Proceedings of the 31st Meeting

WORKING GROUP on PROLAMIN ANALYSIS and TOXICITY

Edited by
Peter Koehler

28 - 30 September 2017
Minden, Germany

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Impressum

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Cover picture* and picture of participants
Thomas Mothes

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* Cover picture: Impressions of the tour of the sourdough factory of Ernst Böcker GmbH & Co KG
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Preface

Markus Brandt from Böcker GmbH has been attending the meetings of the Working Group on Prolamin Analysis and Toxicity (PWG) since many years. Several years ago he approached me and asked if it would be possible for the Böcker Company to host the PWG meeting. In 2016, I informed him that this would become reality in 2017 and Markus and his team started organising the 31st PWG meeting with great enthusiasm. Together with Karoline Schreiber, Markus Düsterberg and the entire Böcker family, he succeeded in preparing an unforgettable conference. The meeting was held in the Lindgart Hotel in Minden, Germany, from 28 to 30 September, 2017. Markus and his team were present during the entire meeting. As in previous meetings in Germany, the German Coeliac Society (DZG), namely Judith Glöggler, perfectly managed the registration of the participants. Apart from the group members the audience comprised invited speakers, guests from academia, industry, and international coeliac societies. Representatives from cereal starch producers, producers of gluten-free foods, as well as manufacturers of kits for gluten analysis and of kits for antibody tests in the serology of coeliac disease participated from industry. The participants had very interesting one-and-a-half days of presentations, discussions and networking.

As far as gluten analysis is concerned, the 2017 meeting was focussed in particular on liquid chromatography/mass-spectrometry (LC-MS) as an evolving technique that is still waiting for approval in routine analysis up to now. Analytical and clinical work in the field of coeliac disease (CD), non-coeliac gluten sensitivity (NCGS), wheat allergy and gluten done in the labs of PWG members as well as results of guests and invited speakers were presented in 19 talks and lively discussed at the meeting. As usual, legal and regulatory aspects of gluten analysis and labelling concluded the meeting. A symposium with presentations looking at the importance of the small intestinal microbiome for CD and NCGS from different perspectives was organised and perceived very positively by the audience.

The year 2017 was very challenging for me because I changed my job after serious problems in and with my former institute, which appears to throw away the long tradition of cereal research in this instant. This situation made me think of resigning from the position of chairman of the PWG, but my wife, friends and colleagues encouraged me to continue. Another consequence was that I am no longer able to distribute the PWG reference material and it has turned out a real challenge to find an organisation that is willing to take over this task. I would like to express my special thanks to Markus Brandt who became a fried during the meeting, to his team, as well as to Judith Glöggler of DZG for her professional help in the organisation of the meeting. Finally, I express my gratitude to all friends, colleagues, sponsors and participants for their inspiration and ongoing support of the PWG and the meeting.

Esslingen, March 2018 Peter Koehler

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1 Executive summary

Nineteen presentations covered all aspects related to gluten, coeliac disease and legal issues. Starting with food technological aspects and aspects of analytical gluten tracing, the programme included breeding and genetic aspects of wheat, post-translational gluten modification by processing, antibodies and serology, alternative therapies of coeliac disease, non-coeliac gluten sensitivity, aspects of the patho-mechanism of coeliac disease and the significance of the small intestinal microbiome in the diet of coeliac disease patients. Finally, legal issues of gluten were discussed.

Analytical session

Seven presentations dealt with analytical challenges of gluten, four of them used liquid chromatography/mass-spectrometry and this showed the increasing importance of this technique. Also the question, which antibody would be most suitable to quantitate gluten in routine analysis was intensely discussed by the participants. It appears to be a fact that different antibodies yield different results in the quantitation of gluten. More action is necessary in this field and the PWG was asked to start scientific action regarding gluten determination by immunochemical methods. Finally, breeding and genetics were on the agenda. For the first time, an approach using CRISPR/Cas9 to modify and/or remove gliadins from wheat was presented.

Clinical session

This session included five presentations that started with an overview on gluten in coeliac disease and non-coeliac gluten sensitivity. A rapid HLA DQ2/DQ8 test was presented that appears to be a promising tool to perform mass screening of coeliac disease. A novel therapeutic approach for coeliac disease therapy by specific inhibition of tissue transglutaminase, a study to get more insights into gluten-specific T cells and a review of serological studies to diagnose coeliac disease completed the clinical session.

Symposium: The role of intestinal microbiota in coeliac disease

Three presentations on the role of microorganisms in coeliac disease and non-coeliac gluten sensitivity were given. In one talk the modification of gluten peptides as affected by microorganisms was described. The second presentation gave an extensive overview on the small intestinal microbiome of healthy and coeliac disease patients and left the question whether the microbiome is the reason for or the consequence of the disease. Finally, the influence of the food constituents gluten and FODMAPS on the small intestinal microbiome was discussed. Al in all, the symposium showed that research on the small intestinal microbiome is an exciting new field in coeliac disease research.

Participants of the 31st Meeting of the Working Group on Prolamin Analysis and Toxicity (PWG), Minden, Germany, 28 – 30 September 2017

2 List of Participants

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3 Programme

THURSDAY, 28 September 2017

20:00 Arrival of Prolamin Working Group and all participants
Informal get-together with dinner
Welcome by Georg Böcker
Location: Lindgart Hotel, Minden

FRIDAY, 29 September 2017

09:00 Opening of the meeting (Peter Koehler)

09:05 Sourdough in Gluten-Free Bread Making
Markus Brandt, Minden, Germany

09:20 Gluten Quantitation by LC-MS: Method of Choice?
Katharina Scherf, Freising, Germany

10:00 Analytical research reports

- *Chirdo, Ciclitira, Feighery, Koehler, Koning, Lundin, Mothes, Schuppan, Smulders, Tranquet; guests*

11:00 Coffee break

11:30 Analytical research reports (continuation)

12:30 Clinical research reports

- *Catassi, Chirdo, Ciclitira, Feighery, Koning, Lundin, Mothes, Schuppan, Troncone, Valenta; guests*

12:50 Lunch

14:00 Clinical research reports (continuation)

15:40 Coffee break

15:40 The Prolamin Working Group Executive Meeting (members only)

17:30 Bus departure to Böcker sourdough factory and tour of production site in small groups

19:30 Joint dinner for all participants
Location: Remontenstall (Campus MTZ),
Artilleriestraße 17, Minden

SATURDAY, 30 September 2017

SYMPOSIUM

09:00	"The role of intestinal microbiota in coeliac disease" *Chair: Fernando Chirdo, La Plata, Argentina*
09:05	Role of bacterial metabolism in gluten peptide modification *Elena Verdú, Hamilton, Canada*
09:45	Gut microbiome trajectory and coeliac disease risk *Yolanda Sanz, Valencia, Spain*
10:25	Influence of diets on the microbiome from patients with NCGS *Walburga Dieterich, Erlangen, Germany*

10:45 Coffee break

11:15 Discussion of current developments concerning gluten analysis, clinical and legal aspects

Statements by participating organisations, representatives from industry, and guests

Outline: action plan 2018 of the Prolamin Working Group

13:00 Lunch and farewell

Afternoon

Extra time for informal meeting and additional Prolamin Working Group meeting concerning action plan

Tour of Minden and joint dinner

SUNDAY, 1 October 2017

Departure of the Prolamin Working Group

4 Analytical research reports

4.1 Sourdough as leavening agent in gluten-free bread making

Markus Brandt, Markus Düsterberg

Ernst Böcker GmbH & Co. KG, Minden, Germany

History of sourdough fermentation

Fermentation for processing of cereals is probably in use since at least 12.000 years [1], and the typical sourdough genera *Saccharomyces* and *Lactobacillus* were identified in fermented cereals excavated in Xinjiang (China) [2]. After being harvested, cereal grains are in a resting state, hard and dry. Only after comminution or addition of water and subsequent soaking they can be consumed. Addition of water activates endogenous enzymes and microbiota – a fermentation that changes the chemical composition of the grain starts, e.g., proteins and carbohydrates are degraded [3]. Until World War I, sourdough was the predominant leavening agent for bread production. Lactobacilli and yeasts are – in variable amounts – the dominating microorganisms in sourdough and both produce the leavening agent carbon dioxide. From the 15th century on, top-fermenting brewer's yeast was introduced for bread baking and further developed. At the end of 18th century, industrial production of baker's yeast on cereal substrates begun. With the introduction of aeration of the mash in 1870, production of yeast was economically advantageous. Lactic acid bacteria were used as "protective cultures" for preventing infections of the yeast. With the short supply of cereals during World War I, molasses as carbon source and malt seedlings as nitrogen source were introduced as alternatives to cereals [4]. In the 1920ies, malt seedlings were replaced by ammonia salts because the availability of ammonia increased strongly after introduction of the Haber-Bosch-process and baker's yeast replaced sourdough as main leavening agent for bread production in the following years. The time, in which bread dough underwent fermentation, decreased from hours or days to only 30 min proofing time in, e.g., the Chorleywood bread process.

Sourdough fermentation of gluten-free doughs

Sourdough fermentation is one of the strategies proposed to reduce or prevent coeliac-immunogenicity through food [5], because in contrast to baker's yeast the microorganisms in sourdough need to grow in the dough and do not only produce carbon dioxide from sugar. Growth requires a broad spectrum of available sugars, amino acids, peptides which are released from the cereal matrix by endogenous or microbial enzymes. The most traditional technique for sourdough fermentation is backslopping. This is the inoculation of the raw material with a small amount of dough

from a previous successful fermentation. This results in a well-adapted microbiota. Natural pure cultures (“Reinzucht” starters) are derived from these continuously propagated fermentations and have stable associations of several lactic acid bacteria and usually one yeast strain. As an example, Fig. 1 shows the leavening activity of baker’s yeast and a rice sourdough starter. Volume was measured in a graduated cylinder. Dough with microbial inactive dried rice sourdough was used as control. Leavening with sourdough resulted in the same volume as leavening with baker’s yeast and needed around 1 h more proofing time for bread production.

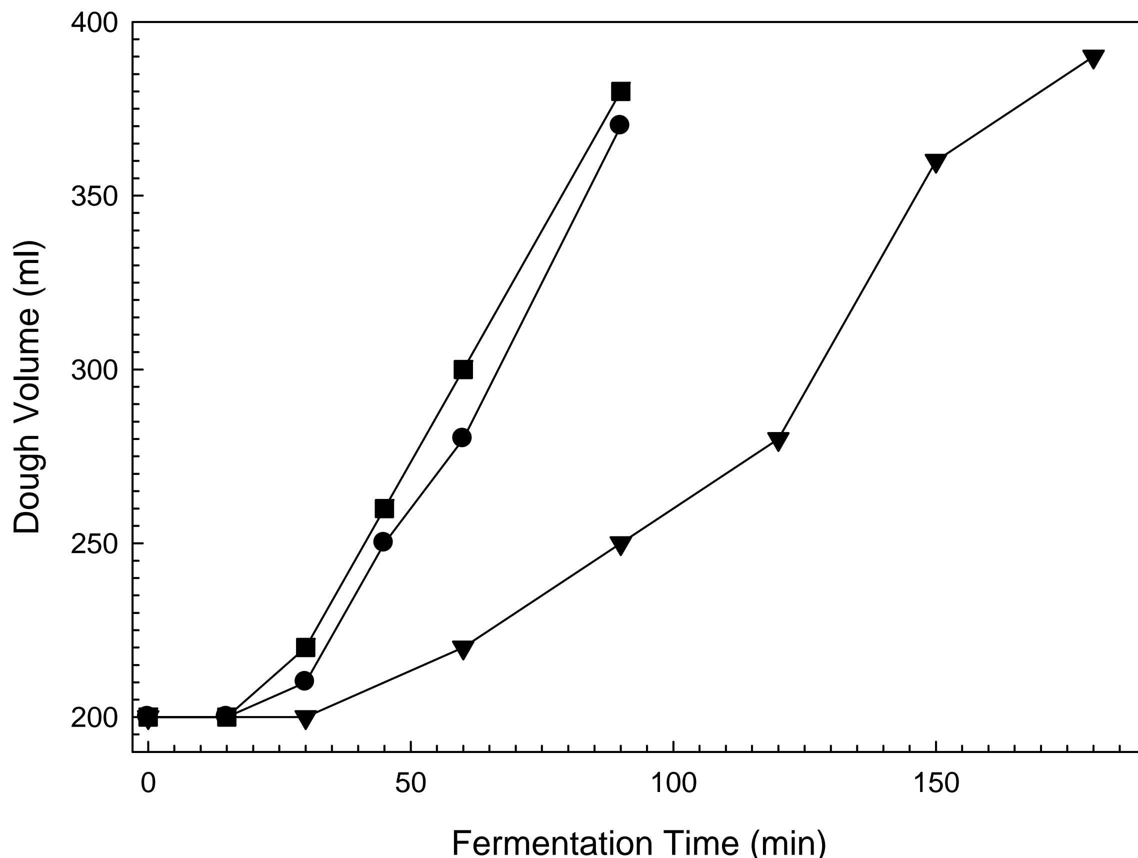

Figure 1. *Increase in dough volume of oat bread doughs (dough yield 218) at 28 °C. The doughs were leavened with (■) 2 % baker’s yeast without dried sourdough, (●) 2 % baker’s yeast with 5 % dried rice sourdough and (▼) 5 % liquid rice sourdough (Böcker Reinzuchtsauerteig Reis)*

The resulting sourdough-leavened oat breads had lower initial hardness (Fig. 2) and a porosity comparable to the breads leavened with baker’s yeast (data not shown). Other gluten-free cereals and pseudo-cereals can also be leavened by sourdough, if the microbiota fits with the carbohydrate composition of the substrate.

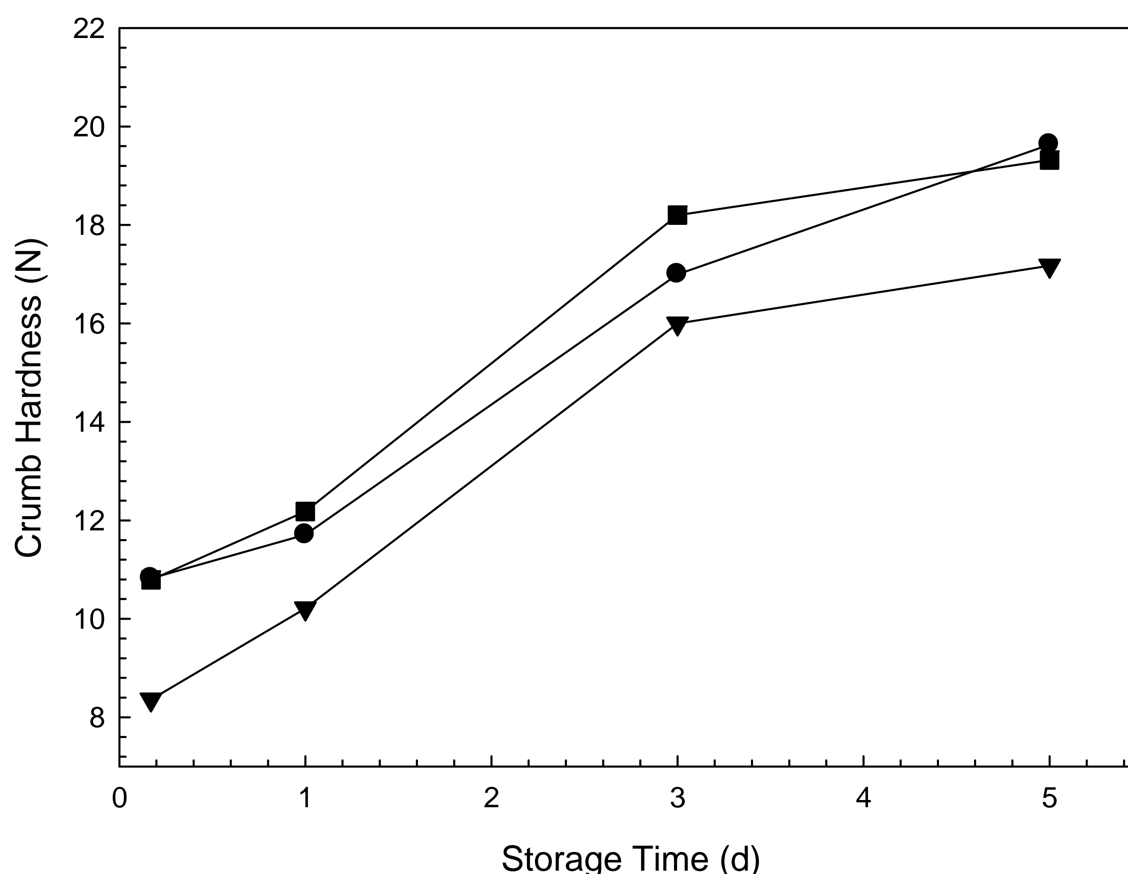

Figure 2. *Staling kinetics at 20 °C of oat breads leavened with (■) 2 % baker's yeast without dried sourdough, (●) 2 % baker's yeast with 5 % dried rice sourdough and (▼) 5 % liquid rice sourdough (Böcker Reinzuchtsauerteig Reis). Hardness was measured with a SMS TA.XT Plus Texture Analyser with a 12 mm spherical probe at 30 % compression on 2 cm bread slices.*

Conclusions

Traditional leavening without baker's yeast needs longer proofing times than conventional bread making, on the other hand, a larger amount of the used flour is subject of the positive effects of fermentation: improvement of flavour, shelf-life or nutritional composition.

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4.2 Gluten quantitation by LC-MS: method of choice?

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Introduction

Any quantitative analytical method relies on the measurement of a certain signal, e.g., UV absorption, and comparison of the signal to that of a known substance, which serves as a reference material. This approach works well in case of analytes with a defined chemical structure, but is not as straightforward in case of polymeric substances or compound mixtures. Gluten, a highly complex mixture of mono-, oligo- and polymeric proteins, is a challenging analyte, because the composition of gluten shows great variability depending on species, cultivar, origin and growing conditions [1]. Despite these issues, analytical methods for gluten should be capable of detecting all gluten proteins, ideally with comparable specificity and sensitivity. Enzyme-linked immunosorbent assays (ELISAs) fulfil the requirement of adequate sensitivity with limits of quantitation down to 3 mg gluten per kg of the food that makes quantitation of gluten contents possible well below the regulatory threshold of 20 mg/kg for gluten-free products [2]. Regarding specificity, ELISAs are known to experience some difficulties due to slight cross-reactivity to oats using the G12 antibody or due to overestimation of rye and barley in comparison to wheat with the R5 antibody [3]. Furthermore, it is not possible to identify the source of gluten (i.e., wheat, rye or barley) with the current commercially available ELISA kits. Most antibodies are specific to certain prolamin types, but do not react to all gluten protein types (Fig. 1) in a similar way. Despite these disadvantages, ELISAs are fast and comparatively cheap, suitable for routine analyses and can be performed without specialised equipment. That is why ELISAs are most commonly used for gluten analysis to assess regulatory compliance. However, there is no independent protein-based reference method for verification of ELISA results, although this is an urgent need.

Liquid chromatography-mass spectrometry (LC-MS) is a versatile, specific and sensitive technique to detect all sorts of analytes including peptides. When using LC-MS for gluten analysis, several points have to be considered. First of all, gluten proteins have to be extracted and digested to peptides and the choice of enzyme (trypsin, chymotrypsin or pepsin) has a substantial influence on the results [4]. Then, the gluten digest is typically cleaned-up prior to analysis using solid phase extraction or other desalting methods. For LC-MS, there are several choices to be made, including the instrument itself, data-dependent vs. data-independent acquisition, untargeted vs. targeted analysis and subsequent data evaluation using bioinformatics tools [5] and an approach for conversion of peptide contents to gluten content using

suitable reference materials. Ideally, LC-MS should detect all coeliac disease (CD)-immunogenic and toxic peptides known to date, but in case of wheat allergies and non-coeliac gluten sensitivity other peptides from non-gluten proteins, such as amylase-trypsin-inhibitors, would have to be considered as well. Several LC-MS methods to detect CD-active peptides have been published [6-8], but most of those focused on two to ten marker peptides, a selection that appears rather small compared to the about 1000 known CD-active peptides. Selection criteria for relevant peptides have to be defined carefully and well-characterised gluten reference materials are essential.

Materials and methods

Isolation of gluten reference materials

Gluten reference materials can be obtained from wheat, rye and barley flours following the strategy depicted in Fig. 1. After defatting the flours, the albumins and globulins were removed by extraction with salt solution, the prolamins extracted with 60 % (v/v) aqueous ethanol and the glutelins with 50 % aqueous propanol at pH 7.5 and 60 °C under reducing conditions (1 % dithiothreitol, w/v).

