IgA-EmA (Tab. 1), oesophagus and liver sections showed comparable sensitivities (0.946 vs 0.953) and specificities (0.981 vs 0.972). The predictive values (PPV and NPV) calculated for the prevalence of CD in our study cohort (34%) exhibited also no significant difference estimating IgA-EmA (0.962 vs 0.947 and 0.972 vs 0.976, respectively). These data show that liver is a substrate as good as oesophagus for assay of IgA-EmA.

Quite contrary to this, an additional number of 23 CD patients were positive for IgG-EmA on liver tissue (Tab. 2). This results in a significantly increased sensitivity (0.517 vs 0.629; p = 0.006).

Table 2. *Performance of IgG-EmA on oesophagus and liver tissue sections and of ELISA for IgG-tTG.*

	IgG-EmA		***p***	**IgG-tTG**
	Oesophagus	**Liver**		
TP	154	187		87
TN	571	553		570
FP	3	21		2
FN	144	111		213
Sensitivity ($CI_{95\%}$)	0.517 (0.459-0.575)	0.628 (0.569-0.682)	*0.006*	0.290 (0.240-0.345)
Specificity ($CI_{95\%}$)	0.995 (0.983-0.999)	0.963 (0.944-0.977)	*0.0002*	0.996 (0.986-0.999)
$PPV_{34\%}$ ($CI_{95\%}$)	0.981 (0.941-0.995)	0.899 (0.848-0.935)	*0.002*	0.977 (0.914-0.996)
$NPV_{34\%}$ ($CI_{95\%}$)	0.799 (0.767-0.827)	0.832 (0.802-0.859)	*0.1*	0.727 (0.695-0.759)

χ^2 test was used to describe the differences between liver and oesophagus. $C_{95\%}$ = 95% Wilson confidence interval, FN = false negative, FP = false positive, $NPV_{34\%}$ = negative predictive value for a prevalence of 34%, $PPV_{34\%}$= positive predictive value for a prevalence of 34%, TN = true negative, TP = true positive

Simultaneously, we observed a significant decrease in the specificity (0.995 vs 0.963; p = 0.002) and PPV (0.981 vs 0.899; p = 0.0002) on liver compared to oesophagus caused by a 7-fold higher rate of control patients with positive IgG-EmA test on liver tissue. Despite the significant increase in sensitivity, liver tissue should not be applied for IgG-EmA detection due to the noticeable decrease of PPV.

The low sensitivity of IgG-EmA assay on both tissues of 52 to 63%, however, is still superior to IgG-tTG assay by means of ELISA (29%). These data do not support the strategy of most laboratories to assay IgG-tTG instead of IgG-EmA.

Autoantibody binding patterns can be misinterpreted due to occurrence of other "unspecific" autoantibodies which overlap with the CD typical staining patterns. This was also observed during assessment of possible EmA staining. Typical staining patterns of EmA both on liver and oesophagus are illustrated in Fig. 1A and D.

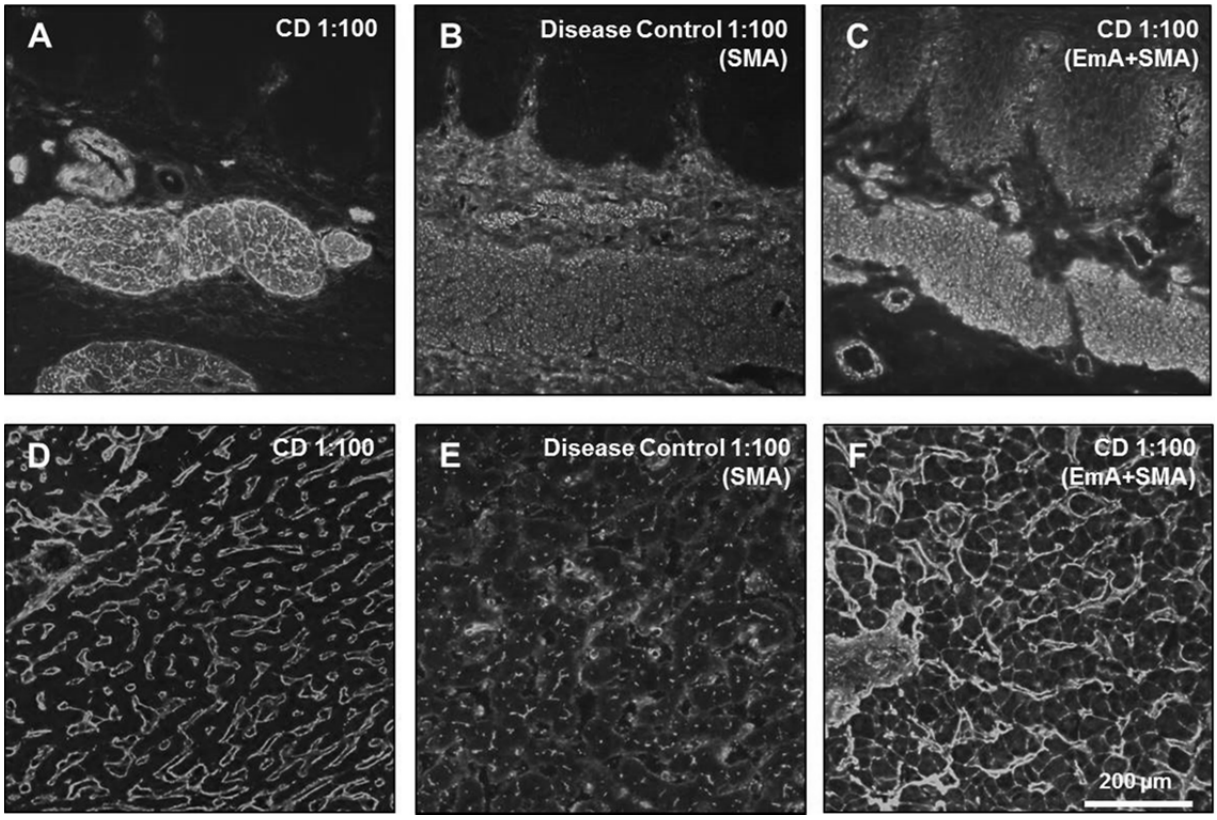

Figure 1. *Staining patterns of IgA autoantibodies on liver and oesophagus sections. Primate oesophagus (A-C) and primate liver (D-F) sections after incubation with serum of a coeliac disease (CD) patient with high autoantibody concentrations (A and D), of a patient with only anti-smooth muscle antibodies (SMA, B and E), and of a CD patient with both, IgA-EmA and SMA (C and F)*

Using oesophagus, other autoantibodies such as anti-smooth muscle antibodies (SMA) bind to structures similar to those recognized by EmA (Fig. 1B). This mimics EmA and can produce a false positive result. The staining pattern of SMA on liver tissue

was clearly different from that of EmA (Fig. 1E). In detail, SMA stained intracellular filaments whereas EmA bound to endothelia of sinusoids. If both antibodies co-exist in one serum sample, and in case of high SMA concentration, these antibodies may hide EmA staining on oesophagus (Fig. 1C). On liver tissue, both antibody species can be clearly distinguished (Fig. 1F).

Conclusions

The abundance of tissue transglutaminase in endothelia cells has been known since a long time. Therefore, the use of liver, which is rich in capillaries, might be a good substrate for autoantibodies against tissue transglutaminase. We show that the performance of the IgA-EmA test on liver and oesophagus is comparable, whereas the assay of IgG-EmA on liver has higher sensitivity, but slightly lower specificity as well as a noticeably lower PPV than on oesophagus. Therefore, IgG-EmA on oesophagus should still be favoured. The IgG-tTG ELISA seems to be unsuitable due to its low sensitivity. Instead, IgG-EmA on oesophagus is recommended.

Further, an advantage of the liver sections may be that interpretation of the staining is easier than on oesophagus sections, since there is less interference on liver by other autoantibodies (e.g. SMA). To sum up, our study shows that liver is an alternative substrate to oesophagus in detection of IgA-EmA.

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5.5 Mass cytometry: high-dimensional dissection of the immune system reveals previously unrecognized heterogeneity

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Our immune system protects us from infections by invading pathogens such as bacteria, viruses and helminths [1]. To achieve this task our immune system is not only capable of producing antibodies that will help to neutralize and/or eliminate pathogens from the circulation, it is also capable of recognizing and destroying virus-infected cells. In addition, our immune system develops memory: upon a first encounter with a particular pathogen, the immune system evolves such that upon re-exposure to the same or a highly related pathogen a more rapid response can be mounted, often eliminating the invader before any harm can be done. However, the immune system can also mount unwanted responses directed to self-antigens, resulting in a variety of immune-mediated diseases like type 1 diabetes and rheumatoid arthritis. Also, exaggerated immune response to the microbiota in the intestine can lead to chronic inflammatory bowel diseases like Crohn's disease, often with grave consequences. Finally, inadvertent immune response to food antigens can result in a variety of diseases, including coeliac disease, caused by immune responses to the gluten proteins in wheat [2].

To execute its functions the immune system uses a variety of cell types, each with their own specialized function, in concert with soluble factors, again each with its own well-defined function [1]. "Classically" the immune system can be subdivided in 6 major lineages: CD4 T cells, CD8 T cells, γδ T cells, B cells, myeloid cells and innate lymphocytes (including NK cells) [1]. The innate lymphocytes form a first line of defence. CD4 T cells are known as helper T cells, they orchestrate the immune responses. CD8 T cells are efficient in killing pathogen-infected cells. B cells produce antibodies, soluble factors that help to eliminate pathogens from the circulation and myeloid cells can internalize and kill pathogens. γδ T cells exert a variety of functions, not all properly understood. Every cell subset is distinguished by the expression of particular cell surface molecules like CD4 in the case of CD4 T cells and CD8 in case of CD8 T cells. Thus, generally CD4 T cells will express CD4 but not CD8 and vice versa. Over 250 CD markers have now been identified. In addition, a large number of intracellular markers are known, including highly lineage-specific transcription factors. Immunohistochemistry and flow cytometry are the two major techniques that are employed to determine the expression of CD and intracellular antigens by immune subsets of interest. The power of immunohistochemistry is that cells can be studied in their natural environment: in tissue sections. The downside is that only a few marker

proteins can be visualized simultaneously. Flow cytometry allows the simultaneous visualization of up to 15 markers on single cells and therefore has become a much used technique in both diagnostics and research. Through flow cytometric analysis of the immune system it has become evident that its composition is much more complex than described above. All 6 major lineages can be subdivided into multiple sublineages, each with a distinct marker expression profile. For example, several distinct CD4 subsets can readily be identified through the expression of a membrane protein that distinguished naïve CD4 T cells from memory CD4 T cells. Similarly, pro-inflammatory and regulatory CD4 T cell subsets can be defined. More recently, the innate lymphocyte compartment was found to harbour a variety of cellular subsets with regulatory properties as well [3,4].

However, flow cytometry has its limitations as the number of antigens that can be monitored simultaneously is limited due to the spectral overlap between fluorochromes used to label the antibodies used. This limitation has recently been overcome due to the introduction of mass cytometry in which flow cytometry is combined with mass spectrometry. Instead of labelling the antibodies with fluorochromes, rare earth metals are used [5,6], of which some 100 are available, all of which can be distinguished from each other by mass spectrometry. In principle, therefore, up to 100 antibodies can each be uniquely labelled and combined to stain (immune) cells, followed by data acquisition on the CyTOF, the mass cytometer. At present up to 36 antibodies can be routinely applied through this methodology, offering a significant advance over conventional flow cytometry.

We have applied a 32 antibody panel to analyse the composition of the immune system in detail. In the analysis, we compared peripheral blood samples with matched intestinal samples from the intestine. Single cells suspensions were prepared and stained with the antibody panel and analysed on the CyTOF. Special computational tools and a data driven approach were applied to accommodate the high-dimensional data sets acquired. The results reveal previously unrecognized heterogeneity within the immune system as over 100 distinct immune subsets were identified, some of which were tissue- or disease-associated. Currently, the antibody is being optimized and expanded which is expected to increase the resolution of the approach even more. It is anticipated that through this technology diagnostic procedures can be significantly improved. Such analyses may also help to predict and/or monitor the response to therapeutic approaches, an important step towards personalized medicine.

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5.6 Gliadin and avenin toxicity studies of duodenal biopsy tissue in organ culture experiments

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Introduction

Oats is phylogenetically more distantly related to the other food cereals. This led to the original *in vivo* oats challenge studies, which reported that oats did not cause activation of coeliac disease [1,2]. Further studies of paediatric and adult coeliac patients supported these earlier observations. However, other investigators reported that oats could activate coeliac disease in some patients and suggested that if oats were taken in sufficient quantity over a longer time period, the cereal might prove to be toxic [3,4]. Because of the continuing interest in possible oats toxicity, we performed a further year long *in vivo* oats challenge study in 54 patients and none showed adverse effects to this cereal [5]. In addition, we carried out *in vitro* studies examining the possible toxic effect of wheat gliadin or oats avenin (as peptic/tryptic digests) when added to duodenal biopsy tissue in organ culture experiments. These latter experiments are reported here.

Materials and methods

Patients

Six control patients and five treated coeliac patients were recruited for the study and approval was given by the St. James's Hospital Ethics Committee. All patients gave informed consent. Duodenal biopsy tissue from each were cultured as described below. In addition, two biopsies from each patient were placed directly into formalin as time 0 controls.

Prolamin fractions

The prolamin fractions used in the study were kindly prepared by Prof. Peter Koehler (Deutsche Forschungsanstalt für Lebensmittelchemie, Freising, Germany). Wheat gliadin was a gift from the Working Group on Prolamin Analysis and Toxicity ("PWG-gliadin"). The prolamin fraction from oats (avenin) was derived from the German oats cultivar "Scorpion". Peptic-tryptic (PT) digests of each prolamin were prepared and stored as a freeze-dried product. In preparation for culture, 5 mg/mL PT gliadin and avenin were re-suspended separately in RPMI-1640 medium supplemented

with 15% heat inactivated, filtered foetal calf serum and a 1% antibiotic/antimycotic solution.

Organ Culture

Pairs of biopsies collected in RPMI were orientated villous side up on a nylon mesh filter. 1.5 mL of PT avenin, PT gliadin or RPMI was added to the central well of a culture dish and the filter with biopsies was placed in the central well so that the biopsies were in contact with the surface of the medium. The remainder of the aliquot of PT avenin, PT gliadin or RPMI was dotted around the outer well of the culture dish. Culture dishes were placed in a modular incubator chamber, gassed with 95% O_2 / 5% CO_2 and placed in a 37 °C incubator. Following 24 h of culture, biopsies were transferred to histology cassettes and immersed in formalin. All 8 biopsies were formalin-fixed and paraffin-embedded.

Immunofluorescent staining

4-µm-thick tissue sections were incubated with a mouse monoclonal antibody to E-cadherin conjugated to Alexafluor^{488} and a rabbit monoclonal antibody to Cytokeratin 20 at 4 °C, overnight. Sections were then incubated with an Alexafluor^{568}-conjugated secondary GAR antibody for 1 h at RT. Tissue sections were incubated with phosphate-buffered saline (PBS) instead of Cytokeratin 20 and a mouse monoclonal antibody to alpha-actin conjugated to Alexafluor^{488} instead of E-cadherin conjugated to Alexafluor^{488}, to check that staining was specific. To visualize nuclei, all sections were incubated with Hoechst 33258. All sections were examined and imaged under a confocal microscope at 63x magnification.

Results and discussion

General effects of biopsy culture

The culture process affected all biopsies, regardless of whether they came from a control or a coeliac patient. Immediately after biopsy, the tissue had a plump and rounded appearance and biopsies placed directly into formalin retained that configuration. However, after 24 h culture on a mesh filter, the other biopsies were visibly flattened and generally difficult to separate from the mesh filter. In these biopsies the villi were generally shorter and wider and often folded over on themselves. Enterocytes were also observed to be much shorter in cultured biopsies compared to time 0 biopsies.

Control patients

All time 0 biopsies from control patients displayed healthy, elongated enterocytes (Fig. 1). Orderly patterns of E-cadherin staining were present with narrow, generally straight lines of green fluorescence visible between enterocytes. In some biopsies cytokeratin 20 staining was visible throughout the enterocytes; however, in others the

quality of staining was suboptimal (the cytokeratin staining is not visible in the greyscale photographs used in this publication). Following 24 h in culture, although enterocytes were considerably shorter, no patient showed any signs of a reaction to culture with PT gliadin or PT avenin. The appearance of enterocytes and nuclei remained organised and the pattern of E-cadherin expression remained similar to that observed at time 0. Although, cytokeratin 20 staining was of good quality only in some biopsies, there was no evidence of an effect caused by prolamins.

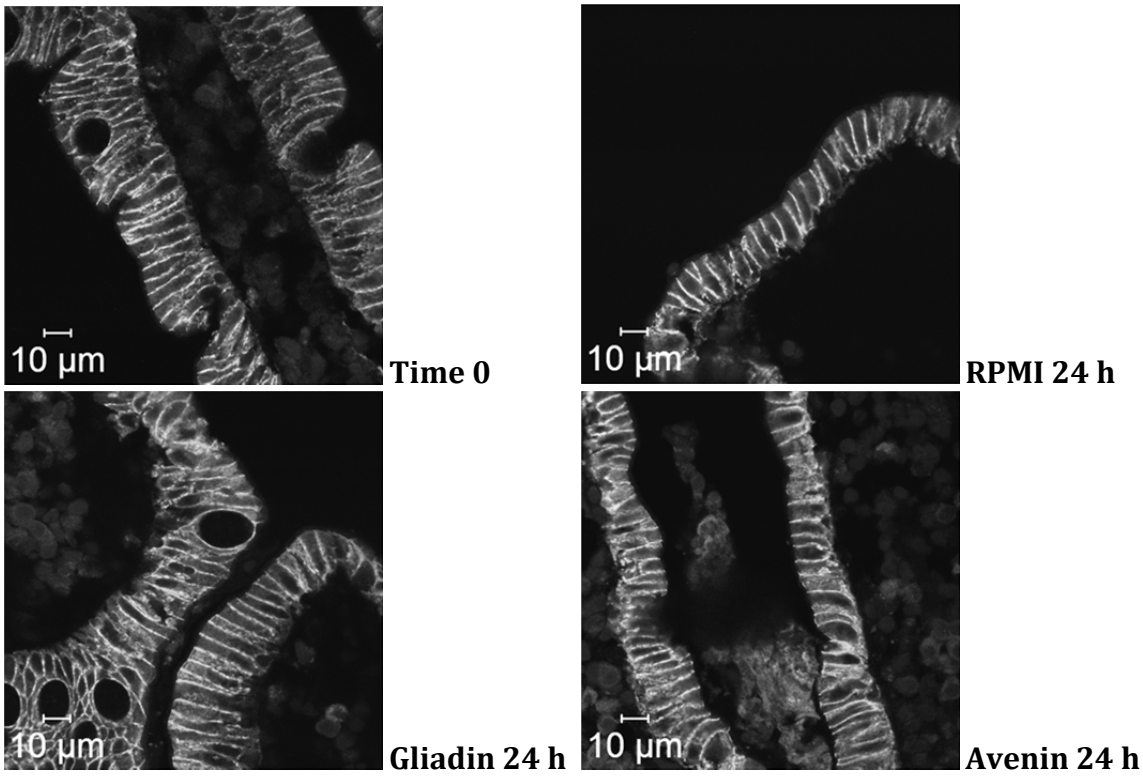

Figure 1. *Control patient. E-cadherin, cytokeratin 20 and Hoechst staining at time 0 and after 24 hours in culture with RPMI, PT gliadin or PT avenin*