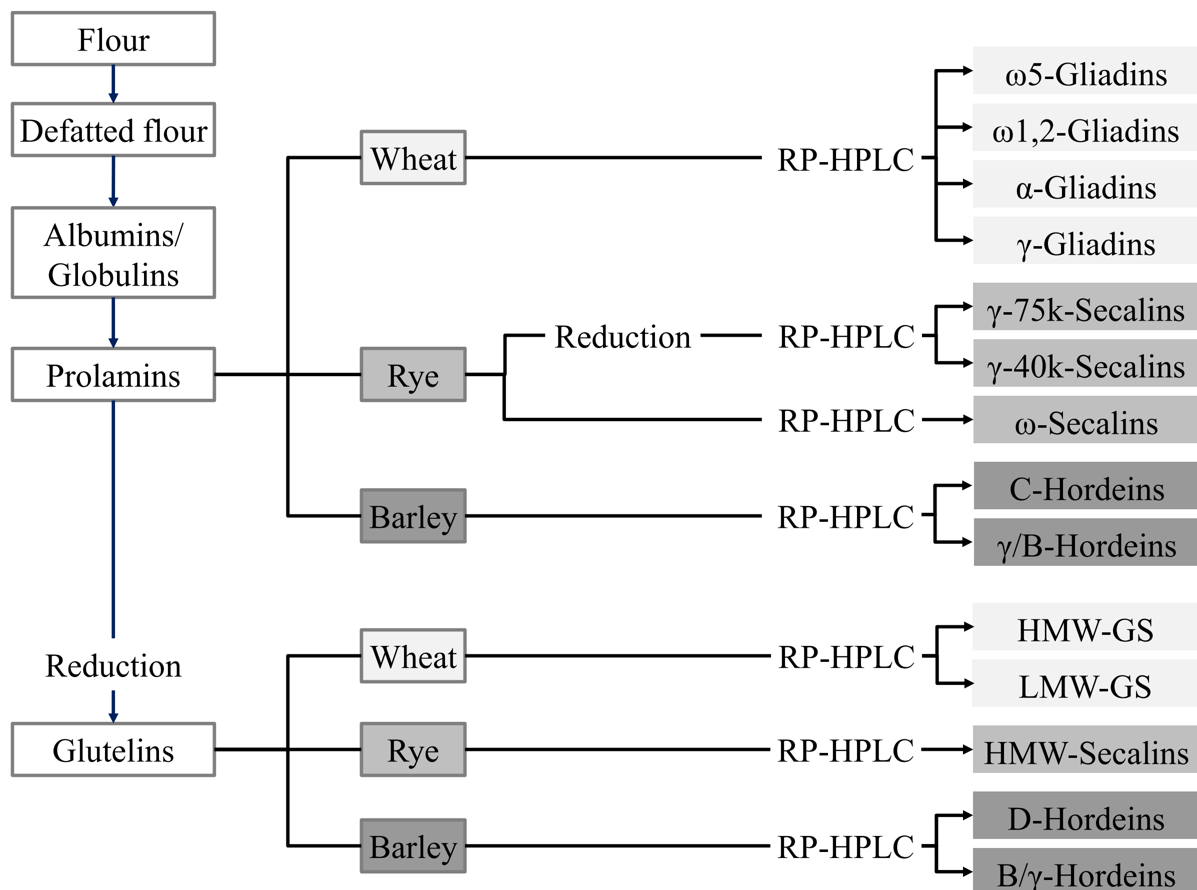

Figure 1. *Preparation procedure for gluten protein fractions (prolamins and glutelins) as well as gluten protein types from wheat, rye and barley flours.*

The prolamin and glutelin fractions were concentrated to about half of the original extract volume using a rotary evaporator, dialysed against distilled water and lyophilised. The fractions were reconstituted in the respective extraction solvents and the gluten protein types separated using preparative reversed-phase high performance liquid chromatography (RP-HPLC). After lyophilisation, the gluten protein types were profoundly characterised with analytical RP-HPLC, sodium dodecyl sulphate gel electrophoresis (SDS-PAGE), N-terminal sequencing, LC-MS of the intact proteins and LC-MS of chymotryptic digests [9]. All methods confirmed that the respective gluten protein types had been highly enriched. Only B- and γ-hordeins could not be separated, because they were present in both the prolamin and glutelin fractions and also had similar retention times on the RP-HPLC column.

Strategy for targeted LC-MS/MS

The purified gluten protein types were used as reference materials for gluten quantitation by LC-MS/MS. First, the gluten protein types were digested with chymotrypsin to identify specific peptides that uniquely occur within each protein type using untargeted LC-MS/MS. Then, the corresponding prolamin and glutelin fractions and gluten extracts from wheat, rye and barley flours were also analysed to select peptides that were consistently detected in all three protein isolates (type, fraction and total gluten extract). From this pool of peptides, the final 16 wheat, 7 rye and 7 barley marker peptides were required to contain 8-20 amino acids and no cysteine. One ^{13}C- and ^{15}N-labelled peptide standard per cereal species was used that allowed quantitation by stable isotope dilution assay for the respective peptide and internal calibration for the other peptides. The most abundant precursor to product ion transitions were chosen, the collision energies optimised and response factors determined (Fig. 2).

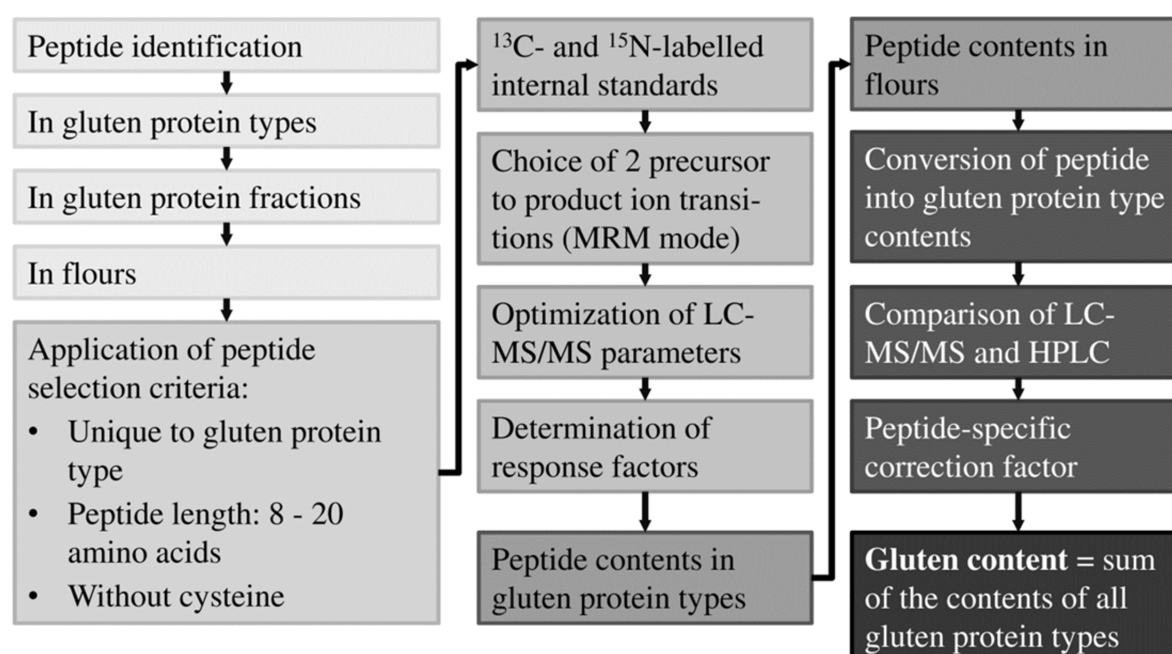

Figure 2. *Strategy to develop a comprehensive LC-MS/MS method for gluten from wheat, rye and barley.*

Multiple reaction monitoring (MRM) on a triple-stage quadrupole mass spectrometer (TSQ Vantage, Thermo Fisher Scientific, Dreieich, Germany) was used in the ESI positive mode to detect the 30 selected marker peptides and the 3 stable isotope labelled internal standards. The quantitation of marker peptide contents in gluten protein types and the gluten extract from flour allowed a conversion of peptide contents into gluten contents. The results of LC-MS/MS were verified by comparing the gluten contents to those determined by RP-HPLC and introduction of peptide-specific correction factors that accounted for losses during digestion. Finally, the gluten contents of wheat starches and raw materials for rye-based sourdoughs determined by LC-MS/MS were expressed as sum of all gluten protein types. The LC-MS/MS results were compared to those of other methods, namely R5 ELISA, RP-HPLC and gel permeation HPLC with fluorescence detection (GP-HPLC-FLD) [10].

Results and discussion

The wheat, rye and barley gluten marker peptides included at least one peptide per gluten protein type to enable comprehensive gluten monitoring also in foods from which gluten is partially removed during processing (Tab. 1).

Table 1. *Amino acid sequences of wheat, rye and barley gluten marker peptides.*

Protein type		Peptides
LMW-GS	P1-4	QQQPLPPQQTFPQQPL, GQQPQQQQL, VQQQIPVVQPSIL, SIILQEQQQGF
HMW-GS	P5-7	LQPGQGQQGY, TASLQQPGQGQQGHYPASL, HVSVEHQAASL
γ-gliadins	P8-10	ASIVAGIGGQ, NIQVDPSGQVQW, LQPQQPQQSFPQQQQPL
α-gliadins	P11-13	LQLQPFPQPQLPYPQPQPF, FQPSQQNPQAQGF, RPQQPYPQPQPQY
ω5-gliadins	P14	QQYPQQQPSGSDVISISGL
ω1,2-gliadins	P15-16	GSSLTSIGGQ, FPHQSQQPF
γ/B-hordeins	P17-19	AIDTRVGV, QQPQPQQGQQQQVPQSVF, AQQQPSIEEQHQL
D-hordeins	P20-21	GGGLTTEQPQGGKQPF, TQQKPGQGYNPGGTSPL
C-hordeins	P22-23	IIPQQPQQPFPLQPHQPY, RQLNPSSQEL
γ-75k-secalins	P27-29	ASIETGIVGH, SQLEVVRSL, QQFPQQPQQPFPQQPL
ω-secalins	P30-31	RQLNPSEQEL, AQQPEQLISQQPFPL
HMW-secalins	P32-33	LTSPQQPGQGQQGY, STSPRQPGQGQQEY

HMW-GS, high-molecular-weight glutenin subunits, LMW-GS, low-molecular-weight glutenin subunits, underlined peptides were used as stable isotope labelled standards

The concentrations of the marker peptides were determined in each gluten protein type and the results ranged from 0.5 µg/mg ω-secalins (P30) to 48.8 µg/mg D-hordeins (P21), indicating that some peptide isoforms occurred more frequently than others and that the efficiency of the enzymatic digest was also highly dependent on the specific

peptide and protein type. The marker peptide concentrations were also analysed in the gluten extract from the flour mixture and the values lay between 0.1 µg/g flour (P31 from ω-secalins) and 1794 µg/g flour (P17 from γ/B-hordeins), again depending on the overall content of the protein type within gluten and peptide yields. Peptides P6, P16 and P32 were below the respective limits of detection in the gluten extracts. These peptide concentrations were used to calculate the content of each protein type in flours. For verification of the LC-MS/MS results, the content of each protein type determined by RP-HPLC was defined as 100 % and the recoveries by LC-MS/MS were calculated. The recoveries ranged from 1.5 % (HMW-secalins determined using P33) to 224 % (ω1,2-gliadins determined using P15) and also varied for the different peptides that were derived from the same protein type (e.g., from 64 % to 156 % using either P19 or P17 to determine γ/B-hordeins). To account for these differences, peptide-specific correction factors were introduced to ensure 100 % recovery of each protein type.

The LC-MS/MS method together with the final calculation procedure was used to quantitate gluten in samples of wheat starch (Tab. 2) and raw materials for rye sourdough fermentation (Tab. 3) as well as the resulting sourdoughs. The gluten contents determined by LC-MS/MS were expressed as sum of the contents of individual protein types provided that at least one peptide was detected. In comparison, the gluten contents determined by RP-HPLC or GP-HPLC-FLD are the sum of prolamin and glutelin fractions. The gluten contents obtained using ELISA are based on duplication of the prolamin content as recommended by Codex [2].

Table 2. *Contents of wheat marker peptides and resulting contents of gluten protein types and gluten, respectively, analysed by LC-MS in two wheat starch samples and comparison of the results to GP-HPLC-FLD and R5 ELISA.*

Pep-tide	**Protein type**	**Peptide content (µg/g)**	**Content of gluten protein type (µg/g)**	**Gluten content (µg/g)**		
		LC-MS	LC-MS	LC-MS	GP-HPLC-FLD	R5 ELISA
Wheat starch 1				117.5^{A}	103.6^{B}	82.5^{C}
P4	LMW-GS	0.9 ± 0.1	92.2 ± 20.1			
P8	γ-gliadins	0.9 ± 0.1	25.3 ± 9.7			
Wheat starch 2				2665.7^{A}	6543.3^{B}	7022.0^{B}
P4	LMW-GS	8.5 ± 0.6	755.7 ± 56.6			
P7	HMW-GS	7.7 ± 1.1	743.7 ± 107.8			
P8	γ-gliadins	19.0 ± 2.5	554.2 ± 71.8			
P11	α-gliadins	2.3 ± 0.2	479.4 ± 40.2			
P15	ω1,2-gliadins	0.7 ± 0.1	132.7 ± 10.8			

Different superscript letters denote significant differences (ANOVA, Tukey‘s test, $p < 0.05$) between results of different methods within one sample, n = 3, mean ± standard deviation, GP-HPLC-FLD, gel permeation high performance liquid chromatography with fluorescence detection, Scherf *et al.*, 2016 [10], HMW-GS, high-molecular-weight glutenin subunits, LMW-GS, low-molecular-weight glutenin subunits, R5 ELISA, Ridascreen Gliadin, R-Biopharm, Darmstadt, Germany

Table 3. *Contents of rye marker peptides and resulting gluten contents analysed by LC-MS in different rye samples and comparison of the results to GP-HPLC-FLD and R5 ELISA.*

Sample	**Peptide**	**Protein type**	**Peptide content (µg/g)**	**Gluten content (µg/g)**		
			LC-MS	LC-MS	RP-HPLC	R5c ELISA
RSB1	P27	γ-75k-secalins	31.9 ± 1.3	8.9^{A}	23.3^{B}	191.1^{C}
RSB2	P27	γ-75k-secalins	36.5 ± 4.3	10.2^{A}	21.0^{B}	134.3^{C}
RSB3	P27	γ-75k-secalins	43.6 ± 2.5	12.2^{A}	24.5^{B}	230.6^{C}
RWF1	P27	γ-75k-secalins	22.2 ± 0.4	6.2^{A}	12.5^{B}	155.8^{C}
RWF2	P27	γ-75k-secalins	42.9 ± 3.5	12.0^{A}	25.1^{B}	314.6^{C}
RWF3	P27	γ-75k-secalins	38.2 ± 2.1	10.7^{A}	25.0^{B}	178.1^{C}

Different superscript letters denote significant differences (ANOVA, Tukey's test, $p < 0.05$) between results of different methods within one sample, n = 3, mean ± standard deviation, RP-HPLC, reversed-phase high performance liquid chromatography with UV detection, R5c ELISA, Ridascreen Gliadin competitive, R-Biopharm, Darmstadt, Germany, RSB, rye semolina bran, RWF, rye wholemeal flour

Significant differences between the three analytical methods were found for wheat starches 1 and 2, rye semolina bran samples 1-3 and rye wholemeal flours 1-3. All three values for gluten content lay in the same range for wheat starch 1, but the LC-MS/MS values for wheat starch 2 were only about half the values determined by ELISA and GP-HPLC-FLD. One possible explanation is that other gluten proteins may have been present that did not contain the specific peptides for which the targeted LC-MS/MS method is selective. The gluten contents determined in the rye-based raw materials for sourdough fermentation showed a certain tendency with LC-MS/MS yielding the lowest and R5 competitive ELISA the highest values. The R5 antibody is known for its high affinity to rye and resulting overestimation of rye gluten when the assay is calibrated to wheat [3], so that the high values observed here were not unexpected. The LC-MS/MS results were about half the values analysed by RP-HPLC, most likely because only one peptide from γ-75k-secalins was detected and none from the other rye protein types. All peptides were below the limits of detection in rye sourdough samples, indicating that they had been hydrolysed during fermentation.

When thinking back to the considerations that have to be made when establishing an LC-MS method for gluten analysis, the results of this study showed that the conversion of peptide contents to gluten contents is challenging even if well-defined reference proteins are available. Furthermore, LC-MS requires expensive and specialised instrumentation that can only be operated by skilled personnel while method development and data analysis require a high level of expertise. In case of targeted LC-MS, only pre-selected peptides are detected and those with amino acid substitution, deletion or insertion are missed, although they may still be CD-active. This selectivity for a set of pre-defined peptides is the most likely reason why LC-MS values tended to be lower in wheat starch and rye samples compared to the other

methods. One major obstacle for LC-MS data evaluation is the lack of curated plant protein databases that are needed for proper identification of peptide sequences and linking those back to the intact proteins.

On the positive side, LC-MS is capable of differentiating between wheat, rye, barley and other cereals, thus allowing the identification of the source of gluten. The method is applicable to samples from which gluten was partially removed by processing, e.g., in beers or wheat starches. Low limits of detection down to 0.03 mg peptide/kg of food can be achieved using state-of-the-art instrumentation, so that regulatory compliance may be assessed at the 20 mg/kg level of gluten. It is possible to quantitate specific peptides very accurately using stable isotope labelled peptide standards. Depending on the LC-MS instrument, different setups can be used such as various separation procedures, ionisation techniques and full-scan, single- or multiple-reaction-monitoring or advanced possibilities like sequential window acquisition of all theoretical mass spectra. Such untargeted LC-MS analyses generate a huge amount of data in a comparatively short time that can be evaluated retrospectively in various ways. As such, LC-MS is a highly versatile technique that can be adapted to different research questions and offers the possibility of detecting gluten and other allergens in one multi-analyte run, especially as further advances in LC-MS instrumentation will contribute to enhancements in selectivity and sensitivity for high-throughput analyses.

Conclusions

The study presented here is the first LC-MS method to use well-defined reference proteins to detect all gluten protein types from wheat, rye and barley and calculate peptide-specific yields that can be related back to the intact proteins. The entire procedure also highlighted the difficulties inherent in this procedure, some of which can be overcome by using an LC-MS instrument with higher selectivity and sensitivity and employing stable isotope labelled peptide standards for each marker peptide. However, some challenges remain like the lack of curated plant protein databases and the conversion of peptide concentrations to gluten contents, as required by legislation. Therefore, LC-MS can be seen as a complementary method to ELISA, especially for samples where ELISA is known to experience difficulties, such as heat-treated or partially hydrolysed foods.

Acknowledgement

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4.3 Use of LC-MS to detect gluten

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No manuscript provided.

4.4 Assessing the effect of fermentation and gluten-reduction strategies using LC-MS

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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. Gluten measurement in fermented products remains controversial [1-3]. The industry standard, a competitive ELISA, may indicate gluten values <20 mg/kg, which is deemed safe for people with CD. A recent study on the antibody response to gluten-reduced beers found that serum from active-CD patients bound to residual gluten peptides in conventional beers and that a subset of the patient sera reacted to gluten-removed beers [4]. The authors concluded that residual peptides in gluten-reduced beers may be specifically recognised by people with CD.

In this study, the hydrolysis of gluten was examined by investigating a range of barley-based beers crafted to remove gluten using proprietary precipitation and/or application of commercially-available enzymes, e.g. prolyl endopeptidases (PEP) that degrade the proline-rich gluten molecules. Proteomic profiling of beers was conducted on the untreated beers, but also after proteolytic digestion. The beers were also subjected to size-fractionation to determine the size range of gluten-derived peptide fragments. LC-MS analyses revealed gluten peptides derived from hydrolysed fragments, many >30 kDa in size. Barley gluten (hordeins) were detected in all beers analysed with peptides representing all hordein classes detected in conventional beers, but also alarmingly in many gluten-reduced beers. Gluten digestion was incomplete in all commercial beers employing PEP and peptides comprising missed cleavages were identified [5] warranting further optimisation of PEP application in an industrial setting.

Materials and methods

Products tested in this study

A selection of beers was purchased internationally from commercial liquor stores based on their ingredients and gluten status according to their packaging and/or company website. All beers selected were barley-malt based products rather than gluten-free beers based on non-gluten containing grains such as rice, sorghum, millet or tef. A number of regular beers that had previously [6] been shown to contain gluten were selected as positive controls, C1-C4. The gluten-reduced (or low gluten, LG) beers, LG1-LG7 and LG9-LG11 are PEP-treated. LG8 is manufactured by an undisclosed proprietary process. LG12 is brewed with a novel ultra-low gluten barley [7]. Chemicals, including formic acid (FA), ammonium bicarbonate, dithiothreitol, iodoacetamide, were purchased from Sigma-Aldrich (Sydney, NSW, Australia). Acetonitrile were purchased from ChemSupply (Gillman, SA, Australia). Enzymes used for digestion (trypsin and chymotrypsin) were purchased from Promega (Sydney, NSW, Australia).