Coeliac patients

At time 0, three of the five coeliac patients had a routine histological appearance consistent with that of treated CD. Two further patients were reported to have increased IELs and villous blunting. All five coeliac patients had negative tTG serology. Following culture with RPMI, strong E-cadherin staining was still visible between enterocytes. Again, cytokeratin 20 staining was of poor quality in all five subjects. After 24 h culture in the presence of PT gliadin four out of five patients showed signs of damage (Fig. 2, arrows). In addition to shortening of enterocytes, E-cadherin staining was less consistent. Although in some areas, biopsies still displayed healthy looking enterocytes, in other areas the pattern of E-cadherin expression was altered, with loss of the nice, straight line of green E-cadherin staining between enterocytes.

The addition of PT avenin did not appear to have any significant effect on biopsies from two of the five coeliac patients (Fig. 2). Similarly to culture with RPMI, enterocytes were shortened but remained generally healthy looking and had normal expression of E-cadherin. In contrast, biopsies from three of the coeliac patients showed evidence of change; although there were some healthy looking enterocytes, there were also areas where enterocytes were less well organised and they lacked the consistent expression of E-cadherin (Fig. 3, arrows). This appearance was similar to that observed with PT gliadin.

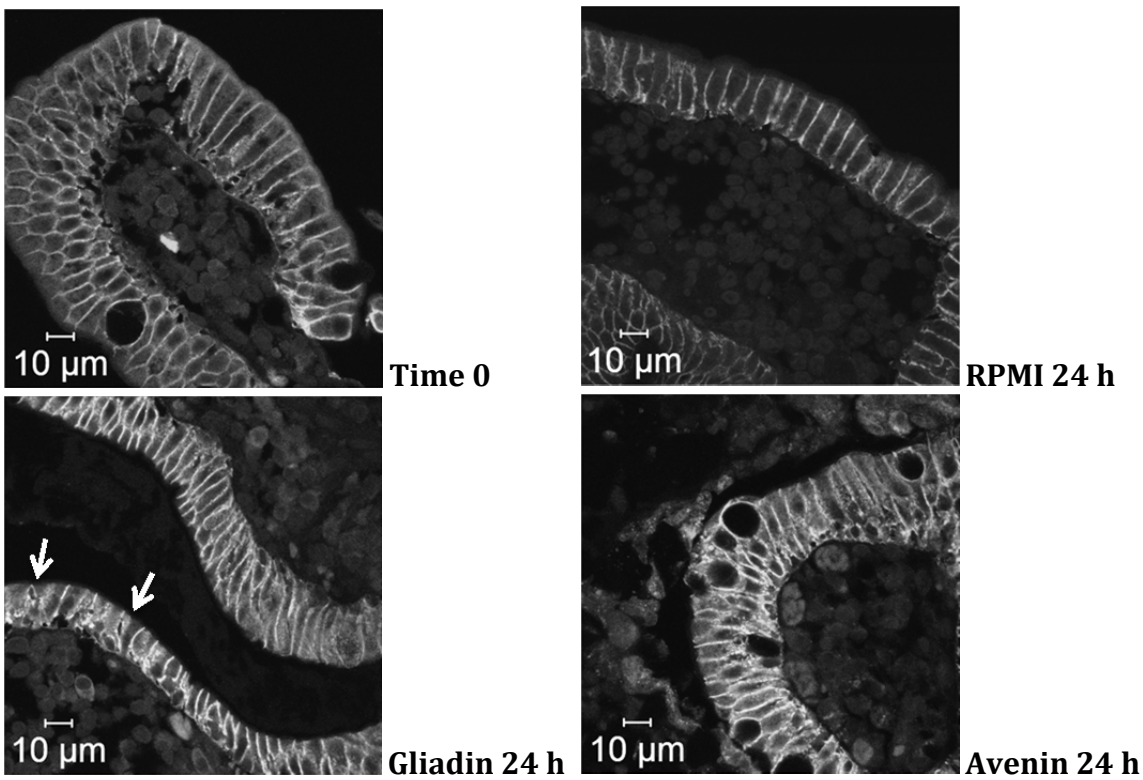

Figure 2. *Coeliac patient. E-cadherin, cytokeratin 20 and Hoechst staining at time 0 and after 24 hours in culture with RPMI, PT gliadin or PT avenin. Arrows indicate shortening of enterocytes and patchy E-cadherin staining*

In this study, we attempted to develop an *in vitro* assay which would examine the question of whether a prolamin fraction from oats caused damage to coeliac biopsy tissue in culture. Since enterocyte damage is an early feature of gliadin mediated damage, we employed E-caderin and cytokeratin 20 staining of enterocytes with confocal microscopy to investigate this question. In the case of tissue from normal controls, there appeared to be no evidence of damage caused by the prolamin fractions. Gliadin caused changes in E-cadherin staining in four of the five coeliac subjects. However, avenin appeared to cause similar changes in three of the five coeliac biopsy experiments. Unfortunately, cytokeratin 20 staining was too unreliable to add meaningful information to the studies.

Conclusions

The experimental technique described here may be useful in determining the potential toxicity (as opposed to immunogenicity) of prolamin fractions or cereal peptide sequences. However, further optimisation of the technique is required and there is a need to overcome the inherent subjectivity, involved in the interpretation of tissue changes.

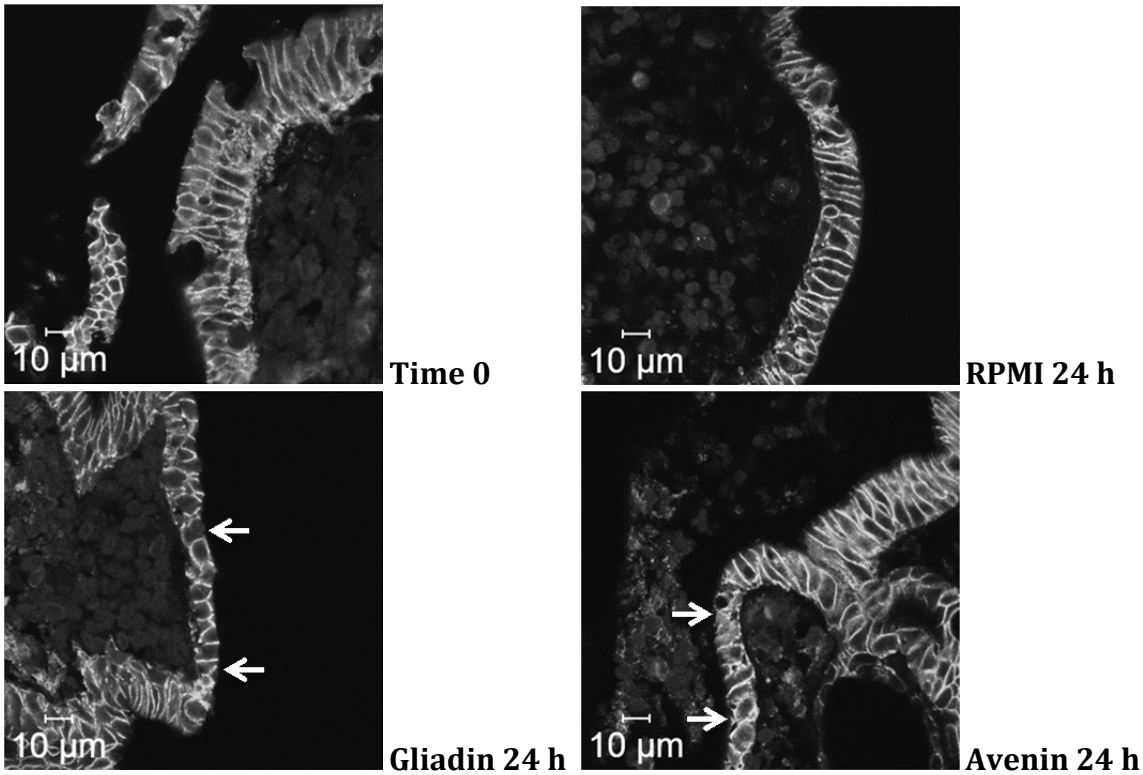

Figure 3. *Coeliac patient. E-cadherin, cytokeratin 20 and Hoechst staining at time 0 and after 24 hours in culture with RPMI, PT gliadin or PT avenin. Arrows indicate disorganization and shortening of enterocytes and patchy E-cadherin staining*

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6 Antigen receptors in coeliac disease

6.1 Gliadin peptides as trigger of the stress/innate immune response of the coeliac small intestine

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Introduction

Ingested food can cause tissue inflammation through different mechanisms. In the intestine, and particularly in the enterocyte, nutrients are modulators of various cellular functions and may be involved in tissue immune response and inflammation [1]. An example of an intestinal inflammatory and remodelling response of the intestine to food is the small intestinal coeliac lesion induced by gluten, an alimentary protein present in wheat and other cereals. Coeliac disease (CD) is characterized by inflammatory and structural changes resulting in remodelling of the small intestinal mucosa [2].

Gliadin, the major storage protein component of wheat, is a peculiar protein very rich in glutamine and proline. Several gliadin peptides are recognized by T cells (TC) of the coeliac intestine, and can induce the adaptive immune response, but most of them are digested by gastric, pancreatic and intestinal peptidases. Several gliadin peptides remain undigested [3]: among these the 33-mer (P55-87) and the 25-mer (P31-55).

The inflammation of the intestinal mucosa is due not only to the adaptive, but also to the innate immune responses to wheat gliadin. The P31-43 peptide, which is contained in the 25-mer, is not recognized by TC in the coeliac intestine and is able to damage the coeliac intestinal mucosa *in vitro* and *in vivo* [3,4]. Moreover, the P31-43 gliadin peptide is able to initiate both a stress and an innate immune response with interleukin-15 (IL-15) as a major mediator [3].

P31-43 is also known to exert a number of biological activities. Although the structural changes of the coeliac mucosa are considered a consequence of sustained mucosal inflammation due to the Th1-TC response, recent data have shown that gliadin peptides, in particular P31-43, induce proliferation of coeliac enterocytes. This process is epithelial growth factor- (EGF) and IL-15-dependent, and has profound effects in inducing the crypt hyperplasia, which is characteristic of the remodelling of the coeliac mucosa [5-8]. Moreover, gliadin peptides induce alterations of structure (cell shape, actin modifications, increased permeability, vesicular trafficking alterations, signalling and proliferation and stress/innate immunity activation in several cell lines [3].

In this paper we will discuss the P31-43 activities that are linked to the activation of the stress/innate immunity activation in the intestinal epithelial cell line (CaCo2) and in intestinal biopsies from CD patients.

Role of P31-43 in enterocyte proliferation, structural changes and innate immune response

Alterations of the intestinal mucosa in CD are mediated both by inflammation due to the adaptive and innate immune response to gliadin and by proliferation of crypt enterocytes causing crypt hyperplasia [3]. The coeliac intestine is characterized by an inversion of the differentiation/proliferation program of the tissue, with a reduction in the differentiated compartment, up to complete villous atrophy, and an increase in the proliferative compartment with crypt hyperplasia [3].

Gliadin peptides and P31-43 induce cell proliferation and actin rearrangements [8,5] in several cell lines. We have already reported [3] that in CaCo2 cells, mimicking the effect of gliadin peptides can induce actin remodelling, proliferation and activation of the EGF-receptor (EGFR)/extracellular signal-regulated kinases (ERK) pathway [5] (Fig. 1). P31-43 can also induce increase of IL-15/IL-15R alpha in CaCo2 cells [3]. Interestingly, levels of IL-15 do not change after P31-43 treatment when analysed by quantitative polymerase chain reaction (PCR) [3], but the levels of the protein present on the cell surface increase rapidly after P31-43 treatment, as measured by fluorescence-activated cell sorting (FACS) and western blot analysis (Fig. 1). The increased IL-15 on the cell membrane worked as a growth factor for TLL2 cells, a cell line that depends on IL-15 for its growth [3]. Moreover, IL-15 can induce epithelial proliferation both in intestinal biopsies and in CaCo2 cells [8]. Interestingly, P31-43 was able to induce activation, again in CaCo2 cells, of tissue transglutaminase (tTG) and of glucose-regulated protein 78 (GRP78), both markers of cellular stress [9]. Finally, intestinal biopsies from CD patients in the active phase of the disease, but not from controls, increased proliferation after P31-43 treatment (Fig. 1).

Taken together all these data indicate that gliadin peptide P31-43 is able to induce proliferation, increase of stress markers and of innate immunity activation in an intestinal epithelial cell line and in intestinal biopsies from CD patients [3,10].

Mechanisms of IL-15 and EGF upregulation in CD induced by P31-43: role of vesicular trafficking

The mechanisms through which P31-43 might induce the coeliac intestinal innate immune response and EGF- and IL-15-mediated enterocyte proliferation have recently been investigated. Recent evidence points to an effect of P31-43 on the endocytic compartment [3].

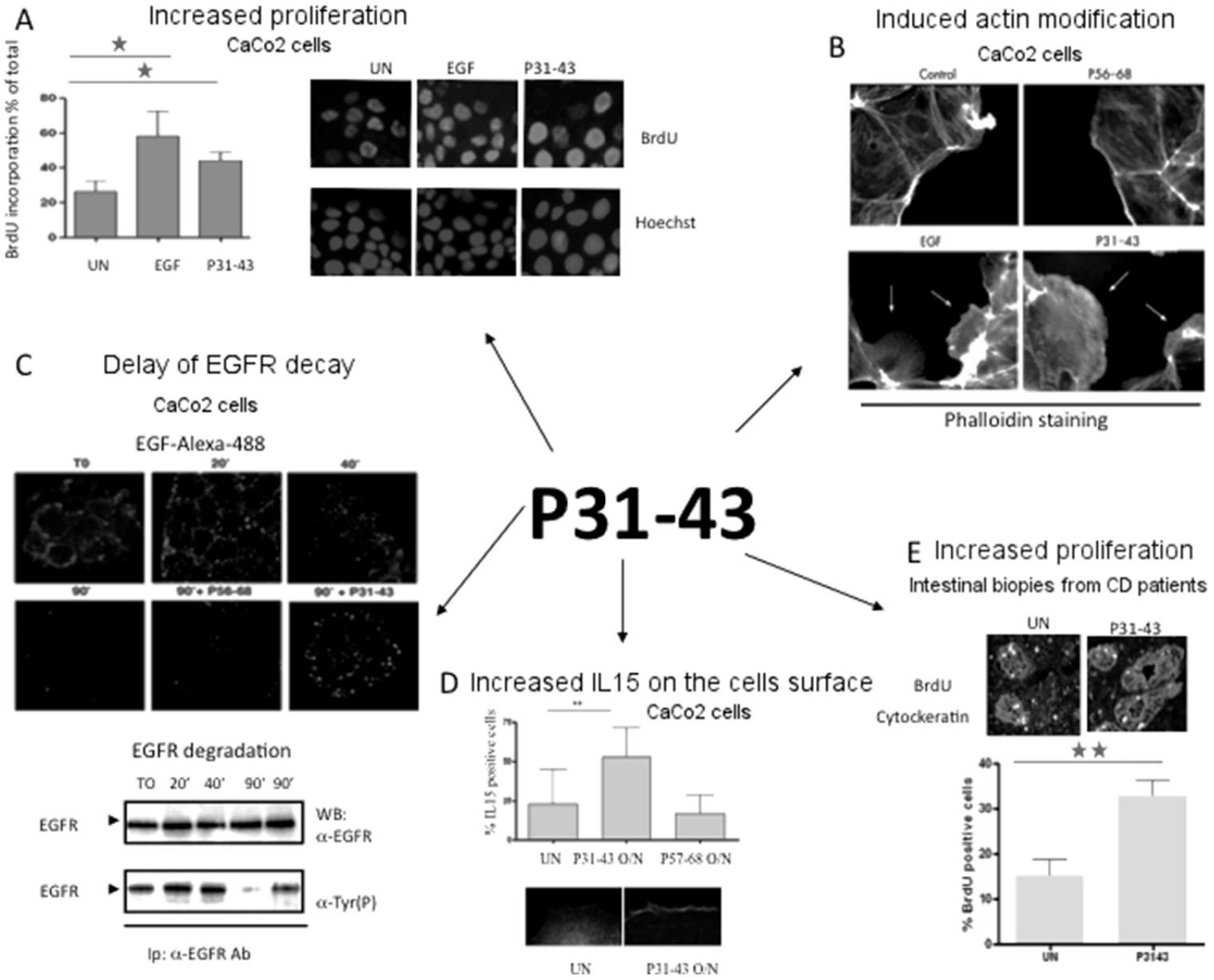
A
Increased proliferation
CaCo2 cells
BrdU incorporation % of total
UN
EGF
P31-43
BrdU
Hoechst
B
Induced actin modification
CaCo2 cells
Control
P56-68
EGF
P31-43
Phalloidin staining
C
Delay of EGFR decay
CaCo2 cells
EGF-Alexa-488
T0
20'
40'
90'
90' + P56-68
90' + P31-43
EGFR degradation
TO 20' 40' 90' 90'
EGFR
WB: α-EGFR
EGFR
α-Tyr(P)
Ip: α-EGFR Ab
P31-43
D Increased IL15 on the cells surface
CaCo2 cells
% IL15 positive cells
UN
P31-43 O/N
P57-68 O/N
UN
P31-43 O/N
E Increased proliferation
Intestinal biopies from CD patients
UN
P31-43
BrdU
Cytockeratin
% BrdU positive cells
UN
P3143

Figure 1. P31-43 effects on CaCo2 cells and intestinal biopsies.