Protein size fractionation, digestion and analysis

Each beer (n=4 replicates per digest) was either: left whole or applied to a 30 kDa or 10 kDa molecular weight cut-off filter as described in [2]. The beers (whole, >30 kDa, 10-30 kDa, <10 kDa) were reduced by addition of DTT and subsequently alkylated by addition of iodoacetamide (IAM) as described in [5]. To each solution, either trypsin or chymotrypsin was added and the samples incubated at 37°C overnight. The digested peptide solution was lyophilised and stored at -20°C until analysis. The samples were reconstituted in 100 µL of 1 % formic acid. All beer samples generated were analysed by LC-MS/MS and proteins identified by automated database searching as described in [5]. Selected proteins (and their peptide fragments) were further analysed by targeted proteomics employing multiple reaction monitoring (MRM) MS [5].

Results and discussion

Identification of gluten in control and gluten-reduced beers

Four control beers (C1-C4) that were previously analysed [6] by LC-MS and contained gluten proteins typical of barley malt beer were analysed. A range of peptides derived from B-hordeins (examples: I6SJ22; P06470; Q4G3S1), C-hordeins (Q41210; Q40053), D-hordein (I6TRS8), γ-hordeins (I6TMV6, I6TEV2) and avenin-like proteins (ALP: F2EGD5, M0VEH1) were detected.

Many of the same proteins (examples: B-hordeins I6SJ22, P06470; C-hordeins Q40053, Q40037; D-hordein I6TRS8; γ3-hordein I6TEV2; ALP M0VEH1) were detected in the gluten-reduced beers. Peptides spanning the entire length of the single gene product D-hordein (I6TRS8) were detected, implying that either the full-length protein persisted in the gluten-reduced beers or that multiple protein fragments that

harboured the detected peptides were present. In order to examine the potential size of the protein fragments, size fractionation of the beers was undertaken followed by repeat tryptic digestion and LC-MS/MS analysis. Examining D-hordein (expected MW 75.0 kDa) as detected in a beer crafted to remove gluten (beer LG7), peptides spanning the entire protein length were detected in both the 10-30 kDa and >30 kDa fractions. The results were qualitatively similar (LG7, 40.0 % sequence coverage) to that observed for a control barley beer (C1, 37.9 % sequence coverage) that underwent no gluten-reduction processes (Fig. 1).

(A) D-hordein as detected in >30 kDa fraction of gluten-reduced beer LG7

MAKRLVLFVAVIVALVALTTAEREINGNNIFLDSRSRQLQCER**ELQESSLEACRRVVDQQLVGQLPWSTGLQMQC**
CQQLRDVSPECRPVALSQVVRQYEQQTEVPSKGGSFYPGGTAPP**LQQGGWWGTSVK**WYYPDQTSSQQSWQGQQGY
HQSVTSSQQPGQGQQGSYPGSTFPQQPGQGQQPGQRQPWSYPSATFPQQPGQGQGQQGYYPGATSLLQPGQGQQG
PYQSATSPQQP**GQGQGQQETYPIATSPHQP**GQWQQPGQGQQGYYPSVTSPQQSGQGQQGYPSTTSPQQSGQGQQL
GQGQQPGQGQQGYPSATFPQQPGQWQQGSYPSTTSPQQSGQGQQGYNPSGTSTQQPGQVQQLGQGQQGYYPIATS
PQQP**GQGQQLGQGQQPGHGQQL**VQGQQQGQGQQGHYPSMTSPHQTGQGQKGYYPSAISPQQSGQGQQGYQPSGAS
SQGSVQGACQH**STSSPQQQAQGCQASSPK**QGLGSLYYPSGAYTQQKPGQGYNP**GGTSPLHQQGGGFGGLTTEQP**
QGGKQPFHCQQTTVSPH**QGQQTTVSPHQGQQTTVSPHQGQQTTVSPHQGQQTTVSPHPGQQTTVSPHQGQQTTVS**
PHPGQQTTVSPHQGQQTTVSPHQGQQTTVSPHQGQQTTVSPHQGQQTTVSPHQGQQPGEQPCGFPGQQTTVSLHH
GQQSNELYYGSPYHVSVEQPSASLKVAK**AQQLAAQLPAMCR**LEGGGGLLASQ

(B) D-hordein as detected in 10-30 kDa fraction of gluten-reduced beer LG7

MAKRLVLFVAVIVALVALTTAEREINGNNIFLDSRSRQLQCER**ELQESSLEACRRVVDQQLVGQLPWSTGLQMQC**
CQQLRDVSPECRPVALSQVVRQYEQQTEVPSKGGSFYPGGTAPP**LQQGGWWGTSVK**WYYPDQTSSQQSWQGQQGY
HQSVTSSQQPGQGQQGSYPGSTFPQQPGQGQQPGQRQPWSYPSATFPQQPGQGQGQQGYYPGATSLLQPGQGQQG
PYQSATSPQQP**GQGQGQQETYPIATSPHQP**GQWQQPGQGQQGYYPSVTSPQQSGQGQQGYPSTTSPQQSGQGQQL
GQGQQPGQGQQGYPSATFPQQPGQWQQGSYPSTTSPQQSGQGQQGYNPSGTSTQQPGQVQQLGQGQQGYYPIATS
PQQP**GQGQQLGQGQQPGHGQQLVQGQQQGQGQQGHYP**SMTSPHQTGQGQKGYYPSAISPQQSGQGQQGYQPSGAS
SQGSVQGACQH**STSSPQQQAQGCQASSPK**QGLGSLYYPSGAYTQQKPGQGYNP**GGTSPLHQQGGGFGGLTTEQP**
QGGKQPF**HCQQTTVSPHQGQQTTVSPHQGQQTTVSPHQGQQTTVSPHQGQQTTVSPHPGQQTTVSPHQGQQTTVS**
PHPGQQTTVSPHQGQQTTVSPHQGQQTTVSPHQGQQTTVSPHQGQQTTVSPHQGQQPGEQPCGFPGQQTTVSLHH
GQQSNELYYGSPYHVSVEQPSASLKVAK**AQQLAAQLPAMCR**LEGGGGLLASQ

(C) D-hordein as detected in <10 kDa fraction of gluten-reduced beer LG7

*MAKRLVLFVAVIVALVALTTA*EREINGNNIFLDSRSRQLQCERELQESSLEACR**RVVDQQLVGQLP**WSTGLQMQC
CQQLRDVSPECRPVALSQVVRQYEQQTEVPSKGGSFYPGGTAPPLQQGGWWGTSVKWYYPDQTSSQQSWQGQQGY
HQSVTSSQQP**GQGQQGSYPGSTFPQQP**GQGQQPGQRQPWSYPSATFPQQPGQGQGQQGYYPGATSLLQPGQGQQG
PYQSATSPQQP**GQGQGQQETYPIATSPHQP**GQWQQPGQGQQGFYPSVTSP**QQSGQGQQGYPSTTSPQQSGQGQQL**
GQGQQPGQGQQGYPSATFPQQPGQWQQGSYPSTTSP**QQSGQGQQGYNPSGTSTQQP**GQVQQLGQGQQGYYPIATS
PQQP**GQGQQLGQGQQPGHGQQLVQGQQQGQGQQGHYP**SMTSPHQTGQGQKGYYPSAISPQQSGQGQQGYQPSGAS
SQGSVQGACQHSTSSPQQQAQGCQASSPKQGLGSLYYPSGAYTQQKPGQGYNPGGTSP**LHQQGGGFGGLTTEQP**
QGGKQPFHCQQTTVSP**HQGQQTTVSPHQGQQTTVSPHQGQQTTVSPHQGQQTTVSPHQGQQTTVSPHQGQQTTVS**
PHPGQQTTVSP**HQGQQTTVSPHQGQQTTVSP**HQGQQPGEQPCGFPGQQTTVSLHHGQQSNELYYGSPYHVSVEQP
SASLKVAKAQQLAAQLPAMCRLEGGGGLLASQ

Figure 1. *Identification of gluten-derived peptides from D-hordein in glutenreduced beer, LG7. Protein sequence coverage of D-hordein (I6TRS8) as detected in the >30 kDa size fraction (A); and the 10- 30 kDa fraction (B); revealing peptide identifications (bold, underlined >95 % confidence) spanning the length of the protein. Analysis of the <10 kDa fraction (C) that was not subjected to any sample preparation (reduction, alkylation or digestion) clearly indicated the use of a prolyl endopeptidase (PEP) in the production of this beer.*

The undigested filtrates (<10 kDa) were also analysed revealing a range of internal gluten peptide fragments. As no digestion was employed, the termini of the peptides detected may reflect the processes during the brewing of the beers. Many of the peptides identified in the sub-10 kDa fraction of PEP treated beers were the result of PEP action with cleavages occurring at P-X, but this was not an efficient process as there were many "missed" cleavages, that is peptides present that contained X-P-X motifs (Table 1). The control beers (C1-C4) showed no obvious pattern of protein cleavage with values ranging from 5-11 % of gluten peptide fragments resulting from hydrolysis of P-X bonds and >90 % of the gluten peptides detected in C1-C4 contained P-X sites within their sequences. Examining LG1-LG7 and LG9-LG11, the majority of peptide fragments resulted from cleavage at P-X (52-73 %) indicating the use of PEP in the production of these beers.

Table 1. *Analysis of gluten fragments detected in the sub-10 kDa fraction of control (C) and low gluten (LG) beers (no enzymatic digestion). The percentage of total termini that resulted from cleavage at P-X are presented. The number of missed cleavages (at P-X) within the gluten peptides is also shown.*

Beer	Gluten peptides[a]	% cleaved at P-X	Missed cleavages (P-X)	Number of P-X in peptides	% peptides with P-X
C1	22	9.1	50	0-5	90.9
C2	49	6.1	90	0-7	93.9
C3	58	11.2	113	0-5	91.4
C4	21	4.8	49	0-5	95.2
LG1	28	71.4	20	0-2	60.7
LG2	22	54.5	26	0-2	86.4
LG3	33	63.6	47	0-3	93.9
LG4	30	71.7	33	0-3	76.7
LG5	40	58.8	42	0-3	70.0
LG6	57	54.4	84	0-3	84.2
LG7	53	51.9	70	0-5	77.4
LG8	29	3.4	38	0-3	82.8
LG9	30	70.0	27	0-3	66.7
LG10	22	72.7	17	0-2	63.6
LG11	19	68.4	9	0-2	42.1
LG12	7	0.0	5	0-2	57.1

[a] Gluten peptide fragments detected with >95 % confidence.

Within the identified gluten peptide sequences in the beers treated by PEP revealed that 60-94 % contained additional PEP cleavage sites. In fact, only LG11 contained more completely digested peptides than partially digested (42.1 % missed cleavages). The untreated beers contained a higher number of P-X sites within an individual peptide (up to 5 missed cleavages) compared to PEP treated beers where peptides

typically only contained 1-2 missed cleavage sites. The exception was LG7 which also contained a peptide with 5 missed cleavages: QQAELIIP↓QQP↓QQP↓FP↓LQP↓HQP. Notably, this peptide also contained the QQPFP epitope recognised by the Mendez R5 antibody. For LG8, PEP activity was not apparent with ~3 % of gluten-derived fragments cleaved at P-X similar to that seen for the control beers. LG8 also yielded a high proportion of gluten peptide identifications containing P-X sites (82.8 %). As with all the beers in this study, LG8 was brewed using barley, but the gluten is claimed to be removed by a proprietary process. Alongside a handful of B- and γ-hordeins, D-hordein was identified confidently in LG8 by 11 peptides that all clustered in the C-terminal region of the protein suggesting that a C-terminal fragment persists after brewing. In LG12, which is a gluten-free beer brewed using a novel gluten-free barley [7], only γ3-hordein (I6TEV2) was detected by seven peptide fragments resulting from non-specific cleavage (hydrolysis during brewing).

The beers were sequentially passed through 30 kDa and 10 kDa MWCO filters aimed at a crude size-based fractionation of the proteins. The retained protein or filtrate for the sub-10 kDa fraction were then digested with trypsin. Fig. 2 shows the proportion of the signal as detected in the three size fractions. The B- and γ-hordeins were detected in both the 10-30 kDa and >30 kDa fractions, which was not a surprising result given that their expected MW were in the range 28-33 kDa. The D-hordein was most abundantly detected in the 10-30 kDa fraction (~50 % of D-hordein peptide signal present, Fig. 6E-H) despite having an expected MW of 75.0 kDa implying that hydrolysis of the protein had occurred, but this was not dissimilar to that observed for the control beers where non-specific hydrolysis was also noted to occur.

Conclusions

The application of PEP during the brewing process reduced the gluten content of beer, but gluten digestion was not complete in any of the PEP-treated beers analysed with many missed cleavage sites detected. The treatment with PEP was effective in reducing the gluten to very low levels in some beers (LG11) and the gluten peptides that were detected contained a lesser proportion of missed cleavage peptides. The analysis presented here represents a snap shot in time with only one batch of each beer tested, but because of the incomplete nature of the treatment it is possible that different peptides and relative amounts of these peptides would be observed in different batches.

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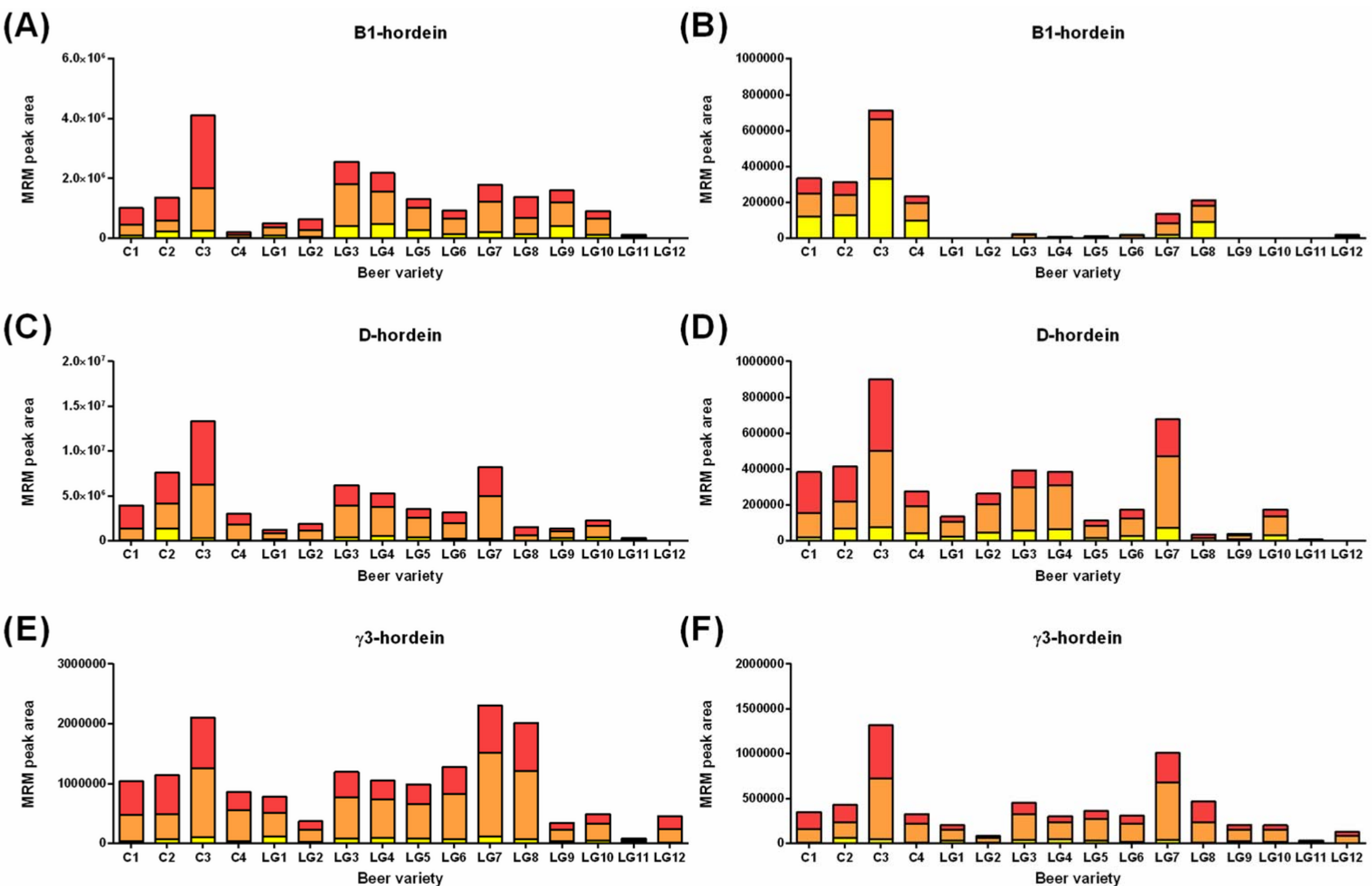

Figure 2. *Detection of hydrolysed gluten in size-fractionated beers. The control beers are labelled C1-C4 and the gluten-reduced beers LG1-LG12. The beers were subjected to size-fractionation prior to yield fractions that were expected to contain proteins >30 kDa (red); 10-30 kDa (orange); and <10 kDa (yellow). Data were analysed by LC-MRM-MS and the integrated peak area for 6 gluten peptides derived from B1-hordeins (A-B), D-hordein (C-D) and γ3-hordein (E-F) are presented.*

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4.5 Influence of food processing on the extractability and composition of wheat protein fractions

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Introduction

Currently, immunological methods, i.e. enzyme-linked immunosorbent assays (ELISA), are recommended by legislation for gluten detection in routine analysis [1]. However, food matrix and the degree of processing can impair gluten quantitation. Especially, the detection of gluten in heated or extruded products like bread and pasta [2] and in products containing partially hydrolysed gluten is challenging [3]. Food processing changes the structure of gluten proteins, which affects their solubility properties [4] as well as antigen-antibody interactions [5]. The objective of this research project is to systematically investigate possible causes for the reduced ELISA sensitivity in processed foods using the bread baking process as model system. The focus lies on the extractability of different wheat protein fractions from bread (crumb and crust), analysed by reversed-phase high-performance liquid chromatography (RP-HPLC), as well as on the proteome composition of the fractions, analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and a shotgun proteomics approach.

Materials and methods

Sample preparation

Kernels of wheat (cv. Akteur, harvested 2015) were milled according to AACCI Method 26-50.01. The flour was sieved (200 µm) and stored at room temperature (RT) for 2 weeks. For dough preparation, flour (800 g) was mixed with 3 % baker's yeast and 2 % NaCl using a kitchen aid (1 min, level 1). After addition of water (amount depends on the water absorption which was analysed in a 10 g Farinograph), the dough was mixed first for 3 min at level 1, then for 3 min at level 3. The dough was placed on a baking sheet and fermented for 20 min (30 °C, 90 % relative humidity (RH)). About 300 g of dough were hand-shaped, put into a tin pan for proofing for 40 min (at 30 °C, 90 % RH) and baked (25 min at 230 °C). At the beginning of the baking process 2 x 25 mL steam was injected. After baking, the bread was cooled for 2 h at RT, separated into crumb and crust, lyophilised and milled (6000 rpm, 200 µm sieve) using an Ultra Centrifugal Mill ZM 200 (Retsch GmbH, Haan, Germany).

Protein extraction

Protein fractions (albumins/globulins, gliadins, glutenins) were extracted using a modified Osborne fractionation. For analytical purposes (analysis by RP-HPLC), protein extraction was carried out on a microscale (100 mg; 3 technical replicates) according to Wieser *et al.* [6].

For preparative purposes (analysis by SDS-PAGE and LC-MS/MS), protein extraction was carried out on a macroscale (15 g) according to Schalk *et al.* [7]. For the latter, the sample material was defatted with pentane/ethanol (95/5, v/v; 4 x) prior to extraction. Extracts were lyophilised for further analyses.

Protein content

The protein content (3 technical replicates) of the extracts (from the modified Osborne fractionation, microscale and macroscale) was determined by RP-HPLC [6]. Lyophilised extracts were re-suspended in their corresponding extraction buffer (1 mg/mL). The protein content (3 technical replicates) of the sample material and the extraction sediments was determined according to ICC Standard Method 167 (Dumas Combustion Principle).

SDS-PAGE

SDS-PAGE was performed according to the protocol described by Lagrain *et al.* [8]. The amount of lyophilised extracts and extraction sediments analysed corresponded to 1 mg protein/mL extraction buffer and 5 µL were loaded onto the gels.

LC-MS/MS

Lyophilised extracts (1 mg protein/mL; 3 technical replicates) were re-suspended and chymotryptically hydrolysed according to Rombouts *et al.* [9]. Protein digests were purified by solid phase extraction on Discovery® DSC-18 SPE tubes (Sigma-Aldrich Inc., Steinheim). The eluates were evaporated and re-suspended in 1.0 mL 0.1 % formic acid for LC-MS/MS analysis. Nanoflow LC-MS/MS was performed by using an Eksigent nanoLC-Ultra 1D+ system (Eksigent, Dublin, CA) coupled online to a LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany). 1 µg of peptides were injected for each measurement. Peptide samples were first loaded on a trap column (75 µm inner diameter x 2 cm, packed in house with 5 µm, Reprosil ODS-3; Dr. Maisch, Ammerbuch, Germany) in 100 % loading solvent (0.1 % FA in HPLC grade water). Peptides were transferred to an analytical column (75 µm x 40 cm, C18 column, Reprosil Gold, 3 µm: Dr. Maisch, Ammerbuch, Germany) and separated using a 110 min gradient in solvent A (0.1 % FA and 5 % DMSO in HPLC grade water) and B (0.1 % FA and 5 % DMSO in acetonitrile) from 4-32 % solvent B at a flow rate of 300 nL/min. MS measurements were performed in data-dependent acquisition mode, automatically extracting the ten most prominent precursor ions in the full MS spectra for high energy collision induced dissociation fragmentation at 30 % collision energy. Full MS spectra and MS/MS spectra were acquired at 30.000 resolution and 7.500 resolution, respectively. Dynamic exclusion was set to 60 s.

Label-free quantitation was performed using MaxQuant (version 1.5.3.30) by searching MS data against a *Pooideae* database (31.10.2016, 317384 entries) using the search engine Andromeda. Variable modifications included oxidation of methionine and N-terminal protein acetylation. Chymotrypsin+ was specified as proteolytic enzyme with up to two allowed miscleavage sites. Precursor tolerance was set to 10 ppm and fragment ion tolerance was set to 0.05 Da. Label-free quantitation and match-between-runs options were enabled and results were filtered for a minimal length of seven amino acids, 1 % peptide and protein false discovery rate as well as reverse identifications. Statistical analysis (volcano plots) was carried out using Perseus (version 1.5.6.0).

Results and discussion

The influence of the bread baking process on protein extractability is shown in Fig. 1. The amount of extracted gliadins was drastically reduced, whereas the amount of glutenins was increased to a similar extent. The change of the distribution of the protein fractions is due to the polymerisation of glutenins and gliadins mainly through heat-induced thiol-/disulphide (SH-SS) interchange reactions [10], triggered by the baking process. As a result, gliadins were not soluble in aqueous alcohol solutions anymore and extracted together with the glutenins. Furthermore, the overall extractability of the total protein was decreased. Thus, the formation of non-SS crosslinks also needs to be taken into account [11].

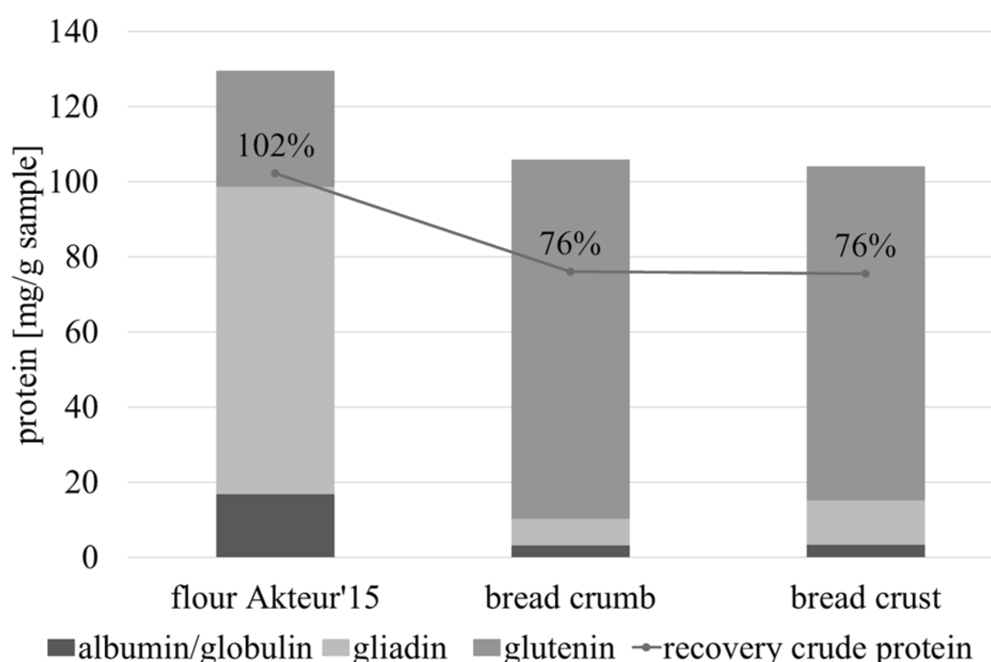

Figure 1. *Protein content of different protein extracts as calculated from RP-HPLC and Dumas data. Differences of all fractions between flour and bread samples are significant ($p < 0.05$, ANOVA). Triplicate determination. Standard deviation < 10 %.*

The composition of the gliadin and glutenin fractions was investigated in detail by SDS-PAGE and untargeted LC-MS/MS analysis.

SDS-PAGE was performed to examine the protein band pattern of the lyophilised extracts and extraction sediments (Fig. 2). In the gliadin fraction, the amount of proteins containing cysteine residues such as alcohol-soluble high-molecular weight glutenin subunits (HMW-GS), α- and γ-gliadins was reduced in the crumb and crust samples in comparison to the flour sample, whereas cysteine-free ω-gliadins accumulated. These findings confirmed the involvement of cysteine containing alcohol-soluble proteins in SH-SS interchange reactions during the baking process. The formation of alcohol-insoluble gluten protein aggregates via SS bonds was reflected in the glutenin fraction by an increase of certain low-molecular-weight glutenin subunits (LMW-GS) in the crumb and crust samples. However, the proportion of HMW-GS as well as of some LMW-GS was decreased in the glutenin fraction substantiating the assumption that insoluble (non-reducible) gluten protein aggregates were formed and remained in the extraction sediments of the processed samples. The SDS-PAGE gel of the sediments demonstrated, that those gluten aggregates could not be extracted with SDS-buffer either. The extractability of proteins from the crust sample was remarkably reduced. Thus, the degree of heat treatment seemed to correlate with the extent of the formation of insoluble gluten protein aggregates.

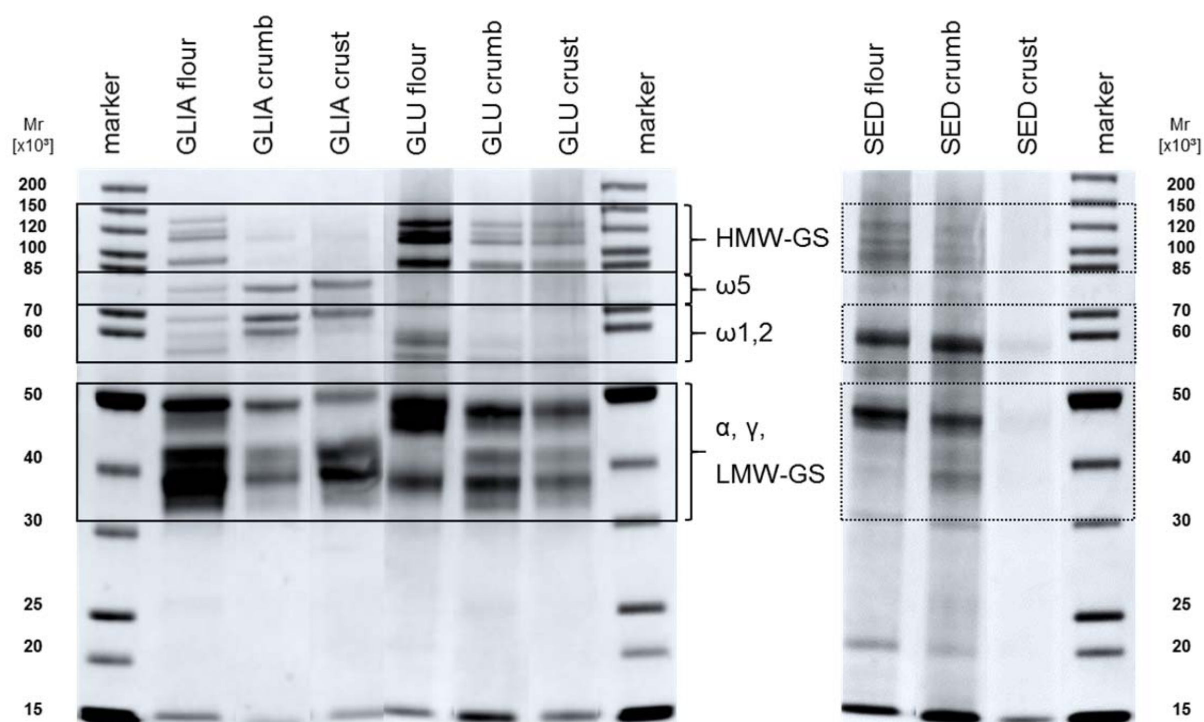

Figure 2. *SDS-PAGE of gliadin (GLIA) and glutenin (GLU) fractions isolated from flour, bread crumb and crust, and their extraction sediments (SED). HMW-GS, LMW-GS: high- and low-molecular-weight glutenin subunits. ω5, ω1,2, α, γ: gliadins.*

The proteome of the gliadin and glutenin fractions from flour, crumb and crust samples was analysed by LC-MS/MS. Tab. 1 comprises all gluten proteins, whose concentration in the gliadin and/or glutenin fraction from processed samples significantly ($p < 0.05$, t-test) changed in comparison to the flour sample.

Table 1. *Log2-fold change of concentrations of proteins in the gliadin and glutenin fraction from bread crumb and crust in comparison to flour.*

Name	1. ID of protein group[1]	Mr x10^3	Gliadin fraction		Glutenin fraction	
			crumb	crust	crumb	crust
α/ß-gliadin (fragment)	D2T2K3	33	-1.17	-0.35	1.57*	1.77*
α-gliadin	A5JSA6	35	-1.12	-0.26	2.41*	2.62*
α/ß-gliadin	I0IT58	31	-0.36	-0.65	2.09*	1.95*
α/ß-gliadin	A0A0K2QJU7	35	-0.28	-0.07	2.05*	1.88*
α-gliadin	A5JSA8	33	-0.96	0.58	1.57	1.82*
α-gliadin	K7X0Q3	34	-0.85	1.21*	2.16*	2.29*
Pseudo α/ß-gliadin	A0A0K2QJX0	27	-0.52	0.93*	1.70*	1.76*
α/ß-gliadin	I0IT51	34	0.17	1.23*	1.54	1.99*
α-gliadin	K7WV47	37	0.34	0.20	1.24*	1.10*
α-gliadin	D2X6D5	35	0.90*	1.32*	2.00	1.73
α-gliadin (fragment)	A0A0E3Z6T7	33	0.03	0.46	1.54*	1.98*
γ-gliadin	B6DQB8	32	-0.79	0.03	1.47*	2.18
γ-gliadin (fragment)	Q9FTC4	27	1.85*	2.79*	nd	nd
γ-gliadin (fragment)	B6DQC4	33	nd	nd	0.75	0.88*
ω1,2-gliadin (fragment)	A0A060N0S6	43	4.08*	2.93*	nd	nd
ω1,2-gliadin (fragment)	D6QY47	39	4.32*	3.42*	nd	nd
LMW (fragment)	Q9XGF0	42	-0.79*	-3.22*	0.60*	-0.05
LMW (fragment)	D6RVY4	42	0.26	-0.61	0.73*	0.72*
LMW	A0A0A0QYP7	40	-0.37	-1.10	-0.11	0.45*
LMW	A8IEC3	34	-1.14	-2.74*	0.35	0.50
LMW	A0A0U3A4B9	41	-1.05*	-1.66*	-0.11	-0.89
HMW y-Typ	Q7Y1U1	32	-1.12	-2.36*	-1.42	-0.07
HMW y-Typ	Q0Q5D3	70	-0.86	-2.95*	-0.11	-0.13
HMW 1By9	Q03871	76	-0.55	-1.45*	-0.36*	-0.05
HMW	A9YSK4	89	-0.27	-1.64*	-0.87*	-0.34
HMW	S5SCQ8	85	-0.26	-1.10*	-0.45*	-0.32

[1]defined by MaxQuant, Mr: relative molecular weight, *significant difference in comparison to flour ($p < 0.05$, t-test), nd: no difference
negative values indicate a decrease of protein concentration in comparison to the flour sample
positive values indicate an increase of protein concentration in comparison to the flour sample

The solubility properties of certain α-gliadins, LMW-GS and HMW-GS were altered. Seven α-gliadins (D2T2K3, A5JSA6, I0IT58, A0A0K2QJU7, A5JSA8, K7X0Q3, A0A0K2QJX0) and four LMW-GS (Q9XGF0, D6RVY4, A0A0A0QYP7, A8IEC3) were present in lower concentrations in the gliadin fraction and in higher concentrations in the glutenin fraction from crumb and crust samples, i.e. shifted from the gliadin to the glutenin fractions as seen in the RP-HPLC data. The amount of five HMW-GS (Q7Y1U1, Q0Q5D3, Q03871, A9YSK4, S5SCQ8) as well as of one LMW-GS (A0A0U3A4B9) was reduced in both fractions from the processed samples. This is in agreement with the SDS-PAGE results. The LC-MS/MS further revealed, that α-gliadins and LMW-GS present in the glutenin fractions could not be clearly distinguished on the SDS-PAGE gel.

The other α-gliadins (I0IT51, K7WV47, D2X6D5, A0A0E3Z6T7), γ-gliadins (B6DQB8, Q9FTC4, B6DQC4) and especially ω-gliadins (A0A060N0S6, D6QY47) accumulated in the gliadin and/or glutenin fractions. Beside the lack of cysteine residues (ω-gliadins), mechanisms why these proteins did not seem to participate in the gluten network formation need to be further elucidated.

Conclusions

These findings contribute to a better understanding of the influence of food processing (heat treatment) on the gluten protein structure relevant for ELISA detection. Research regarding the structure of selected proteins to figure out reaction mechanisms involved in gluten crosslinking is ongoing.

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4.6 Variations in gluten content estimations revealed by FAPAS interlaboratory proficiency reports: Dependence on the use of analytical products, users and range of concentrations

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Introduction

Following a gluten-free diet (GFD) is currently the only treatment for coeliac patients. However, gluten exposure (voluntary or involuntary) is not uncommon, even when following a GFD. This implies that damage to the gut mucosa as well as very severe damage to other bodily structures keeps happening even when the patient is asymptomatic [1,2]. In addition to the obvious reasons why coeliac patients are exposed to gluten, such as cross-contaminations, omissions, food temptations or mistakes, the role of the accuracy of analytical techniques to assess the gluten content in food samples remains unanswered. Food industry players must produce below the legal limit of 20 mg/kg to be allowed to label their products as "gluten-free" and the analytical methods they use (mostly immunomethods, ELISA and lateral flow devices) need to be accurate, repetitive and reproducible.

Currently, there is a wide variety of kits from different manufacturers that differ mainly in the antibody used. The first generation of polyclonal antibodies (like the401.21 or Skerrit antibody) which showed limitations in the recognition of certain gluten types, was then followed by the monoclonal antibody (mAb) R5 [1], which has a poor recognition of the most immunotoxic peptide, the α-gliadin 33-mer. The last generation of antibodies comprise the mAbs G12 and A1 [2], which show a strong recognition of gluten immunogenic peptides (GIP) [3]. Methods based on the R5 mAb are more widely used than any other, since the sandwich ELISA based on R5 was endorsed by the Codex Alimentarius as type 1 method [4].

FAPAS is an institution that organizes proficiency tests in which laboratories take part to verify their quality assurance procedures, monitor their laboratory performances and compare their methods and results to those of the rest of participants. The data obtained from every proficiency test are rigorously analysed and a report with statistical evaluation of results is published by the provider. Those tests of gluten analysis in different "blind" matrices have been performed since more than a decade. Laboratories can take part using any of the commercially available ELISA or LF methods. FAPAS sends out a quality control material with unknown gluten concentration for the recipient laboratories and subsequently the results submitted are statistically analysed to provide an assigned value. This assigned value is derived from

the consensus of the results obtained by the participant laboratories [5]. Where possible, FAPAS segregates the results into subsets according to the ELISA kit used.

In this study, we aim to analyse the variability of results obtained by different analytical kits based on FAPAS reports, and also the results obtained by the most widely used kit, the Ridascreen Gliadin R5 Sandwich (R-Biopharm), when used by different laboratories and users.

Materials and methods

We used the reports from FAPAS proficiency tests in which Biomedal's analytical laboratory had participated and compared them to the data obtained in our laboratory by analysing the same FAPAS reference material using the GlutenTox A1/G12 Sandwich ELISA kit (KT-5196, Biomedal, Spain) and the Ingezim Gluten R5 Sandwich ELISA kit (30.GLU.K.2, Ingenasa, Spain). Some data obtained using Biomedal's GlutenTox PRO A1/G12 lateral flow kit (KT-5660, Biomedal, Spain) are also shown.

Results and discussion

Up to 7 FAPAS proficiency tests were analysed (Table 1). Most of the participants used Ridascreen R5 Sandwich ELISA kit from R-Biopharm (62 participants-75 %), likely due to the favourable position as Type I method for gluten analysis by Codex Alimentarius. Despite one of the purposes of a reference method is to contribute to decrease variations in analytical results, a wide range of values was obtained with this kit by the different participant laboratories. The minimum values ranged from as low as 30 % of the assigned value (Ridascreen) to as high as 266 % of the assigned value (Ridascreen) (Table 1).

Table 1. *Analysis of the data obtained after analysing seven FAPAS samples by different Sandwich ELISA methods*

ID FAPAS test	Gluten content (mg/kg)				
	Ingezim R5	GlutenTox A1/ G12	Assigned value (Ridascreen)	Ridascreen $Value_{max}$- $Value_{min}$	% Ridascreen analysis/total reported results
27121	20.8	22.9	21.0	33.8 - 10.5	75 % (129)
27138	14.6	15.4	15.0	34.0 - 6.3	66 % (88)
27142	21.9	24.0	27.3	45.4 - 8.1	75 % (109)
27172	25.3	18.8	15.4	32.0 - 9.4	65 % (48)
27183	45.7	25.1	28.5	54.6 - 10.1	63 % (137)
27191	25.5	20.6	30.1	80.0 - 15.4	62 % (99)
27194	41.5	22.3	24.9	40.8 - 12.8	75 % (55)

Biomedal analytical laboratory is accredited to the ISO/IEC 17025 standard for its gluten assay with the Ingezim Gluten R5 Sandwich ELISA kit (Ingenasa) and participated in those studies using this kit. In parallel, the same reference materials were analysed by Biomedal using the GlutenTox A1/G12 Sandwich ELISA method (Biomedal) to study the comparability of the gluten assays. The comparison of the results for Ingezim Gluten R5 and GlutenTox A1/G12 methods showed less than 25 % of deviation in 5 out of 7 samples. Furthermore, the results obtained by GlutenTox A1/G12 kit differed less than 15 % from the assigned value (Ridascreen) in 6 FAPAS tests, and 32 % in one of them while two results higher than 60 % from the assigned value were obtained by the Ingezim Gluten R5 kit in two tests.

Another recent FAPAS interlaboratory proficiency test was analysed (report 27204, Tables 2 and 3) where the GlutenTox PRO A1/G12 lateral flow test kit was assessed in Biomedal for qualitative estimation of gluten content in two test materials. The assigned value of each test material calculated from the results obtained by the Ridascreen R5 Sandwich ELISA test method by FAPAS, TMA24 mg/kg and TMB 41 mg/kg, was correctly estimated by the GlutenTox PRO lateral flow test by using different cut off by dilutions (>20 mg/kg < 40 mg/kg for TMA; and >40 mg/kg for TMB). At least one fourth (26.3 %) of the reported values with the Ridascreen kit might have prompted to the labelling of the analysed material as gluten-free, with less than 20 mg/kg of gluten (Table 2).

Table 2: *Results of collaborative study TMA (Data obtained from FAPAS report 27204 not excluding any result, calculated by Biomedal)*

	Neogen-Veratox for Gliadin R5 (8510)	**NH Foods Ltd. Fastkit ELISA Ver. III Wheat**	**R-Biopharm Ridascreen Gliadin (R7001+R7002)**
Number of analyses	11	7	99
Average (mg/kg)	25.9	29.8	25.6
Standard deviation (mg/kg)	9.8	18.9	14.2
Variation coefficient (%)	38	63	56
Number of results <20 mg/kg	3	2	26
Minimum value (mg/kg)	13.7	18.8	11.8

The other laboratory results with other methods (Veratox R5 and NH Food FastKit ELISAs) had similar proportion of <20 mg/kg reported results (29 % and 27 %, respectively). The coefficients of variation of the TMA (38 % to 63 %) results were significantly higher than those of the TMB (37 % to 31 %) when the NH Food FastKit was used. The lower gluten concentration in a test material, the higher probability of a variation coefficient increase in the performance of an assay.