A) *P31-43 increased proliferation in CaCo2 cells. Increase of Bromodeoxiuridine incorporation (BrdU) after treatment with P31-43 and EGF, used as positive control, respect to the untreated (UN) sample. Statistical analysis of five independent experiments and immunofluorescence images of CaCo2 nuclei that incorporate BrdU (red) respect to total nuclei (blue)*

B) *P31-43 induced actin modifications in CaCo2 cells. Immunofluorescence images of CaCo2 cells stained with phalloidin to highlight F actin*

C) *Pulse-chase determination of EGFR activity. Endocytic vesicles containing EGF-Alexa488 persist longer after P31-43 treatment of CaCo2 cells. Cells were pulsed for 1 h at 0°C with EGF-Alexa488 and chased after temperature shift to 37°C, with P31-43 or control P56-68 for the times indicated. 63x objective. Representative results from 3 independent experiments. Western blot of immunoprecipitated EGFR from CaCo2 cells which were pulsed for 1 h with EGF at 0°C and chased for the indicated times after temperature shift to 37 °C. In PTG and P31-43 treated samples, the EGFR is still activated 90' after temperature shift, by which time all activated EGFR has been degraded in control cells. Densitometric analysis of Western blot experiments. EGFR phosphorylation is expressed as fold-increase over the corresponding untreated line. UN=untreated*

D) *Gliadin peptide P31-43 increased IL-15/IL-15R alpha complex on the cell surface in CaCo-2 cells. P31-43 increased IL-15 on the cell surface in CaCo-2 cells. FACS analysis of IL-15 on the cell surface after overnight (O/N) P31-43 or control gliadin peptide P57-68. Statistical analysis of ten independent experiments. Confocal images of IL-15 R-alpha expression on CaCo2 cell surfaces. 63x objective*

E) *P31-43 increased proliferation in intestinal biopsies from CD patients. Statistical analysis of three independent experiments. Columns represent means and bars are the standard deviations. *= $p<0.05$ (Student's t-test), **= $p<0.01$ (Student's t-test).*

Endocytosis has many effects on signalling; in fact, signalling pathways and endocytic pathways are regulated in a reciprocal manner. It is now widely accepted that the "endocytic matrix" is a master organizer of signalling, governing the resolution of signalling in space and time. Consequently, endocytosis affects several cell functions, ranging from proliferation to actin organization, cell motility and stress/innate immunity activation [3].

P31-43 is strikingly similar to a region of hepatocyte growth factor regulated substrate kinase (HRS), a key molecule regulating endocytic maturation, which is localized on the membranes of early endocytic vesicles [11]. The sequence similarity between gliadin peptide P31-43 and HRS is in a small area of the proline/glutamine-rich domain of HRS. The COOH terminal of HRS contains a clathrin-binding domain that binds clathrin to clathrin-coated vesicles and is one of the domains needed to localize HRS to the vesicle membranes. Both in CaCo2 cells and in the coeliac enterocytes, P31-43 localizes in the early endosome and delays vesicular trafficking [3].

In CaCo2 cells, P31-43 interferes with the correct localization of HRS at the level of the early endosomes, interfering with HRS-mediated maturation of early endosomes. By interfering with the localization to the endocytic membrane of HRS, P31-43 induces two important effects: a) it delays endocytic maturation and b) it alters the recycling pathway. By delaying the maturation of endocytic vesicles, P31-43 reduces the degradation of EGFR and other RTKs (receptor tyrosine kinases) that are endocytosed in these vesicles, and prolongs their activation, resulting in increased proliferation, actin remodelling and other biological effects. The alteration of the recycling pathway is able to direct more transferrin receptor and IL-15R alpha to the cell surface, allowing more IL-15/IL-15R alpha trans-presentation in epithelial cells. Moreover, the trans-presented IL-15 is able to activate IEL *in vitro* in an IL-15-dependent way, demonstrating that the effects of P31-43 on enterocytes can activate signalling in lymphocytes [3]. Interestingly, more IL-15R alpha is expressed in CD enterocytes and in patients on a gluten-free diet, indicating that in the CD mucosa, a constitutive alteration of IL-15R alpha trafficking could be present (see below).

The production of IL-15 is tightly controlled at multiple levels, not only at the level of intracellular trafficking but also of transcription and translation. P31-43 increased IL-15 mRNA levels only after prolonged incubation, whereas the increase of the trans-presented IL-15/IL-15R alpha complex on the cell surface was an early effect [12]. By increasing the synthesis of IL-15 and the amount of the cytokine that is trans-presented to the neighbouring cells, P31-43 affects both enterocyte proliferation, which is EGFR-IL-15 dependent, and the activation of innate immunity (Fig. 2) [12].

Constitutive alterations in CD cells

Recent observations suggest an effect of P31-43 on the maturation and function of early endocytic vesicles and consequently on EGFR signalling, enterocyte proliferation and IL-15 trans-presentation and synthesis [3]. However, the explanation

for why the stress/innate immune and proliferative responses to certain gliadin peptides (i.e., P31-43) in the CD intestine are so intense and disruptive has not been elucidated. For this reason, it is interesting to review the recent literature regarding constitutive alterations in CD biopsies and cells. Several reports note constitutive, gluten-independent alterations of the CD cells. They have been studied in the normalized intestinal biopsies of patients in the remission phase of the disease on a gluten-free diet and in cells obtained from tissues far away from the intestine, the primary site of inflammation.

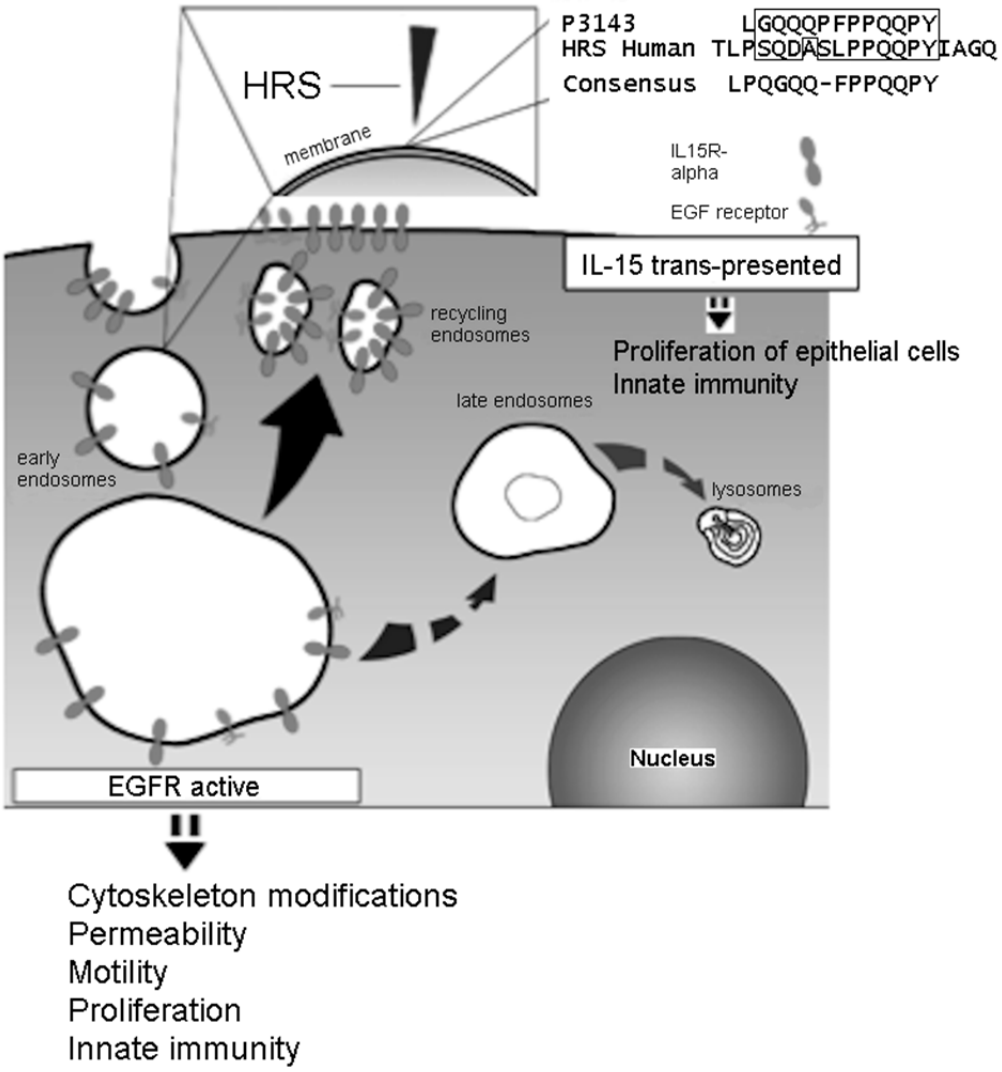

Figure 2. Overview of the P31-43 effects on the endocytic pathway. *Due to a sequence similarity with HRS, P31-43 interfered with its correct localization to the endocytic membranes. The biological effects of this interference were: a) delayed endocytic maturation, and b) increased recycling to the cell surface. P31-43-induced delay of the active EGFR decay could activate several downstream signals with different biological effects. The increase of the recycling pathway can direct more transferrin receptor and probably other recycling receptors and IL-15 to the membranes*

Structural alterations

Different studies have identified the importance of the disruption of the integrity of the epithelial layer in CD. One of the first structural alterations identified is the alteration of the cell-to-cell junctional complexes that regulate intestinal permeability. Patients show enhanced intestinal permeability and altered tight junction (TJ) morphology. These disruptions persist in patients who are on a gluten-free diet (GFD) with a normalized intestine, suggesting that permeability may play a driving role in the development of CD. Moreover, epithelium integrity is impaired in the early stage of the disease [3]. Polymorphisms in the TJ genes PARD3 and MAGI2 have been associated with disease susceptibility in a Dutch cohort [3]. Interestingly, serine/ threonine-protein phosphatase 2A regulatory subunit B" subunit alpha (PPP2R3A), implicated in the negative control of cell growth, division and TJ regulation, remains downregulated at the intestinal level in patients on a GFD. These observations suggest a role for this pathway in the pathogenesis of CD [3].