***Table 3.** Results of collaborative study TMB (Data obtained from FAPAS report 27204 not excluding any result, calculated by Biomedal)*

	Neogen-Veratox for Gliadin R5 (8510)	**NH Foods Ltd. Fastkit ELISA Ver. III Wheat**	**R-Biopharm Ridascreen Gliadin (R7001+R7002)**
Number of analysis	13	7	97
Average (mg/kg)	42.90	48.13	42.36
Standard deviation (mg/kg)	15.78	14.69	13.60
Variation coefficient (%)	37	31	32
Number of results < 20 mg/kg	0	0	2
Minimum value (mg/kg)	23.9	38	17.39

Conclusions

The interlaboratory reproducibility of the analytical results for the same test material appeared to be more dependent on the different performances by the same user than on the type of the analytical method used. ELISA methods using different antibodies with the same laboratory users may produce more similar results than the results obtained from different laboratories with the same kit. ELISA methods using antibodies other than R5 antibody can also produce quantitative results of gluten concentration consistent with the assigned value from the most used ELISA kit.

Coefficient of variation (VC) of the analytical results from the different laboratories and gluten ELISA kits were considerable higher for gluten concentration close to 20 mg/kg (VC: 38-63 %) than when the gluten concentration was almost two times higher (VC: <37 %). This high VC in low gluten content close to the legal requirement may be relevant due to the frequent discrepancies and interpretations of the analytical results that arise in quality control tests, where the uncertainty of measurement of every laboratory assay may also play an essential role in food certifications and labelling. The selection of the same antibody did not solve the problem of discrepancies in analytical results of same tested material (see also [6]).

Lateral flow immunoassays, as the GlutenTox PRO A1/G12 test kit, could produce semi quantitative results of gluten concentration in the tested materials consistent with the assigned value from the most used ELISA kit.

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4.7 Are we friends with einkorn (*Triticum monococcum*)?

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Introduction

Gluten related disorders are a well-studied research area. With the advancement of cereal diseases, one of the most important goals of the plant research is to identify setting genotypes of grain with a special flour protein composition beneficial for patients.

Wheat seed storage proteins are considered as key players in triggering of different wheat-related health problems. They contain immune responsive peptides (epitopes) responsible for the autoimmune reaction of coeliac patients.

Today the only effective cure for coeliac disease is a lifelong gluten-free diet. Although there are several studies published about the disease-triggering proteins in case of bread wheat, rye or barley, only a limited information is available about the epitope content, distribution, and frequency of the diploid wheat's, *Triticum monococcum ssp. monococcum and T. monococcum ssp. aegilopoides.*

Einkorn (*Triticum monococcum ssp. monococcum*) is a diploid species of hulled wheat, with tough glumes ('husks') that tightly enclose the grains. The cultivated form is similar to the wild form, except that the ear stays intact when ripe and the seeds are larger [1]. Einkorn is probably the most ancient cultivated wheat species. Its cultivation started approximately 12000 years ago.

It has much greater genetic variability, than the hexaploid bread wheat. Therefore, it has more diverse storage protein composition and epitope content as well.

Einkorn wheat has high carotenoid, tocol, micro-element content and decreased carbohydrate content [2, 3]. It has higher levels of fat, phosphor, sodium, beta-carotene, and pyridoxine than modern wheat species [4]. It can provide easily digestible food. Therefore, einkorn is a promising candidate for development of bakery products and has the main role in functional food production in organic farming. Furthermore due to the simplicity of its genome, einkorn wheat has attracted the

interest of the scientific community on nutrition and health aspects in relation to coeliac disease [5].

The main aim of our research is to characterize the seed proteins of the einkorn collection of the Cereal Gene Bank of our Institute in Martonvásár using bioinformatics, proteomics and immunomics analyses. Using a complex workflow, we can get useful and important information about the toxic protein levels, and distribution of epitopes of the approximately 200 different einkorn genotypes.

By proteomic and immunological tests, we can identify immune responsive proteins, which are unsafe even after digestion.

In addition to providing much more accurate information about the relationship between the storage proteins and the immune response they induce, a large number of sample suggest, that it is possible to identify some *T. monococcum* genotypes, that can be eatable for coeliac patients as well.

Materials and methods

Different *Triticum monococcum* seeds were derived from the Cereal Gene Bank of the Department of Plant Genetic Resources and Organic Breeding, Martonvásár.

Patient's sera suffering from coeliac disease obtained from Dr. Gábor Veres (1st Department of Pediatrics, Semmelweis University of Medicine) and Dr. Ilma Rita Korponay-Szabó (Heim Pál Children's Hospital, Coeliac Centre). In this study, two coeliac negative, three coeliac disease positive patients on a gluten-free diet and ten coeliac disease positive sera were used for the estimation of toxic potential the einkorn seed total protein extract.

In case of *T. monococcum* total protein extracts, proteins were extracted with SDS buffer followed the protocol of Dupont and co-workers [6]

Serological ELISA analyses carried out using high binding plates, were coated with the diluted total protein extracts of the einkorn genotypes (10μg/mL) and incubated overnight in 4°C degree followed wash steps plates were blocked for 1 h with 5 % Casein and 0.05 % TWEEN20. The immune reactivity of proteins was detected with anti-Human IgA (α-chain specific) peroxidase- conjugated antibody produced in goat (Sigma-Aldrich-A0295) in the presence of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, St. Louis, Missouri, United States). For the large-scale, serological ELISA HLA DQ2.5 and HLA DQ8 human sera of patients with different age and gender were used in four replicates.

To map the epitopes to the protein sequences coeliac disease-specific linear T-cell and B-cell epitopes were collected from the ProPepper database [7]. Epitope mapping was carried out using motif search algorithm of the CLC Genomic Workbench (8.5.1), with 100 % motif identity. *In-silico* protein digestion was made with PDMQ software tool [8].

Results and Discussion

In our previous study G12 and R5 commercial available ELISA test kits were used and based on the results of the bioinformatics and immunomics analyses some diploid and tetraploid species were identified to have significantly lower gliadin content (Fig. 1) [9]. In one of the investigated *T. monococcum* genotypes, the toxic protein content normalised to Chinese Spring bread wheat was significantly lower, so we decided to make a large scale serological ELISA screen of 200 different wild- and cultivated einkorn genotypes. There are other studies indicated the lower immunogenicity and easier digestibility of einkorn bread, but only one or few genotypes were tested [10-13]. In the first step, the homology test of the seeds was carried out using the gliadin extracts of the seed endosperms in SDS-PAGE. The seeds of the inhomogeneous genotypes were individually grown in a greenhouse for further analyses.

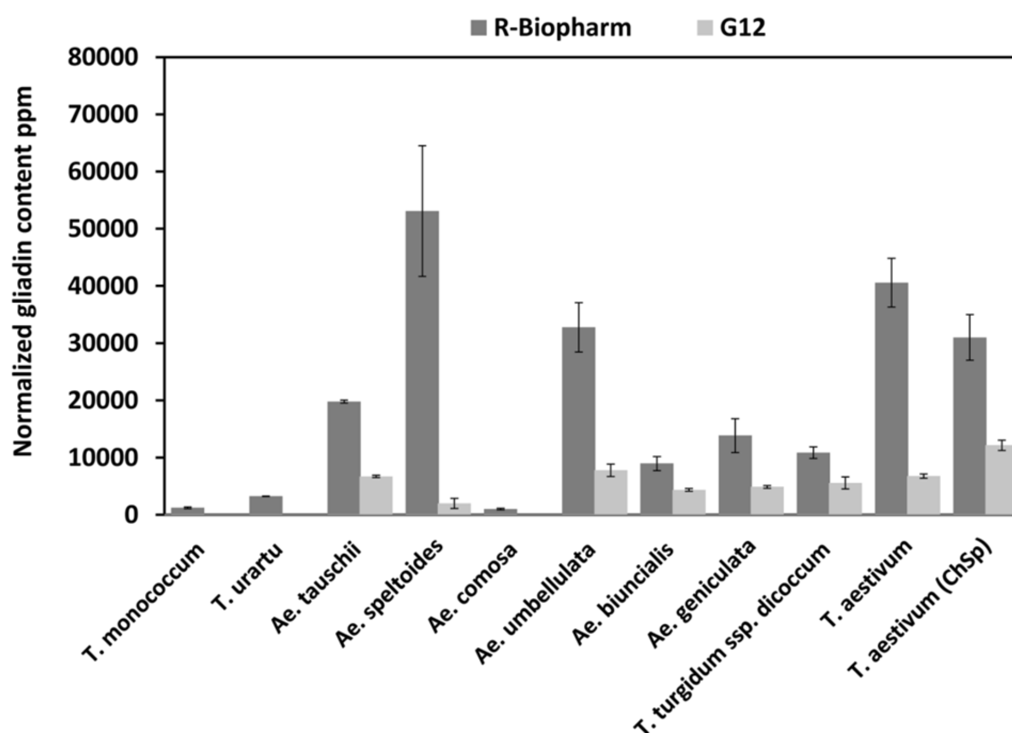

Figure 1. *Measured R5 and G12 gliadin contents in diploid, tetraploid and hexaploid cereal species. Dark grey columns represent the values obtained using the R5 mAb assay, light grey columns show results for G12 mAb. Error bars represent standard deviations calculated based on the results of replicates (Gell* et al.*, 2015).*

Based on the *in-silico* epitope mapping *T. monococcum* sequences retrieved from the UniProt database all of the alpha-, gamma, -and omega – gliadins containing toxic epitopes, to some extent resistant to peptic-tryptic digestion (*in-silico*), but the composition and the distribution of these epitopes are more diverse than in bread wheat (Fig 2, Fig 3).

The results of the serological ELISA analyses revealed that from the nearly 200 cultivated and wild einkorn genotypes four have lower toxic protein contents consequently to Chinese Spring with all of the ten different patient’s sera (Fig 4).

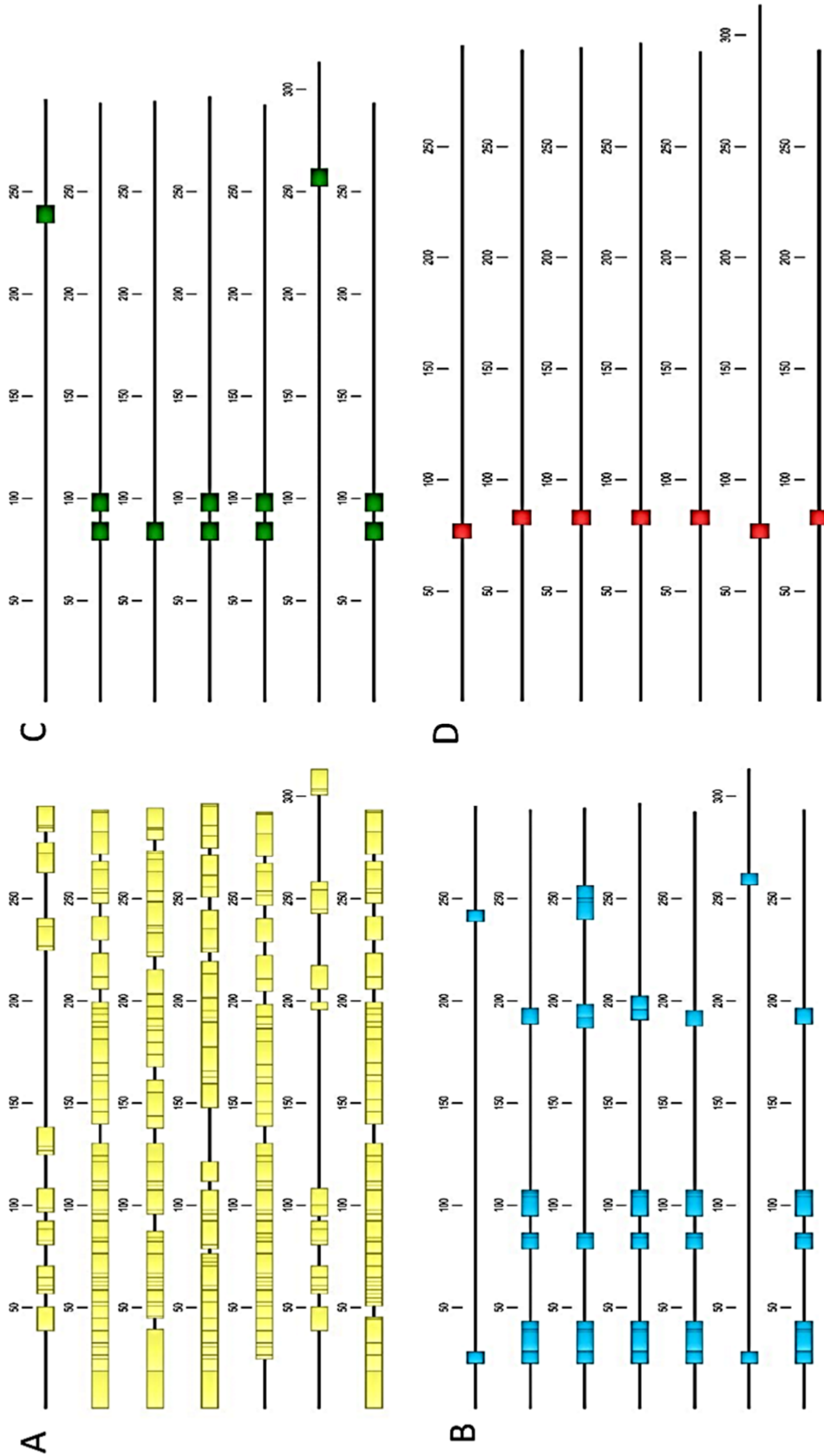

Figure 2. *In-silico epitope mapping of* T. monococcum *α-gliadin sequences retrieved from the UniProt database. A: B cell-specific epitopes; B: B cell epitopes resistant to proteolytic digestion; C: T cell epitopes; D: T cell epitopes resistant to proteolytic digestion.*

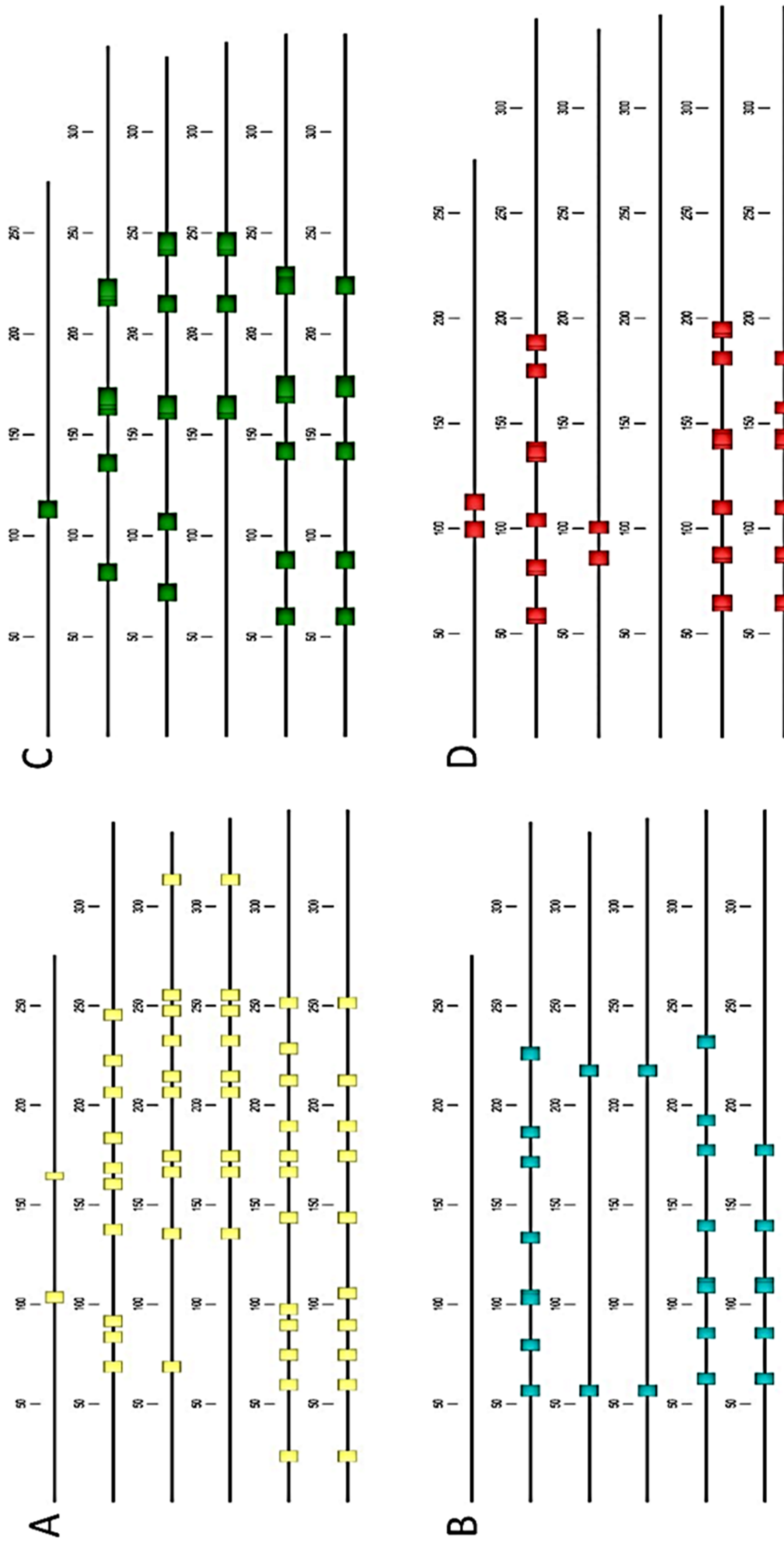

Figure 3. *In-silico epitope mapping of* T. monococcum *ω-gliadins retrieved from the UniProt database. A: B cell-specific epitopes; B: B cell epitopes resistant to proteolytic digestion; C: T cell epitopes; D: T cell epitopes resistant to proteolytic digestion.*

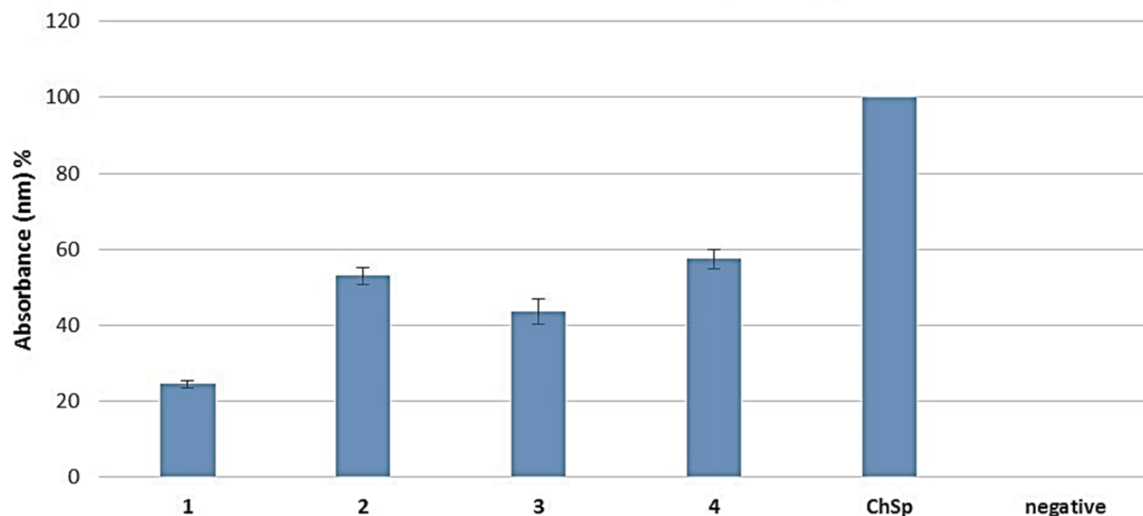

Figure 4. *Serological ELISA test of* T. monococcum *total protein extracts (1 - 4) compared to Chinese Spring (ChSp) bread wheat with ten different coeliac disease sera (DQ8 and DQ2).*

Compared these results with the commercially available test kits it was revealed that the R5 and G12 antibody underestimate the toxic protein levels of the *T. monococcum*. From one hand the underestimation could be a cause of the variable gliadin composition and amino acid sequences of einkorn, and from the other hand in the serological test total extracts were used, could containing non-gluten cross-reactive proteins.