Recent genetic studies point to the importance of polymorphisms of CD genes that are involved in actin remodelling and cell adhesion. Among these, the lipoma-preferred partner (LPP) gene presents the strongest non-HLA association signal, mapped in intron 2 [3]. More recently, it has been suggested that deregulation of transcription binding properties, due to single point mutations, might be the causal mechanism underlying the association of CD with the LPP region [3]. The LPP protein localizes to focal adhesions, which are the site of membrane attachment to the extracellular matrix and cell-cell contact [3]. A constitutive alteration of LPP sub-cellular distribution together with alterations of cell shape, actin cytoskeleton and focal adhesion has been demonstrated in CD fibroblasts from GFD-patients [13]. Moreover, cell shape and actin rearrangements are altered in CD dendritic cells from GFD-patients (data submitted for publication).

Taken together, these data indicate that structural alterations are present in CD cells independently of gluten.

Signalling and proliferation

Alterations in signalling pathways and cell proliferation have been demonstrated in CD biopsies and cells (enterocytes, skin fibroblasts, dendritic/monocytes) of patients on a gluten-free diet.

NF-κB pathway

The nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) pathway is constitutively altered in CD, with more than 20 components of the pathway increased in GFD-CD biopsies. Most of the mRNA overexpressed in GFD-CD was central to the regulation of the pathway [3]. Interestingly, two key mediators of the NF-κB pathway, Rel and TNFAIP3, have CD-associated gene variants [3]. It is widely accepted that NF-κB is a key regulator of inducible gene expression in the immune

system. Both innate and adaptive immune responses, as well as the development and maintenance of the cells and organs that comprise the immune system are, at multiple stages, under the control of the NF-κB family of transcription factors. Moreover, NF-κB is responsible for the transcription of genes encoding a number of pro-inflammatory cytokines and chemokines [3]. It has also been shown that NF-κB is a major mediator of IL-15 [3], which, among its many pleiotropic effects, is also able to decrease claudin-2 levels in epithelial tight junction structures and leads to augmented paracellular permeability, a phenomenon that is relevant and persistent in CD.

EGF receptor/ligand system

Constitutive activation of the EGF receptor/ligand system is also present in CD enterocytes. Increases in EGFR protein levels, EGF mRNA, the downstream effector molecule ERK and proliferation, which is ERK-dependent, have been found in enterocytes from normal biopsies of GFD-CD patients [3].

Stress/innate immunity activation

Cellular stress

Cellular stress has been implicated in the early events of the disease, in particular in the epithelium [3]. Heat shock protein-65 (HSP-65) is increased in CD enterocytes before they develop the disease, indicating that epithelial stress may play a role in the pathogenesis of CD. An alteration of this pathway was confirmed later with the observation that HSP-72 increased [3]. More recently, a marker of ER cellular stress, the molecular chaperone glucose-regulated protein 78 (Grp78), the master negative regulator of the unfolded protein response (UPR), was found increased in enterocytes with moderate and severe enteropathy and after at least two years on a GFD. Remarkably, the increased peri-nuclear Grp78 aggregates co-localize with increased major histocompatibility complex class I-related chain B (MICA/B^{+}) in CD enterocytes, linking cellular stress and innate immunity in CD.

IL-15/IL-15R alpha

IL-15 is elevated in intestinal biopsies from CD patients on a GFD [13]. In dendritic cells from CD patients at all stages of the disease, more IL-15 has been found in the cell membranes, indicating that this key mediator of the immune response is constitutively altered in CD (Zanzi et al., manuscript submitted). In CD patients on a GFD, the IL-15R alpha receptor has been observed at higher levels in intestinal biopsies [4].

In conclusion, the data in the literature point to several constitutive alterations of cell structure, signalling, proliferation and stress/innate immunity in CD cells. These pathways, already constitutively altered in coeliac cells, render them more susceptible to the effects of the gliadin peptides that can act on the same pathways. These same

three sets of metabolic pathways can be triggered in normal cells by P31-43, which mimics the coeliac cellular phenotype in controls.

Conclusion

In this short review we have shown most of the effects of gliadin peptide P31-43 in intestinal biopsies and cells from normal subjects and CD patients at different stages of the disease. We have highlighted the effects of an alimentary peptide, contained in very common foods, that is biologically active on cell structure, signalling/proliferation pathways and stress/innate immune activation. These are also constitutively altered in coeliac cells and biopsies, rendering them more sensitive to the effects of gliadin.

What remains to be understood is the molecular defect explaining the alterations of the coeliac cells, most likely due to a particular genetic make-up. It is possible to hypothesize that coeliac subjects have constitutive alterations that amplify the signals induced by gliadin peptides.

Gluten itself could play a role in the pathogenesis of diseases different from CD, such as type 1 diabetes. In children with insulin-dependent diabetes but not coeliac disease, intestinal inflammation is triggered by viral infections and alimentary proteins [3]. In particular, signs of an altered mucosal immune response to gliadin have been described in type 1 diabetes both by challenging the rectum with gliadin peptides *in vivo* [3], and the proximal small intestine *in vitro* [3]. In conclusion, gliadin and its undigested peptides have biological effects not only in cells and the intestinal mucosa of patients with CD but also in normal subjects or in different diseases. How these effects can affect the health of non-coeliac subjects will be the object of future research.

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6.2 Consumption of wheat alpha-amylase trypsin inhibitors (ATIs) accelerates murine systemic lupus erythematosus

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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disorder characterized by severe and persistent inflammation that leads to tissue damage in multiple organs and pathogenic autoantibodies. Kidneys are particularly affected leading to nephritis and renal failure [1]. Although both genetic and environmental factors play an important role in the disease mechanism, the aetiology remains unclear. However, the role of diet as modulator of autoimmune diseases could help to explain this complex disease mechanism.

We have identified wheat (rye, barley) alpha-amylase/trypsin inhibitors (ATIs) as main nutritional triggers of innate immunity via the toll-like receptor 4 (TLR4)-MD2-CD14 complex in cells of the mononuclear phagocyte system including monocytes and dendritic cells [2]. ATIs are a family of non-gluten wheat proteins that represent 2-4% of the wheat protein and that are significantly present as “contaminating protein” in gluten and gliadin preparations [3]. Importantly, ATIs likely serve as major triggers of non-celiac, non-allergy wheat sensitivity, which we characterize as worsening of a pre-existent, mostly extraintestinal disease with the consumption of gluten- (and therefore ATIs-) containing grains, flours and processed products [3-6].

The MRL-Fas(lpr) mouse develops progressive and spontaneous glomerular, tubulointerstitial and perivascular kidney disease, arthritis, lymphadenopathy and splenomegaly that replicates SLE. Therefore, we examined the effects of gluten-free and gluten- (plus ATI-) containing diets on disease progression using the MRL-Fas(lpr) mouse model.

Materials and methods

MRL-Fas(lpr) mice were placed either on a gluten-free (GFD/ATI-free) diet or a diet containing 25% gluten (containing amounts of ATIs equivalent to the human wheat-

based diet) for at least four weeks. We analysed clinical parameters such as proteinuria, haematuria, haemoglobinuria and serum inflammatory chemokine/cytokine levels throughout the experiment. At sacrifice, myeloid inflammatory cells were quantified immunohistochemically in the intestine, kidneys and spleen.

Results and discussion

Mice on a GFD showed significantly attenuated clinical parameters of kidney dysfunction (proteinuria, haematuria, haemoglobinuria) and serum inflammatory cytokines (IL-6, KC and TNFα) compared to mice on a gluten- (and ATI-) containing diet. Gluten/ATI-fed mice also showed a significant increase in kidney infiltrating $CD4^+$ T cells producing IL-12 and IL-17. Both clinical and immunological findings were in line with a worsened course in patients with SLE. Intestinal $CD68^+$ and $F4/80^+$ myeloid cells and $CD4^+$ T cells were mildly increased in the intestine of mice ingesting ATIs.

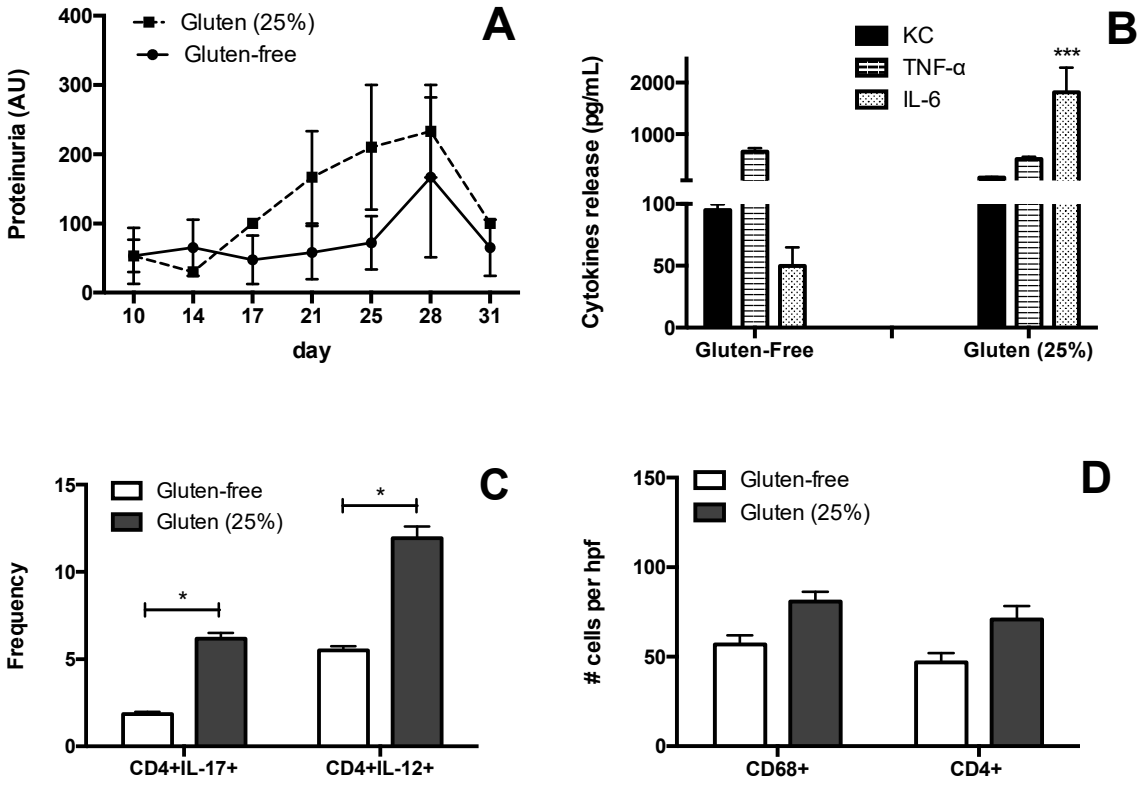

Figure 1. Evaluation of MRL-Fas(lpr) mice on a gluten-free compared to a gluten-(and ATI-) containing diet. *(A) Clinical score (proteinuria) during the experiment. (B) Markers of inflammation (KC, TNF-α, and IL-6) in serum. (C) Isolated single cells from mice's kidneys, labelled with antibodies CD4 plus IL-17 and IL-12 and subjected to FACS sorting. (D) Immunohistological number of intestinal $CD68^+$ and $CD4^+$ cells. Level of significance (*p<0.05;***p<0.001) is represented by asterisks*

Conclusion

We observed that dietary wheat ATIs (here combined with gluten), exacerbate the development of SLE in mice. This effect is mediated by intestinal innate and (secondary) adaptive immune activation. The gluten-free (and thus ATI-free) diet seems to have a protective effect on the development of SLE in MRL-Fas(lpr) mice, confirming the role of ATIs as major trigger of inflammation in autoimmune diseases. Further studies need to dissect how the intestinal immune activation by ATIs exacerbates the extra-intestinal manifestations of SLE.

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7 Statements by participating organisations, representatives from industry, and guests

7.1 News from Codex and regulatory affairs

Hertha Deutsch

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Introduction

AOECS, the Association of European Coeliac Societies, has Observer status in the Codex Alimentarius Commission since 1992. Information about the organisation, the duties and all the extensive work of all Codex Committees is published on their website [1]. From November 2014 till July 2015 three important Codex sessions took place, where issues regarding "gluten-free" were discussed and adopted: Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) [2], Codex Committee on Methods of Analysis and Sampling (CCMAS) [3] and Codex Alimentarius Commission (CAC) [4]. Furthermore, on 13 December 2014 the EU Regulation 1169/2011 came into force with further improvements for coeliacs [5].

CCNFSDU

The thirty-sixth session of the CCNFSDU took place from 24 - 28 November 2014 in Bali, Indonesia, [2] and was attended by 299 delegates representing 54 Member Countries, one Member Organisation and 25 International Organisations, one of them being the AOECS. At the adoption of the agenda the Committee agreed to discuss among other items under agenda item 11 "Other Business" also the proposals of Austria and USA:

> *"Proposal for an extension of the method recommendation in the Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten (CODEX STAN 118-1979) with a method that also accurately detects the toxic fraction in gluten harmful for individuals intolerant to gluten: the ELISA G12 method" (Austria)*
>
> *"Proposal to amend the Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten (CODEX STAN 118-1979) to add the term "khorasan wheat" (USA)*

G 12 method

At agenda item 11, Austria presented the proposal and recommended that since the ELISA G12 method fulfilled all the criteria stated in 5.1 in the CODEX STAN 118-1979, and was supported by inter-laboratory validation data and international

approvals, it should be incorporated into CODEX STAN 118-1979 and therefore proposed to refer the proposal to CCMAS for consideration.

On behalf of AOECS, I recalled that the threshold for "gluten-free" as defined in CODEX STAN 118-1979 was determined by analysing foods with the R5 method. Therefore, I requested that before taking a decision, consideration should be made regarding the labelling consequences for the term "gluten-free" before incorporating the G12 method into the standard, should the results determined by the G12 in the same food samples be different than those determined by the R5 method.

Conclusion - G 12 method

The Committee agreed to ask CCMAS to examine ELISA G12 as a potential additional method.

Kamut

Already at the session of the CCFSDU in November 2013 [6] the representative of Belgium suggested to replace the term "kamut" with "khorasan wheat" in the Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten.

On behalf of AOECS, I reported that "kamut" is a widely used cereal name well known to consumers, food producers and all Codex member states and requested not to remove this name from the list of gluten-containing cereals as it would be understood that kamut does not contain gluten and this would cause very severe and adverse consequences for the health of the gluten-intolerant population worldwide.

In the Conference Room Document (CRD) 26 for the CCNFSDU session of November 2014 [1,2] the delegation of USA acknowledged the concern of AOECS and in order to facilitate consensus, USA proposed to amend the wording: "and khorasan wheat, which is marketed under the trademarks such as KAMUT".

Conclusion - kamut

The Committee modified the proposed wording and agreed to forward the amendment to CODEX STAN 118-1979 to CAC 38 for adoption:

> ***AMENDMENTS TO THE CODEX STANDARD FOR FOODS FOR SPECIAL DIETARY USE FOR PERSONS INTOLERANT TO GLUTEN (CODEX STAN 118-1979)***
>
> ***2.1.1 Gluten-free foods***
>
> ***Gluten-free foods are dietary foods***
>
> *a) 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 khorasan wheat, which is also marketed under different trademarks such as 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

b) consisting of one or more ingredients from wheat (i.e., all Triticum species, such as durum wheat, spelt, ***and khorasan wheat, which is also marketed under different trademarks such as 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.*

2.1.2 Foods specially processed to reduce gluten content to a level above 20 up to 100 mg/kg

These foods consist of one or more ingredients from wheat (i.e., all Triticum species, such as durum wheat, spelt, and ***khorasan wheat, which is also marketed under different trademarks such as KAMUT****), rye, barley, oats* or their crossbred varieties, which have been specially processed to reduce the gluten content to a level above 20 up to 100 mg/kg in total, based on the food as sold or distributed to the consumer.*

The Codex secretary explained that usually it is not possible to have a trademark name in a Codex Standard, however, because this issue is strongly related to a health hazard, the legal adviser of Codex approved this exception.

CCMAS

The thirty-sixth session of the CCMAS took place from 23 - 27 February 2015 in Budapest, Hungary [3] and was attended by 52 member countries, one member organisation and observers from 11 international organisations, one of them being the AOECS.

A few days before the CCMAS session, the Inter-Agency Meeting (IAM) took place. The report is published in CRD 3 [1, 3]. Regarding methods for gluten determination, their conclusion was:

"The participants discussed the status of the R5 and G12 methods for gluten determination. AACCI reported that two separate collaborative trials had been carried out successfully with R5 and G12 on maize- and rice-containing foods, respectively. AACCI has approved each method with scope restricted to the matrices used in the collaborative trials. AACCI proposes to remove the "all food" designation for R5 methods and include G12. In essence both methods had recently been fully validated by collaborative trial and are published by AACCI as:

R5 method: AACC Intl 38-50.01 (immunoassay procedure (validated using maize matrices)) and G12 method: AACC Intl 38-52.01 (immunoassay procedure (validated using rice matrices))"

The Codex Working Group (WG) on the endorsement of methods of analysis and sampling was held on Saturday, 21 February 2015, prior to the plenary session. Among other items, the WG discussed also the method for detection of the toxic fraction in gluten harmful for individuals, which was one of some subjects on agenda item 2, published in document CX/FH 15/36/2 [3]. The delegate of AACCI repeated their suggestion as written above.