According to previous examinations connected to climate change with bread wheat, drought and heat stress and their combinations have a huge impact on the expressed amounts of storage proteins. Based on these findings further abiotic stress analyses will be needed to determine the stability of the lower toxic protein content of the investigated einkorn genotypes.

Conclusions

Due to the large-scale examination of the huge number of einkorn genotypes, highly diverse population screening has been performed.

Using bioinformatics methods our study confirmed, that einkorn seed storage protein sequences have more diverse epitope profiles compared to the bread wheat sequences. In some of the *T. monococcum* genotypes we have measured significantly reduced toxic peptide content, however, they were above the officially determined gluten-free limit. In summary, this result indicates that without further food processing or specific digestion einkorn genotypes are not suitable food alternatives for coeliac patients.

Further analyses are needed to estimate the environmental effects, abiotic stress factors on the einkorn seed storage protein composition and toxic epitope expression level. Therefore it is suggested to perform additional environmental examinations with einkorn wheat as well.

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4.8 Gene editing using CRISPR/Cas9 to modify or remove gliadins from wheat and produce coeliac disease epitope-free wheat

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Introduction

Cereals cause allergies and intolerances in some people [1]. Individuals that express the human leukocyte antigen (HLA) DQ2 and/or DQ8 can become intolerant to gluten proteins from wheat, rye and barley and may develop coeliac disease (CD), a chronic inflammation of the small intestine. The prevalence of CD is 1-2 % of the general population worldwide. Strict, gluten-free consumption is currently the only remedy.

Wheat varieties that are safe for people with coeliac disease cannot be produced by conventional breeding alone [2], as CD epitopes occur in many gliadins and some glutenin proteins [3], these storage proteins are encoded by gene families with dozens of members [4,5] partly clustered on two different chromosomes of wheat, and bread wheat has three sets of chromosomes. Wheat deletion lines, in which parts of chromosome arms with gluten loci have been eliminated by mutagenesis, are useful research tools [6], but they also lack many other genes, which may affect their commercial use [7].

CRISPR/Cas9 is a relatively new technology to perform targeted gene editing, i.e. to induce mutations and deletions at specific locations in the genome [8-11]. It requires the introduction into plant cells of a construct that contains the code for a nuclease (Cas9, or a similar enzyme such as Cpf1) and one or more guide RNA sequences that target the gene sequence to be altered. The nuclease generates sequence-specific double strand brakes in the DNA, that will be repaired by the plant but sometimes by mistake point mutations, indels or other errors are introduced. In wheat this may be used for the selection of plants with simultaneous mutations in the three genomes [13]. Mutating epitope regions in gluten genes will lower the level of CD epitopes and ultimately produce CD-safe wheat lines.

In an alternative approach, genetic transformation is avoided. The nuclease protein, loaded with gRNAs, is added directly to wheat protoplasts or embryogenic cells [16]. This makes the process similar to chemical mutagenesis. However, it is less efficient for multiple targets than using a transformation step because the added nuclease is rapidly degraded. It is therefore unlikely that a regenerated plant can be found in which all extant copies of CD epitopes in gliadin genes have been mutated.

We have edited alpha- and gamma-gliadins of bread wheat using CRISPR/Cas9 with guide RNAs towards CD epitopes and conserved regions of these gene families, based on an alignment of many known gliadins. We discuss the results in view of our final goal of producing a bread wheat variety that has no CD epitopes.

Materials and methods

We stably transformed immature embryos of the spring wheat variety Fielder with Cas9 + sgRNA constructs directed towards epitopes or conserved regions in the alpha-gliadins and gamma-gliadins (Jouanin *et al.*, in prep.). Multiple gRNAs were combined in a single construct. Transgenic wheat plants (generation T0) were regenerated in vitro and transferred to a growth chamber for flowering and grain set. The T1 grains were cut into two parts. One part, containing the embryo, was sown to produce the T1 plant and further generations. The other part was used for an Acid PAGE comparison with seeds of the Fielder control and with selected Paragon gamma-irradiated lines (Jouanin *et al.*, in prep.) and for a proteomics (LC-QTOF-MSMS, [22]) analysis of proteins that were absent and proteins that were mutated in the gene-edited lines compared to Fielder.

For further evaluation, DNA from the leaves of the corresponding T1 plants will be extracted to determine the gliadin gene copy number using a quantitative PCR method (droplet digital PCR), as well as for gluten gene sequencing. As the wheat genome is very large, an exome capture system was designed that enables isolating and sequencing only the gluten genes (Jouanin *et al.*, in prep.). The sequence data will give us additional information about the types of mutations generated by CRISPR/Cas9 in the gliadins and potentially in off-target sites in the gluten genes.

The wheat lines will be self-pollinated several times to produce plant lines homozygous for all gliadin mutations and without the CRISPR/Cas9 construct. CD epitope-specific T cell clones will be used to determine the immunogenicity of the gluten extracted from the mutant wheat grains. Dough rheology will be measured and, if necessary, adjusted with, for instance, oat avenins [7] to ensure a decent quality of bread made using grains from these edited wheat lines. Ultimately, if an edited line completely devoid of CD epitopes is produced, gluten from the edited wheat line will be tested on voluntary CD patients in a food challenge, using the prepared bread.

Results and discussion

The Acid PAGE gels show differences in the gliadin protein profiles between the T1 CRISPR grain and the variety Fielder, including disappearance or shifts of bands in both the alpha-gliadins and the gamma-gliadins, confirming actual mutagenesis. Interestingly, similar changes are observed in some of the gamma-irradiated lines.

Recently, Sánchez-León and colleagues [13] transformed wheat lines with CRISPR/Cas9 constructs with gRNAs targeted against alpha-gliadins. They generated

plants with mutations in the alpha-gliadin genes, namely small and larger deletions leading to frameshift mutations and premature stop codons. In gluten extracts from the T1 grains certain alpha-gliadin bands were absent on Acid PAGE and SDS-PAGE gels. Surprisingly, in some lines also a decrease in gamma- and omega-gliadins was observed. Compensatory effects in the production of different gluten gene protein families was also found in some plants, as previously observed in RNAi lines [14,15]. The total gluten content was up to 85 % lower.

Obviously, both our gene-edited plants and those of Sanchez-León *et al.* are not yet safe for CD patients. Not all members of the targeted gliadin families have been modified, and the epitopes in omega-gliadins and LMW-glutenins [21] were not targeted at all. In addition, in some of their edited lines Sanchez-León *et al.* [13] observed compensation, e.g., higher synthesis of omega-gliadins, which actually increased the G12 signal. Therefore, we can expect that at least two or three rounds of CRISPR/Cas9 gene editing will be necessary to modify all epitopes. Alternatively, parallel modification can be performed for different gluten gene families in different plants of the same cultivar, after which the modified loci can be combined through crossing. This may be especially useful when combining safe(r) alpha-gliadin loci on one or more of the homologous chromosomes 6 with other loci on the homologous chromosomes 1.

Only a limited number of gliadin genes is expressed at a high level [17]. It is therefore possible that it may be sufficient to only modify those genes, in all gliadin gene families to anticipate compensation. However, at this moment we do not know whether a high level of expression of some genes is completely independent of the environment, and we also do not know whether the high expression level will consistently manifest itself in other genotypes after crossing. If so, a mutation that only modifies an epitope in one of those gliadin proteins, such as a substitution of one or two amino acids, would be preferable over a mutation that leads to a frameshift mutation and a truncated mRNA, as this may abolish expression altogether, possibly leading to compensation by expression of a different gene. For such novel variants we will be able to predict their immunogenicity, with varying confidence, based on the knowledge obtained in systematic studies of the immunogenicity of epitope variants [18] and the 3D structures of the details of the DQ2-epitope-T cell and DQ8-epitope-T cell protein complexes [19,20]. Tests with T cell clones will need to be done to verify this.

Whether a gene-edited wheat variety is considered ‘non-transgenic’ in the EU is still unclear. Since the CRISPR/Cas9-containing construct is introduced by genetic transformation, some authorities deliberate that the resulting variety may be considered GM according to the European directive 2001/18/EC, even though the CRISPR/Cas9 construct is removed by segregation in the offspring of the original mutated plant (the so-called “null-segregants”) and the variety produced therefore does not contain any foreign DNA. The absence of foreign DNA also means that the handle used for safety testing (the transgene, its location, and its possible direct and indirect

effects) is absent. Will they forever be considered (according to the *process* applied) as transgenic? A *product*-based safety assessment approach would focus on the traits introduced in the plant and its safe use, as is the current practice for conventionally bred plants. Gene-edited varieties are being considered non-GM in other parts of the world, such as the US, where not the process but the product is evaluated.

Public debates on the use of gene editing technology for improving crop varieties should include the medical and social benefits associated with using the technology, i.e., the health benefits of wheat without CD epitopes for the CD patients, the undiagnosed CD sufferers, and their children. Therefore, CD patients as a prominent stakeholder group should be involved in the discussion.

A CD-safe wheat line with good baking quality may be introduced in the market as a specialty variety that has to be produced, processed and sold in a separate production chain. Such a separate chain will be more difficult to establish, and quality control will be more challenging, than a regular gluten-free production chain, as the grains and the plants are morphologically indistinguishable from regular wheat grains and plants. It would also require an adaptation of the current regulation regarding the 20 ppm gluten threshold for gluten-free products. Notably, new safety assessment assays would have to focus on measuring threshold amounts of CD immunogenic epitopes rather than total gluten.

Conclusions

Mankind has modified many characteristics of wheat during the last 10,000 years, from non-shattering and hull-less grains in the first millennia to semi-dwarf varieties with higher yield in the 1960s. Now we have the gene editing technology to also modify health-impairing plant and food characteristics. Our vision is that of a CD epitope-free wheat which has largely retained its baking quality. Ultimately, this may make coeliac disease a very rare disease.

Acknowledgements

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5 Clinical research reports

5.1 Complementarity of 3 monoclonal antibodies (mAbs) directed against native and deamidated repeat motifs of gliadins to evaluate the extent of gluten modification after chemical or enzymatic deamidation processes

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Introduction

Acid-hydrolysed wheat proteins (a-HWP) were used as ingredients in food and cosmetics. From the 2000's, cases of severe food allergy to HWP have been reported in people tolerant to native wheat proteins. More recently in Japan, a soap containing a-HWP elicited severe skin reactions and food allergy to wheat in over 2000 individuals [1]. Gliadins and glutenin subunits (GS), the main protein fractions of wheat flour, are characterised by homologous domains consisting of repeated sequences of 6 to 8 amino acids rich in glutamines. We have shown that deamidation of these sequences, as the main consequence of acid hydrolysis, generates neo-epitopes responsible for this allergy [2]. The main IgE epitopes involved were identified for French and Japanese cohorts (QPEEPFPE and PEEPFP respectively) [2,3]. The detailed analysis of the IgE reactivity of French patients on all the possibilities of deamidation of the peptide QPQQPFPQ revealed that the position and the extent of deamidation at the scale of this repeated sequence had a strong impact on the IgE binding. Deamidation of 3 on the 4 glutamines allowed the strongest IgE/peptide interaction. The determination of the extent of deamidation of wheat prolamins is essentially based on biochemical methods such as the measurement of ammonium release after extensive acid hydrolysis [4]. Such a measure gives an overall result that provides little information about the rate and distribution of deamidation at the scale of an epitope. A method to assess this rate of deamidation at the epitope level, together with the presence of residual native repeat peptide would be useful for the characterisation of glutens modified by chemical or enzymatic processes of deamidation.

In this work, we evaluated the ability of 3 mouse monoclonal antibodies directed against native and deamidated repeat motifs of gliadins to discriminate between gliadin and gluten samples with different deamidation rates.

Materials and methods

Antibodies

MAb PQQ3B4 has been raised against the peptide YPQQPFPQ and described in Tranquet *et al.* [5]. MAb MCO1 has been raised against the peptide QPFPQPELPYPQPQ and described in Skovbjerg *et al.* [6]. Mab INRA-DG1 has been raised against the peptide LQPEEPFPEQC and described in Tranquet *et al.* [7]. MAbs PQQ3B4, and INRA DG1 were used as cell culture supernatant. MC01 was provided purified by Zedira (Darmstadt, Germany).

Native and deamidated gliadin sub-fractions and total gliadins

Native gluten and native gliadins were previously prepared from wheat flour (c.v. recital) according to Battais *et al.* [8]. Briefly, flour was defatted and gluten was separated from starch by extensive washing of the dough by water. The gliadin fraction was isolated from the gluten with 70 % ethanol (v/v) and further divided into α, γ, ω2 and ω5-gliadins by ion-exchange chromatography and reverse-phase HPLC. Three samples of deamidated gliadins with deamidation rates of 15 %, 35 % and 48 % (referred to as D-GLIA 15, 35 and 48) have been produced by acid hydrolysis of gliadins and characterised in a previous study [7]. Briefly, Gliadins (10 mg) were solubilised in 1 mL of 50 % (v/v) ethanol, 0.1N hydrochloric acid, and heated at 90˚C for 40, 90, and 120 min before dialysis against water and lyophilisation. Purified α-, γ, ω2-, and ω5-gliadins, LMW-GS were deamidated by acidic treatment. Level of deamidation were determined for α-, γ, ω2-, and ω5-gliadins: 52 %, 32 %, 36 % and 51 % respectively [9].

Gluten samples treated by protein glutaminase

Protein-glutaminase (PG) Amano 50 (50 unit/g, 1 % active constituent, 99 % dextrin as bulking agent), was supplied by Amano Enzyme Inc. (Japan). Vital Gluten (Raisio Grain Starch Ltd, Finland) at 10 mg/mL was dispersed with PG at 0.13 Unit/mL in sodium phosphate buffer pH 7 and incubated at 40°C for various periods of time (0-30h). Control sample (Gluten CTL) was treated under the same conditions without PG for 24h. After incubation with enzyme, the samples were freeze-dried. In these conditions, deamidation degrees of 20 %, 45 %, 60 and 72 % were reported for incubation times of 1 h, 3 h, 5 h, and 30 h respectively [10].

Indirect ELISA

Reactivity of the three antibodies on gliadins and LMW-GS, either native or deamidated, was assessed by indirect ELISA as described by Battais *et al.* for sample preparation and coating [8]. Uncoated sites were blocked with 4 % defatted skimmed milk in phosphate-buffered saline (PBS) at pH 7.4 for 1 h. MAbs were diluted in PBS containing 0.1 % skimmed milk (dilution 1:2 for PQQ3B4; MC01 at 0.4 µg/mL; dilution 1:50 for INRA-DG1) and then added and incubated for 1 h at room temperature. After washing with PBS 0.05 % Tween 20, bound antibodies were

detected with horseradish peroxidase anti-mouse IgG (Bio-Rad, Marnes-la-Coquette, France, dilution 1:3000) and ortho-phenylenediamine as the substrate. Color development was stopped with 100 µL of 2 M H2SO4, and the absorbance was read at 492 nm.

Inhibition ELISA

In order to further characterize the reactivity of the 3 mAbs, native or deamidated gliadin and gluten samples were used as inhibitors in competitive ELISA as described for INRA-DG1 in Tranquet *et al.* [7]. For mAb PQQ3B4 (diluted at 1:10), the coating solution was a total gliadin fraction at 0.3 µg/mL. For the mAb MC01 (diluted at 1 µg/mL), the coating solution was deamidated α-gliadins at 0.5 µg/mL.

Results and discussion

The reactivity of the 3 mAbs on native and deamidated gliadin sub-fractions and LMW GS were determined by indirect ELISA (Fig 1). Stars indicated the proteins which contain the immunogenic peptide against which the mAb was raised.

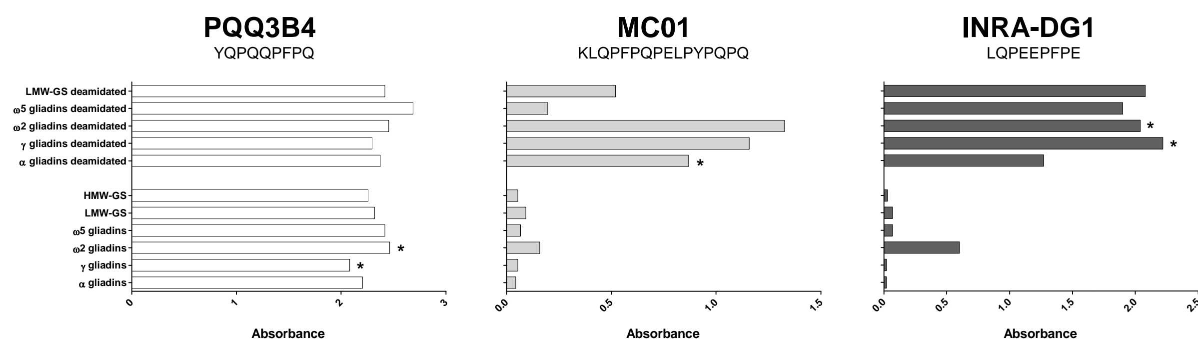

Figure 1. *Reactivity of mAbs PQQ3B4, MC01 and INRA-DG1 towards purified native and deamidated gliadins and GS in indirect ELISA. Stars indicated the proteins in which the immunogenic peptide was present.*

In antigen coated plate ELISA, mAb PQQ3B4 was able to bind to all gliadin classes and LMW-GS, whether native or deamidated. MAb MC01 recognised deamidated α, γ and ω2 gliadins and, to a lesser extent, deamidated LMW GS, while deamidated ω5 gliadins, native gliadins and native GS were not bound. INRA-DG1 recognised all deamidated gliadins and deamidated LMW-GS and showed low but noticeable reactivity to native ω2 gliadins, while the other native gliadins and GS were not bound.

The three mAbs were able to bind gluten proteins which contain their corresponding immunogenic peptide (stars in Fig 1) but they also recognised other gliadins and GS. As gliadins and LMW-GS share homologous sequences in their repetitive domains, the cross-reactivity of mAbs generated against these sequences was expected and has already been described [5,11]. Even considering this point, the wide reactivity of mAb PQQ 3B4 was remarkable. Furthermore, in this assay, the binding of PQQ3B4 to deamidated gluten proteins raises questions. Was the mAb capable of a certain extent

of cross-reactions with deamidated repeated sequences or was its binding only due to native sequences remaining in acid hydrolysed gluten proteins as previously shown for mAb R5 [7,12]? Impact of deamidation on mAb PQQ3B4 binding was tested by pepscan on the peptide QPQQGQQ. The replacement of at least 1 glutamine by glutamic acid abolished PQQ3B4 binding (data not shown). So it can be assumed that mAb PQQ3B4 binding on deamidated gliadins was due to interaction with remaining native epitopes. Both mAbs MC01 and INRA-DG1 have recognised deamidated gliadins and LMW-GS with minimal cross-reactivity to native proteins. A previous characterisation had shown that mAb INRA-DG1 has a restricted reactivity and recognizes strongly deamidated epitopes (XPXEPFPE, where X is Q or E). In the case of MC01, considering that the peptide against which it was generated has only one deamidation site, it can be hypothesised that MC01 would rather bind to weakly deamidated sequences.

In order to go further in determining their ability to discriminate products according to the presence of native sequences or weakly or strongly deamidated sequences, these three mAbs, were implemented in competitive ELISA using modified gliadin and gluten samples whose deamidation rates have been characterised.

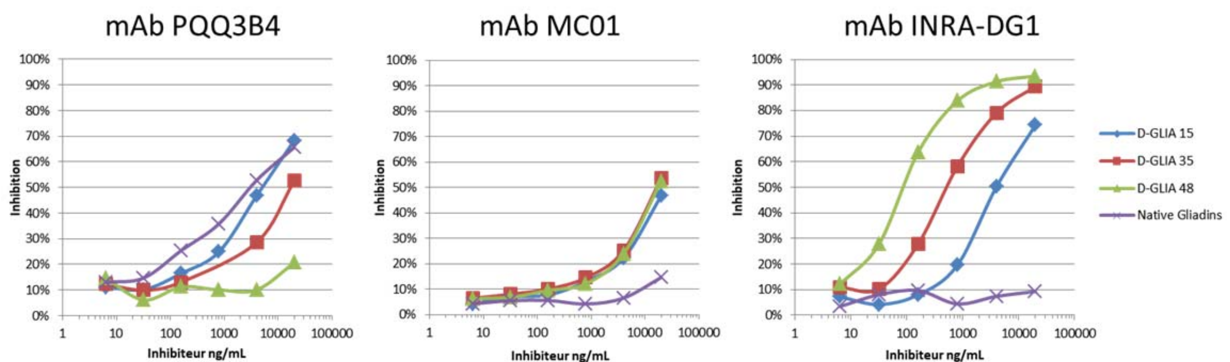

Figure 2a. *Characterisation of mAbs PQQ3B4, MC01 and INRA-DG1 in a competitive ELISA with native and deamidated gliadins (D-GLIA15, 35, 48).*

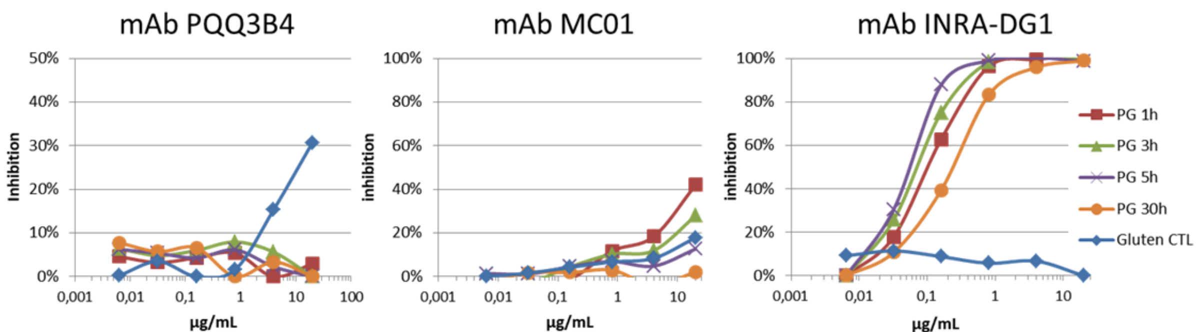

Figure 2b. *Characterisation of mAbs PQQ3B4, MC01 and INRA-DG1 in a competitive ELISA with native and PG-treated gluten 1h, 3h, 5h, 30h.*

In the PQQ3B4 competitive ELISA, native gliadins were the most potent inhibitor. Gliadin samples with low deamidation degree (DD of 15 % and 35 %) were still well detected by this mAb in contrast to the sample with 48 % of DD. The native gluten sample also partially inhibited the mAb binding to native gliadins coated on the plate but none of the PG-treated gluten samples (DD comprised between 20 and 72 %) inhibited PQQ3B4 mAb binding.

Opposite results were observed in the INRA-DG1 competitive ELISA: native gliadins or native gluten showed no inhibitory effect, while INRA-DG1 mAb bound well to all deamidated samples. The inhibitory effect on INRA-DG1 binding gradually increased as the DD of gliadin and PG-treated gluten samples increased. In competitive ELISA, the MCO1 mAb bound equally well to the samples D-GLIA 15, 35 and 48, which displayed an equivalent inhibition capability. In this test, only gluten treated with PG for 1 or 3 h inhibited MC01 (44 % and 28 % respectively); other PG-glutens were not detected neither the native samples.

This combination of three competitive ELISAs indicated that during the kinetic of acid hydrolysis of gliadins, the proportion of native epitopes decreased only significantly for the sample with a DD of 48 %. Despite different DD (15, 35 and 48 %), similar amount of weakly deamidated epitopes remained in the three D-GLIA samples; they much more differ by in their highly deamidated epitopes content. Our combination of mAbs indicated that only two of the PG-treated samples contained a low amount of weakly deamidated epitopes while all contained large amount of highly deamidated epitopes and no native epitopes.

Conclusions

In order to overcome the ambiguity resulting from global determinations of deamidation rates in deamidated gluten, we evaluated the three mAbs, PQQ3B4, MC01 and INRA-DG1 in competitive ELISAs for their capacity to differentiate products based on the extent of deamidation at the epitope level. PQQ3B4 reacts with native epitopes from the repeated domains of gliadins while MCO1 detect weakly deamidated epitopes. INRA DG1 binds to highly deamidated sequences which are the main IgE-binding epitopes involved in allergy to HWP.

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5.2 Increase in plasma interleukin (IL)-2, IL-8, and IL-10 from 2 to 6 hours after oral gluten challenge observed in coeliac disease (CD) but not in non-coeliac gluten sensitivity (NCGS) patients on a gluten-free diet (GFD)

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Introduction

Coeliac disease (CD) is an immune-mediated disease of the small intestine. In patients with this condition, ingestion of dietary gluten drives an immune response where CD4+ T cell recognition of deamidated gluten presented by HLA-DQ2 or HLA-DQ8 molecules is of paramount importance [1]. This disease is diagnosed by clinical signs, serological investigation (IgA against transglutaminase 2) and gastroduodenoscopy where typical histopathological alterations can be found [2]. Many people in the society who do not suffer from CD prefer not to eat gluten due to abdominal problems or feeling of distress. This condition is often referred to as "non-coeliac gluten sensitivity" or "non-coeliac wheat sensitivity" [2]. It has been claimed that this condition caused by innate immune response to gluten [3], yet questions have been raised whether the condition is truly existing [4]. We have done a head-to-head comparison of gluten challenge in these two clinical entities [5, 6]. Clinical evaluation of patients with "gluten sensitivity" is important as CD is associated with significant morbidity and mortality, whereas the NGCS counterpart is not. Importantly, gluten withdrawal normalizes serology and small intestinal histology in CD, so evaluation of individuals already on a gluten-free diet is difficult. In common with many active inflammatory conditions, patients with untreated CD show increased levels of circulating cytokines that are typically associated with activated lymphocytes and/or antigen presenting cells [7]. We therefore performed parallel challenges of treated CeD patients and non-coeliac, but "gluten-sensitive" individuals, who also were on a gluten-free diet.

Materials and Methods

Patients

Two cohorts of patients were studied. CD patients (n=19) had a prior, biopsy-proven diagnosis in accordance with guidelines [5]. They were recruited from our hospital's records, from local support groups or via social media. Prior to challenge, they had a clinical evaluation, reassessment of gluten-free diet was and a control gastroduodenoscopy. Only patients with normalised mucosa (mucosal healing of their diet) could proceed to challenge. NCGS patients (n=49) were also recruited from local support groups and via social media [6]. None of them had a prior specialist evaluation of NCGS, but all were on self-instituted gluten-free diet. HLA typing was performed. Those negative for HLA-DQ2 and HLA-DQ8 were deemed not to have CD. For those positive for these HLA types, we required a previous gastroduodenoscopy with negative result while on a gluten containing diet. The studies were approved by the Regional Ethics Committee and all participants signed an informed, written consent.

Challenge

Both groups were challenged with müsli bars provided by the group of Professor Peter Gibson, Monash University, Melbourne, Australia. The müsli bars are based on quionoa, a cereal devoid of gluten and of non-digestible carbohydrates referred to as FODMAP [8]. The CD patients were challenged, in an open un-blinded manner, with once daily müsli bar spiked with 5.7 g gluten (without FODMAP) for 14 days [5]. The NCGS cohort were challenged, in a double-blind, crossover study with un-spiked müsli bars (placebo), with müsli bar spiked with gluten (same as for the CD patients) and with müsli bars spiked with 2 g fructans [6]. The results of the challenge outcomes are reported elsewhere [5, 6].

Symptom scores, immune activation and cytokine measurements

A müsli bar was consumed in the morning with the patient under observation in the Endoscopy Unit, Oslo University Hospital Rikshospitalet. Symptoms were measured by GSRS or self-administered visual analog scale. CD subjects were assessed for serology, duodenal histology, and frequency of gluten-specific T cells in blood using HLA-DQ:gluten tetramers. The NCGS cohort was followed with symptom scores only. Venipuncture was done and plasma collected before, and 2, 4 and 6 h after intake of the müsli bar. Plasma was first analysed for cytokines with a 27-plex cytokine bead assay from Bio-Rad (Hercules, CA). The advantage of multiplex assays is that they are labour-efficient and require much less amount of sample, but the kits are expensive. We used the recommended 1:4 dilution of all samples and found 6 of 27 tested cytokines to be under the detection limit. The results have been published [5]. We later analysed plasma cytokines with a more sensitive Mesoscale V-plex assay (www.mesoscale.com) (manuscript in preparation).

Results

The müsli bars containing gluten induced mucosal changes (villous atrophy as defined by VH/CrD < 2.0) in 5 of 19 treated CD patients and mobilised HLA-DQ:gluten tetramer positive T cells in 12 of 15 evaluated patients (Fig. 1).

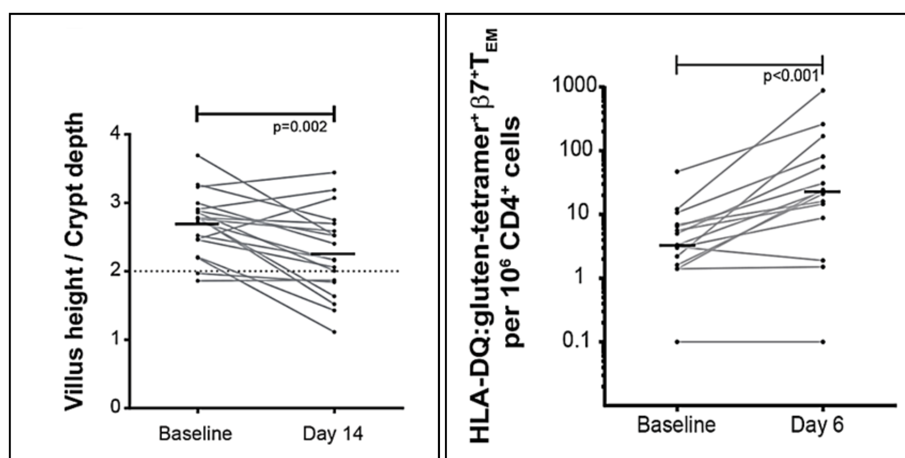

Figure 1. *Gluten ingestion for 14 days induced histopathological deterioration as measured by villous height/crypt depth ratio in the majority of patients (left panel). HLA-DQ:gluten tetramers could detect immune activation in almost all patients (right panel). Data published in [5].*

In the NCGS patients, there were no signs of symptomatic response to the gluten bars versus placebo bars (Fig. 2). The symptom scores after FODMAP (fructan) müsli bars were significantly higher than gluten score for overall GSRS-IBS ($p<0.05$) and GSRS bloating ($p=0.0003$).

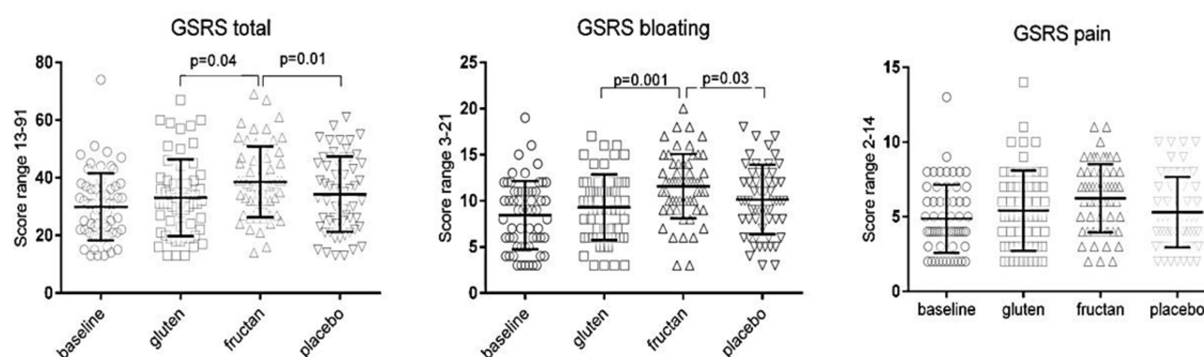

Figure 2. *Individuals that reported gluten-sensitivity (but without proper investigations) were challenged with non-spiked quinoa based müsli bars (placebo) or spiked with gluten or fructans, respectively. Data published in [6].*

Cytokines from challenge of the CD patients were first analysed using the Bio-Rad multiplex system (Fig. 3).

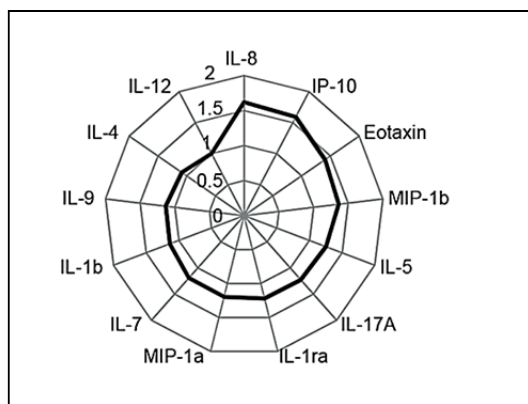

Figure 3. *Results of plasma cytokine levels using the Bio-Rad multiplex system. Shown are fold-change differences compared with baseline levels. Interleukin-2 was also included in the analysis but the levels were below the assay systems detection limit. Figure published in [5].*

We next analysed the cytokine levels with the more sensitive Mesoscal V-plex assay. IL-2 fold changes from pre-challenge were significantly increased in CD compared to NCGS after gluten challenge at 2 h, 4 h and 6 h. Elevations in IL-8 and IL-10 were also significantly increased in CD compared to NCGS at 4 h and 6 h, but median elevations were between 1.2 to 1.8-fold.

Discussion

In CD there is response to gluten with concomitant villous blunting, HLA-DQ:gluten tetramer+ T cells and both "adaptive" and "innate cytokines". Increase in IL-2, IL-8 and IL-10 after oral gluten challenge is specific for CD, but IL-2 is most sensitive. In NCGS there is no clinical response to gluten when this is devoid of FODMAP, but there is clinical response to FODMAP. Measurement of circulating cytokines 4 h after a single gluten challenge may assist in differentiating between CD and NCGS. It may be developed as a clinical tool to distinguish the two clinical entities in individuals already on a gluten-free diet. At present, it should be used in research trials only.

Our findings, and those presented by Tye-Din, Anderson *et al.*, could support a possible approach to diagnostic workup in patients suspected of having coeliac disease who have already adopted gluten-free diet. Conceivably such individuals, who have experienced symptom relief on a gluten-free diet, and who seek investigation of possible CD, could be a target group for the following work-up: Firstly, HLA typing should be performed. Those negative for HLA-DQ2 and HLA-DQ8 can, with a high degree of confidence, be re-assured that they do not have CD. Those who are positive for HLA-DQ2 or HLA-DQ8, can be offered a single gluten challenge with measurement of cytokines after 4 h. Whether this challenge needs to be done with "pure" gluten or if it can be done with regular bread, has not been clarified. Those with a positive cytokine test could be candidates for prolonged gluten challenge followed by regular clinical investigations [2]. Further refinements to this approach or,

alternatively, HLA-DQ: gluten tetramer based diagnostic applications [5,9] may become parts of the clinician's toolbox in the future. At this stage, they serve as research tools only.

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5.3 Re-exploring the iceberg of coeliac disease in children: Preliminary results of a multicenter Italian screening project based on a rapid HLA DQ typing test

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Introduction

Coeliac disease (CD) is an autoimmune inflammatory process of the small bowel, related to the ingestion of particular protein mixture "gluten" found mainly in wheat and similar grains like rye and barley in genetically susceptible individuals [1]. Almost 1 % of the population in Europe and North America are affected with CD [2-4]. Various landmark studies reported a worldwide increase in the prevalence of CD [5-8]. However there are still a high proportion of cases that remain undiagnosed [6]. We aimed to assess the prevalence of CD autoimmunity and overt CD in Italian school age children by using HLA typing as the initial screening test, and to redefine the clinical spectrum of CD.

Materials and methods

Children aged between 5-10 years (49 % males, 51 % females; mean age ±SD: 8.1±0.97 years), attending the primary school from two different regions of Italy, i.e., central part (Ancona) and Northern part (Verona), were invited to participate. No exclusion criteria were formulated. Already known CD subjects data were included during the final study analysis. The diagnostic algorithm of the screening is shown in Fig. 1.

Sample collection

In the school, about 10 µL of blood was collected from each child by a simple prick test for the HLA DQ determination. Positive HLA DQ2/DQ8 subjects were re-invited in the respective hospitals, clinically evaluated and 2 mL blood was collected, serum was separated and kept in -80^0 C in different aliquots until the different serological tests were performed.

HLA DQ2/DQ8 typing

HLA DQ2/DQ8 susceptible subjects were identified by the "Celiac Gen Screen Kit" developed by BioDiagene S.R.L (Palermo, Italy), based on a rapid single PCR reaction

HLA on a single blood drop. The HLA DQ typing takes about 90 min to complete the process and includes following steps:

Step 1. Lysis of blood samples collected using EDTA tubes. Ten µL blood sample was poured in 200 µL of extraction buffer (provided with the Celiac Gene Screen kit) in 1.5 mL Eppendorf tube and incubated at room temperature. 2 µL of this lysate was used further for the amplification.

Step 2. Amplification. In 0.2 mL ready to use PCR-tubes provided with the Celiac Gene Screen kit, 18 µL of ready to use Taq mix (provided with the kit) and 2 µL of isolated DNA were mixed by pipetting. PCR-tubes were vortex PCR-tubes were placed into thermo-cycler. After 1.5 h of PCR run, tubes were ready to use for the analysis.

Step 3. Detection of HLA genes. PCR tubes were placed in the Bio-Run Reader. A software combines and analyses all data at once and gives the interpretation in ~10 – 20 s. Celiac Gene Screen identifies the presence of the alleles that codify for the DQ2 and DQ8 heterodimers that determine the risk of developing CD. depending on the fluorescence value in the tube, the Bio-Run Reader says that CD associated alleles are detected or not in the sample.

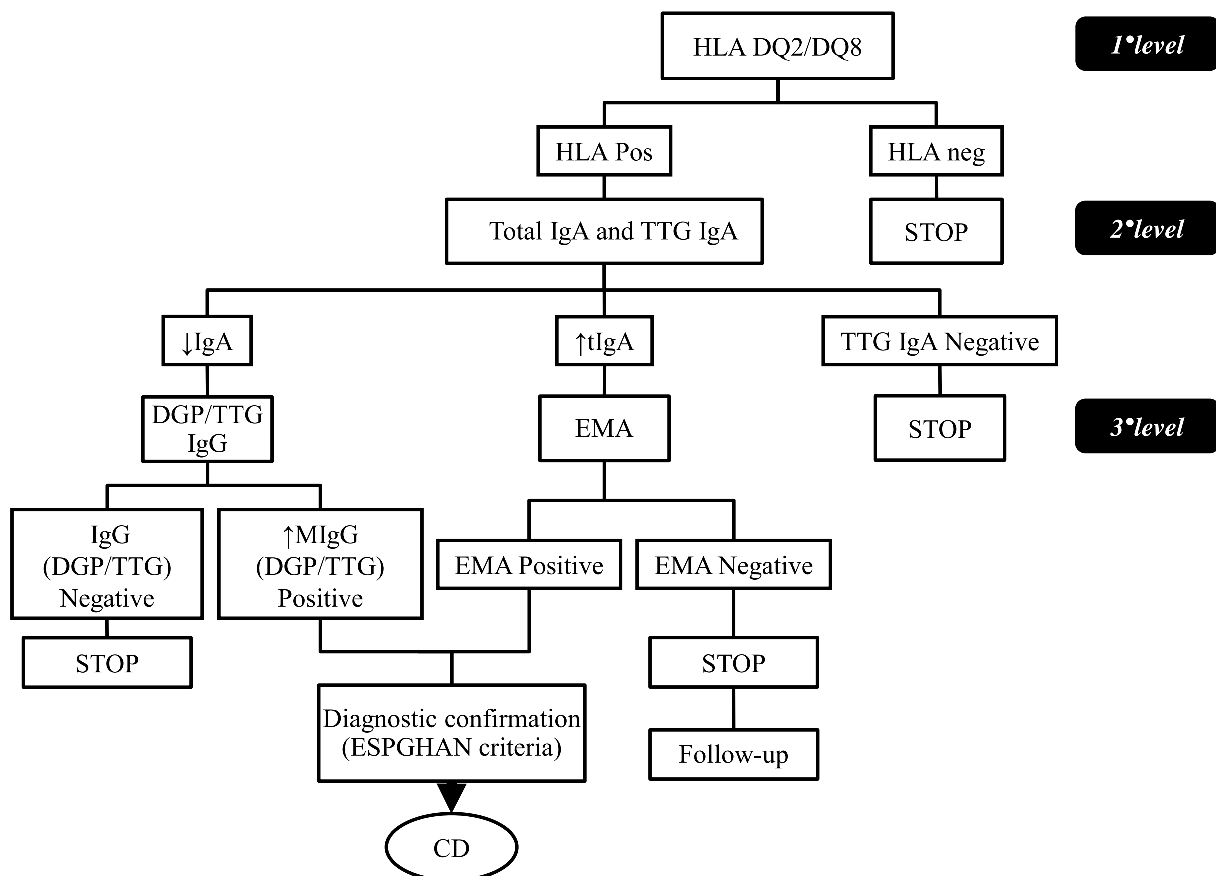

Figure 1. *Diagnostic algorithm of the screening. TTG= anti tTG ab, ↑= increased level of ELISA from the cut off ↓= decreased level of ELISA from the cut off, DGP= Deamidated gluten peptide, EMA= anti endomysial antibody*

Antibody testing

Serum anti-transglutaminase IgA antibodies (TTG) and total IgA were performed in HLA positive patients. Anti-endomysium antibodies (EMA) and anti-deamidated gliadin peptides IgG antibodies were searched in TTG positive and IgA deficient patients respectively. Manufacturer's guideline was strictly followed for every serological test. Biopsy was performed according to the ESPGHAN criteria.