On behalf of AOECS, I did not agree to delete "all foods" because coeliacs need a reliable method for all various kinds of foods in the CODEX STAN 118-1979. To focus on maize matrices and rice matrices is not really helpful in practice because there are a large variety of gluten-free ingredients/foods on the market and the question appears how to deal with potential different analytical results of the same food sample if both methods will be classified as Type 1 method. For the time being we do not have enough data to be sure that both methods show the same results in various food samples. Further on, it is well known that the G12 antibody has a weak cross-reactivity to some oats samples and it needs to be investigated whether the positive signal comes from contamination with wheat, rye or barley or from a specific oats cultivar which cross-reacts with the G12 antibody.

The conclusion of the consideration of the WG on the endorsement of methods of analysis and sampling is published in CRD 2 [1,3]:

> *"Method for detection of the toxic fraction in gluten harmful for individuals*
>
> *The WG noted that it was not possible to have two Type I methods and that if ELISA G12 method were to be added, the provision in the Standard would need to be differentiated to allow for both methods to be included as Type I methods. The WG noted that ELISA G12 had been validated for gluten-free foods, rice matrices, whereas R5 had been validated for gluten-free foods, maize matrices. Both methods had recently been fully validated by collaborative trial and are published by AACCI as:*
>
> *R5 method: AACC Intl 38-50.01 (immunoassay procedure (validated using maize matrices)) and*
>
> *G12 method: AACC Intl 38-52.01 (immunoassay procedure (validated using rice matrices))*
>
> *The WG recommended that decision in this regard should be taken by CCNFSDU."*

In the plenary session during the consideration of this item the comments were the same as in the WG and few other delegations participated in the discussion, but did not have other proposals or suggestions for a solution. Usually the recommendation of the WG is adopted in the plenary session and this was also the case regarding this item.

Conclusion - CCMAS

CCMAS recommended that decision in this regard should be taken by CCNFSDU.

Further items on the agenda important for coeliacs were the consideration of a discussion paper on the development of procedures/guidelines for determining equivalency to Type 1 methods. This paper had not been completed in time to allow for discussion in the electronic WG space, it was presented as a first draft and work will continue.

At the agenda the item “Review and Update of Methods” the R5 was not part of the work package, however, work will go on to review all existing methods.

CAC

The thirty-eight session of the CAC took place from 6 - 11 July 2015 in Geneva, Switzerland [4] and was attended by delegates from 140 Member Countries, one Member Organisation, 33 International Governmental and Non-Governmental Organisations, including UN agencies and AOECS.

The most important task of the Codex Commission is to adopt Codex Standards and Guidelines which are usually taken into national legislation by governments. Also the World Trade Organisation refers to Codex Standards in matters of import, export and legal issues. In the Commission session, also decisions were taken about the budget, the Trust Fund (= financial support for delegates from developing countries to participate in Codex sessions and to implement Codex Standards in their countries), adoption of new work or to revoke existing Standards.

The most important issue on the agenda for coeliacs was item 5 (f) “Amendment to Codex Standards and Related Texts” which concerned the above written amendments to CODEX STAN 118-1979 - see under “Conclusion - Kamut” of this paper.

Conclusion - CAC

The Codex Commission adopted the amendments without any further discussion.

FAO GM Food Platform

During lunch break also “side events” took place. The most important session was the FAO GM Food Platform, which is an online platform to share information on the safety assessment of foods derived from recombinant DNA plants authorized in accordance with the “Guideline for the conduct of food safety assessment of foods derived from recombinant DNA plants”. The FAO GM Foods Platform is open to the public. As of 18 June 2015, a total of 164 members have nominated focal points for the Platform. 105 members have completed their country profile and the Platform hosts 733 national records of the results of GM food safety assessment. After some presentations delegates could make some comments or ask questions.

Also this year, I recalled § 42 and 43 of the “Guideline for the conduct of food safety assessment of foods derived from recombinant DNA plants”, informed about the incidence/prevalence of CD and gluten sensitivity and asked if there is any control

mechanism installed that the text of the Guideline not to transfer genes from gluten-containing cereals to foods gluten-free by nature is really considered in practice. Only the delegate of Australia answered that he was aware of this issue.

Conclusion - FAO GM Food Platform

How is this very imported issue monitored by the rest of the 163 members of the FAO GMO Food Platform?

EU Regulation 1169/2011

This Regulation [5] on the provision of food information to consumers comprises several Regulations and Directives of previous years. The labelling of gluten containing substances or products in pre-packed foods remained unchanged and is published in Annex II. Some new and further improvements for consumers in terms of general labelling information are published, the most important is written in Article 44: National measures for non-prepacked food. This very important item came into force on 13 December 2014 and enables any consumer suffering either from allergy or intolerance to obtain correct information also e.g., in any restaurant in the EU which uses a substance from Annex II in a food. The first item in this Annex II is “cereals containing gluten and products thereof”.

Conclusions

As in the past, also this year some benefits regarding food regulatory improvements for coeliacs were achieved. However, some important items are still open for discussion, consideration and further work.

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6. Report of the thirty-fifth session of the Codex Committee on Nutrition and Foods for Special Dietary Uses, Bad Soden am Taurus, Germany, 4 - 8 November 2013.

7.2 Update on activities CEN/TC 275/WG 12 "Food allergens"

Rupert Hochegger

Austrian Agency for Health and Food Safety (AGES), Vienna, Austria

Abstract

Molecular and immunological analytical methods are currently the recognized tools for determination of DNA and protein analytes in foods.

Sampling and analysis methods used in the context of official controls shall comply with relevant Community rules or, if no such rules exist, with internationally recognized rules or protocols, for example those that the European Committee for Standardization (CEN) has accepted or those agreed in national legislation.

One of the goals of CEN technical committee 275 “food analysis” is to support the harmonization of molecular and immunological methods for detection, identification and quantification of specific DNA sequences and specific proteins in foods, which produce comparable results when performed at different laboratories.

The scope of working group (WG) 12 - food allergens - is to standardize methods for the detection and determination of potentially allergenic substances in foodstuffs or markers for their presence, including but not limited to immunological and molecular-biological methods.

At the last WG meeting it was agreed to draft a document for the definition of minimum performance requirements for ELISA methods. The standard document shall be allergen-specific and define analytical performance criteria of the respective method. The scope of this document is to provide recommendations on how ELISA methods for food allergen analysis shall be evaluated and validated in order to ensure that comparable and reproducible results are obtained in different laboratories.

To be widely used by official control laboratories, there is synergy between the recommendations that will be made within this document and those of the Codex Alimentarius Commission.

8 Perspectives and action plan of the PWG

Peter Koehler

Deutsche Forschungsanstalt für Lebensmittelchemie, Leibniz Institut, Freising, Germany

The Prolamin Working Group executive meeting and joint discussion held on 9 October 2015, led to the decisions and statements outlined below.

Action plan

I. Analytical

- Peter Koehler is responsible for the PWG gliadin reference material (peter.koehler@tum.de).
- PWG gliadin will continue to be the reference material supported by the group. Material for 5 - 10 years is still on stock.
- The group will wait for the first MoniQA reference flours and will then decide how to proceed with novel extracted reference material(s). No additional reference will be prepared before, because a suitable reference material is available.
- An increase of the price was discussed, but without final decision.

II. Clinical

- For the symposium of the 2016 meeting the topic "Enzymatic gluten degradation" has been selected. Speakers will be Andreas Frey and Eva Helmerhorst.
- A speaker on innate lymphoid cells or the importance of the microbiome in gluten intolerances will be invited.

III. Members, Policy

- Olivier Tranquet and Rudolf Valenta have been asked to join the group and have accepted.
- A joint EU research project (Horizon 2020) could deal with all "Wheat hypersensitivities". A suitable call is looked for or will be generated (Troncone, Schuppan, Gilissen, Koning, Koehler).
- The website will be further improved (Chirdo, Koehler).
- This printed, citable book (print run: 300 copies with ISBN number) was made possible by funding of Dr. SCHÄR GmbH/Srl, (Burgstall, BZ, Italy) and by the help of Mrs. Anneliese Stoiber and Dr. Gaby Andersen, Deutsche Forschungsanstalt für Lebensmittelchemie (Freising, Germany). It will be distributed among leaders of opinion in gluten analysis and clinical medicine. An electronic version can be downloaded from the PWG website.

Next meeting: 2016

We are very pleased to announce the venue for our meeting in 2016:

Valencia, Spain

Host:

Prof. Dr. Cristina M. Rosell
Institute of Agrochemistry and Food Technology (IATA-CSIC)
E-mail: crosell@iata.csic.es

Time: 22 - 24 September 2016

Focus of the meeting:

- Enzymatic gluten degradation
- Gluten quantitation (immunochemical/non-immunochemical)
- Gluten reference materials for analytical and clinical studies

The meeting will be limited to 55 participants and attendance is by invitation only. Invitations have been sent by March 2016. Registration deadline will be June 15, 2016.

For registration please contact:

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Very special thanks to the hosts for this kind invitation!