Statistical Analysis

Quantitative variables were summarised as mean ± standard deviation (SD), prevalences and 95 % confidence intervals (CI) were calculated.

Results and discussion

A large number of school children were invited and their screening and further investigation had been done on the basis of the study algorithm mentioned in the methodology part. This study has been recently ended; final analysis is still in progress. At this step we are not able to provide any solid outcome. In coming time final analysis will be completed and the final result will be revealed.

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5.4 Specific TG2 inhibition and clinical study

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No manuscript provided.

5.5 A clinical trial to dissect the transcriptional programme of gluten-specific T cells

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Introduction

Coeliac disease (CD) is caused by an inappropriate immune response to gluten proteins found mainly in wheat, rye and barley. Several types of T cells are involved in CD: a) CD4$^+$ T cells that recognize gluten epitopes presented by HLA-DQ2 or -DQ8 and which orchestrate responses of B cells and other T cells [1, 2], and b) intraepithelial lymphocytes (IEL) composed of CD8$^+$ αβ T cells and γδ T cells that induce tissue damage [1, 3].

Concerning gluten specific CD4$^+$ T cells, Anderson *et al.* have shown that after a gluten challenge over 3 days in treated CD patients, interferon-γ producing, gluten-specific cells measured by ELISPOT can be found on day 6 after challenge [4]. Gluten specific cells CD4$^+$ T cells can also be detected by HLA-DQ:gluten tetramers on day 6 after challenge [5-7]. Notably, HLA-DQ:gluten tetramer positive cells T cells express gut-homing molecules like β7 integrin and CD103 [5] and the activation marker CD38 [8].

Despite several studies employing HLA-DQ:gluten tetramers [5-7], no kinetic analysis of appearance of gluten-reactive T cells in blood after gluten challenge has been performed. In addition, not much is known about functional aspects of HLA-DQ:gluten tetramer staining T cells besides their cell surface markers which are described above.

In this project, we aim to study the kinetics and specific activation of gluten-specific CD4$^+$ T cells. This can then be used to track down potential therapeutic targets for CD. Additionally, we will study the frequency of all gut-homing T cells and their response in blood after gluten challenge.

Materials and methods

CD patients on a gluten-free diet undergo gluten challenge for 3 days with newly in-house developed, standardised gluten-containing cookies (see figure 1). Blood samples are drawn prior to challenge. In the first study (kinetics study), cell kinetics in response

to gluten challenge are examined, and additional blood samples are drawn on day 1 and 4 – 8. For the second study (transcriptome study), that will provide insights into the transcriptomics of gluten-specific T cell, study participants donated blood samples on day 1 and 6 after the first intake of gluten.

Gluten-specific $CD4^+$ T cells are stained with HLA-DQ:gluten tetramers and for the *kinetics study*, their frequency is analysed on flow cytometer for every sample as described elsewhere [7]. In the *transcriptome study*, gluten-specific $CD4^+$ T cells undergo subsequent cell sorting and RNA sequencing [9,10].

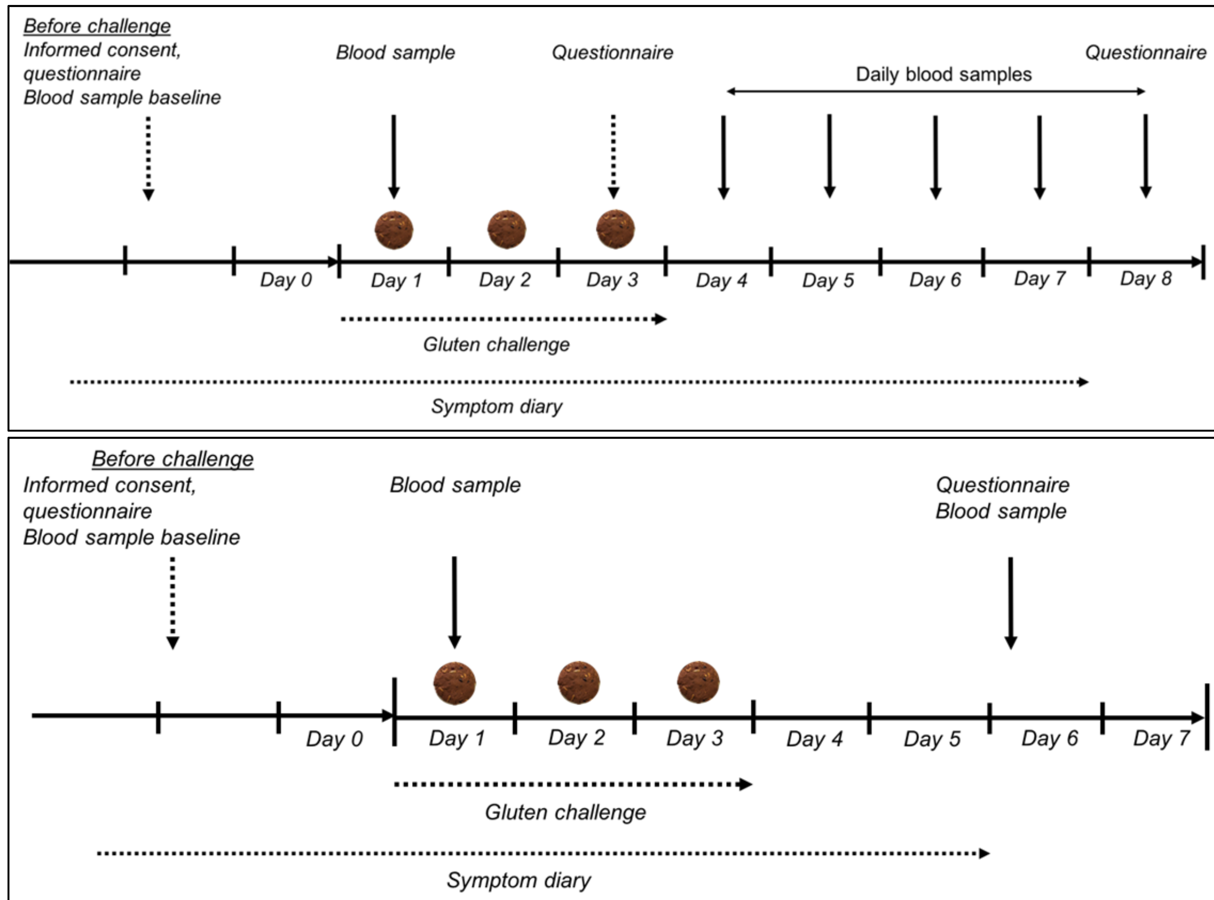

Figure 1. *Timeline for the two gluten-challenge studies: the kinetics study (above) and transcriptome study (below)*

The studies are approved by the regional ethical committee of South-East Norway (ref. 2013/1237) and registered at clinicaltrials.gov (NCT NCT02464150).

Results and discussion

In a first round of experiments, we collected blood samples from five participants in the *kinetics study* and will in a next step analyse the data. For the *transcriptome study*, blood samples from 11 participants have been collected, analysed on flow cytometry and sorted for RNA-sequencing. All of the patients were challenged with gluten for 3 days.

Data from the first patients indicate a clear efflux of gut-homing gluten-specific T cells into the blood after challenge.

Conclusions

We are in the ongoing process of patient inclusion for both the *kinetics study*, as well as the *transcriptome study* to achieve a decent number of participants enabling us to draw reasonable conclusions from our work. The data obtained so far indicate that gluten challenge with newly developed cookies is provoking a specific T cell response in blood.

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5.6 Antibody response to gluten-free diet in paediatric CD is detectable at three months

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Introduction

Until recently, histological investigation of mucosal tissue from biopsies was regarded as a cornerstone of the diagnosis of coeliac disease (CD) [1]. However, it was shown that assays of IgA-antibodies to tissue transglutaminase (IgA-TTG) in blood samples are reliable for diagnosis in a large proportion of patients, meaning that the number of necessary biopsies can be reduced markedly [2-4].

Less is known about the quantitative response of IgA-TTG to a gluten-free diet (GFD). The concentrations of IgA-TTG generally decrease soon after beginning a GFD, but rarely normalize within months and remain high in some patients even after years. We hypothesised that response to GFD can nonetheless be assessed based on short-term decline in antibody concentrations when measured without upper detection limit.

Materials and methods

This is a secondary, exploratory analysis of data of the prospective antibody study (AbCD) [3]. The AbCD study enrolled patients aged 5 months to 18 years if a duodenal biopsy was planned to confirm or refute CD. A baseline blood sample was tested centrally for IgA-TTG and immunoglobulin G against deamidated gliadin (IgG-DGL). A GFD was recommended if there was strong suspicion of CD. Follow-up serology was performed blinded about three months later for central antibody measurements.

To distinguish between a response of antibody concentrations to a GFD from chance fluctuations, the results of central tests were compared with those of local ones (if available). The standard deviation of local vs central measurements of both antibodies in the same patients before GFD was about a factor of two. We define a change of two standard deviations i.e. a factor of four to be a "substantial response" to GFD.

All central antibody tests were performed with test kits of EUROIMMUN (Lübeck, Germany). If antibody concentrations were above the measurement range, sera were serially diluted and values corrected by the dilution factor. For further details see the paper with the primary results [3].

Results and discussion

From 898 patients, 548 had a GFD and 350 had not. After exclusion of children with blood samples taken more than 30 days before beginning a GFD, of children initially on a gluten-reduced, of children without follow-up or with follow-up samples obtained less than 2 months or later than 6 months after beginning the GFD, of children with selective IgA-deficiency and of cases with both IgA-TTG and IgG-DGL below the company cut-off at baseline, 345 children remained for primary analysis (under GFD) and 46 children for secondary analysis (not under GFD).

The results are summarised in Tab.1. The factor of decrease for IgA-TTG during short-term GFD is 14. If we consider only the children with a strict diet, the factor is somewhat higher (15.3). If we consider only children with a less strict diet, the factor is smaller (5.6). Under normal gluten-containing diet, there are no substantial changes. In more than 80 % of the children under GFD the concentration of IgA-TTG is reduced substantially.

The concentration of IgG-DGL decreases during short term-GFD, too. The factor of decrease is smaller than for IgA-TTG. In 28.1 % of children, the concentration is reduced more than fourfold.

Of note, even in case of substantial antibody decrease, IgA-TTG remained above the upper limit of normal in 84 % and above ten times the upper limit of normal (10×ULN) in 27 %, IgG-DGL remained above the upper limit of normal in 38 % and above 10×ULN in 5 % of patients.

A limitation of our study is that we have a lack of long-term data and follow-up biopsies. As a result, we could not follow the progress of “non-responders” nor could we correlate the antibody response with morphological changes in the intestine. A second limitation is that we could not perform a gluten challenge, a third limitation, that it was not possible to assess GFD adherence with an established instrument. We only could use the data provided by the trial paediatrician who could make a cross either for strict, less strict or no diet. Otherwise, assessment of GFD compliance was done according to good clinical practise and the recommendations of the country-specific guidelines for management of CD. Finally, our data are only valid for the test kit of EUROIMMUN.

Table 1. *Short-term response of IgA-TTG and of IgG-DGL to gluten-free diet*

	IgA-TTG		**IgG-DGL**	
	Factor of decrease*	**Children with substantial decrease (%)***	**Factor of decrease***	**Children with substantial decrease (%)***
GFD total (n = 345)	14.0 (12.0-16.4)	80.6 (76.1-84.4)	2.9 (2.7-3.1)	28.1 (23.6-33.1)
very strict (n = 316)	15.3 (13.0-18.0)	83.2 (78.7-86.9)	3.0 (2.8-3.2)	29.7 (25.0-35.0)
less strict (n= 29)	5.6 (3.2-9.7)	-	2.2 (1.7-2.9)	-
Normal diet (n=46)	1.3 (1.0- 1.8)	-	1.0 (0.8-1.3)	-

* Means (95 % confidence interval)

The major strengths of our study are the large number of patients prospectively recruited, the central, blinded measurements of antibodies, and the serial dilution of the blood samples so that even very high antibody concentrations could be measured appropriately.

Conclusions

Measurement of IgA-TTG after 3 months can be useful in confirming the response to a GFD, if initial concentrations are known. Blood samples have to be diluted enough to determine the absolute antibody concentration. For comparability, it is necessary to use of the same test kit for initial and follow-up assays. A decrease by more than a factor of four can be considered a clear indication of a response to GFD. A smaller decrease suggests a rather poor response, perhaps indicating low adherence to the diet and need for clinical or dietetic advice. However, at least on the short-term, high IgA-TTG concentrations at follow-up are consistent with good GFD response.

The concentration of IgG-DGL also decreases in the vast majority of cases, but does not distinguish GFD response from random fluctuations as effectively as IgA-TTG. In standard laboratory procedures, IgG-DGL does not reach the upper bound of the measurement range very often, meaning that it could be a pragmatic albeit inferior alternative to IgA-TTG.

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6 The role of intestinal microbiota in coeliac disease

6.1 Role of bacterial metabolism in gluten peptide modification

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No manuscript provided.

6.2 Gut microbiome trajectory and coeliac disease risk

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No manuscript provided.

6.3 Influence of diets on microbiota from patients with non-coeliac gluten sensitivity

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Introduction

Cereals and especially wheat products are the main carbohydrate sources in Western diets. Interestingly, in recent years patients more often complain about gastrointestinal and extra-intestinal symptoms after eating gluten-containing cereal products. The main symptoms are diarrhoea or obstipation, abdominal pain, flatulence, nausea, and vomiting, as well as muscle, head and body aches, or fatigue. In this context, coeliac disease is well characterised as a destructive intestinal immune response to immunogenic cereal gluten peptides in genetically predisposed individuals, affecting about 1 % of most populations worldwide [1]. In addition, non-coeliac gluten sensitivity (NCGS) is now considered as a distinct clinical entity, caused by wheat consumption [2,3]. In contrast to patients with wheat allergy or coeliac disease no disease specific serological markers are known in patients with NCGS. Furthermore, there is no mucosal damage in patients with NCGS, except of moderately increased numbers of lymphocytes in some patients [3,4]. Since there are no reliable diagnostic markers in NCGS, the prevalence data are vague and range from 0.6 % – 6 % [5,6]. Interestingly, recent double-blind placebo controlled studies showed that gluten trigger the clinical symptoms only in a minority of patients and thus question the causative role of gluten in NCGS [4,7].

In recent years, other elimination diets enjoyed great popularity. In this context, a FODMAP-reduced diet (fermentable oligo-, di- and monosaccharides and polyols) showed a clear improvement of clinical symptoms, especially in patients with IBS [8,9], and a reduction in dietary FODMAPs also improved the gastrointestinal symptoms in most patients with NCGS [7,10]. However, other wheat components like amylase-trypsin inhibitors (ATI), which were shown to stimulate the native immune system [11], or a microbial dysbiosis may also influence the pathogenesis of NCGS. The aim of this study was to evaluate the effect of a low FODMAP diet and a gluten-

free diet (GFD) on the microbial composition from patients with NCGS compared with microbiota pattern from healthy controls.

Materials and methods

Subjects

Nineteen patients with typical clinical presentation of NCGS were enrolled in the study. All patients complained about intestinal and extra-intestinal symptoms which appeared usually within one and twelve hours after ingestion of wheat products, and symptoms resolved under gluten-free diet. Ten healthy controls without any diseases and complaints on standard diets were enclosed. Informed consent was obtained from all participants and the study protocol was approved by ethics committee of University of Erlangen.

Patient characteristics and nutritional analysis

None of our participants adhered to a strict GFD at first presentation. Before study beginning, the age, sex, medication, complaints and underlying diagnoses were requested, and sera were analysed for routine blood parameters, vitamin levels and micronutrients. A nutritional analysis using the nutrition software PRODI® (Nutri-Science GmbH, Germany) was performed for analysis of proteins, fats and carbohydrates.

Exclusion of wheat allergy and coeliac disease

Total and wheat-specific immunoglobulin E (IgE) were determined shortly before study beginning (ImmunoCAP™250, ThermoFisher Scientific, Germany), and a prick test was used to exclude cutaneous wheat sensitisation. Furthermore, coeliac specific serum antibodies for deamidated gliadin and tissue transglutaminase were determined from all study participants using commercially available kits (Eurospital S.p.A., Italy). Genotyping from blood cells for HLA-DQ2 or -DQ8, were performed from all participants according to manufacturer's instructions (Eu-Gen, Eurospital S.p.A., Italy). Patients with NCGS underwent initial gastroduodenoscopy, and 4-5 duodenal biopsies were taken to exclude coeliac disease.

Microbiome analysis

Stool samples were collected and stored at -20°C. Genomic bacterial DNA was isolated from frozen samples with the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany), and the V3-V4 region of bacterial 16sRNA genes was amplified, and DNA was analysed on the Illumina MiSeq.

Study design

All participants ate a Western standard diet for at least 4 weeks before study onset. At study start, the participants were instructed by a professional dietician to consume a

low FODMAP diet for two weeks. After a transition period of at least five days, when participants were requested to eat standard diet, the participants were instructed to consume a GFD for another two weeks. Stool samples were collected under the Western standard diet, and at the end of the low FODMAP and gluten-free diet, respectively. Furthermore, patients completed questionnaires concerning nutrition, gastrointestinal symptom rating score (GSRS), before study start and at the end of each diet.

Statistics

Statistics were performed using GraphPad Prism 6 (GraphPad software Inc., USA). Parametric t-test and non-parametric Mann-Whitney U were used to determine statistical power. $p<0.05$ was considered statistically significant.

Results and discussion

Since the data from this study have not been published, only preliminary results are presented here.

Laboratory and genetics

The laboratory and blood parameters for vitamins and micronutrients did not vary between patients with NCGS and healthy controls.

The HLA-DQ2 or -DQ8 are mandatory to develop coeliac disease, but their role in NCGS is still unclear. Interestingly, our data showed that these genotypes were over-represented in patients with NCGS (52.6 %), compared to controls (30 %) and prevalence data in Western countries, and thus are in accordance with other reports [3,4,12,13].

Dietary effects on clinical symptoms

Wheat containing standard diet caused clinical symptoms in all our patients with NCGS. The main gastrointestinal complaints were flatulence, abdominal pain, and diarrhoea. The severity of gastrointestinal symptoms was significantly different between NCGS patients and healthy controls at the beginning of the study under Western standard diet. Patients with NCGS reported a clear improvement of gastrointestinal symptoms under a low FODMAP diet, and their complaints further improved when they consumed the GFD.

Thus, our study demonstrated a substantial improvement of gastrointestinal symptoms in patients with NCGS already after consuming a low FODMAP diet. Apart from the risk that a non-blinded two-week FODMAP low diet may have caused some placebo effect, our data confirmed the observations from others that FODMAPs at least partially account for the clinical symptoms in NCGS patients [10,14]. However, gluten elimination further improved and almost completely resolved clinical symptoms in all our patients with NCGS, suggesting a multifactorial trigger in NCGS.

Preliminary data of dietary effects on microbiota

It was demonstrated that patients suffering from IBS profit from a low FODMAP diet [8,15,16], but the mechanisms how a FODMAP-restricted diet may affect IBS symptoms is still unclear. An important role of intestinal bacteria and bacterial dysbiosis was suggested in IBS [17].

When we compared the microbiota on phylum level, there was a clear trend to decreased *Bacteroidetes* and increased *Firmicutes* in NCGS patients compared to healthy controls. Interestingly, these data resemble findings from other studies, where a similar dysbalance was described in most IBS patients [18,19]. Whereas, dietary changes had only a minor effect on microbiota in our controls, the diets caused significant variations in our patients with NCGS.

Thus our study confirmed other reports that also showed a higher microbial fluctuation in patients with IBS, and dietary changes resulted in more variations in microbial composition, whereas healthy controls showed higher stability of their microbiota [20,21]. Although there were major variations after dietary changes especially in patients with NCGS, we noticed a substantial clustering of the individual microbiota, even under the different diets. This is in accordance with a prior study showing that a major dietary modification caused microbial shifts within 24 hours, while the enterotypes with their dominant species remained stable during the whole ten-day observation period [22].

Conclusions

Our data suggest a multifactorial ongoing in NCGS caused by FODMAPs, combined with a gluten-related immune reaction and a microbial dysbalance. However, the direct causal role of over- or under-represented microorganisms in NCGS remains unclear and more detailed analyses are required.

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7 Statements on current developments concerning gluten analysis, clinical and legal aspects

7.1 Comparison of analytical data from collaborative studies using R5 and G12 antibodies

Paul Wehling

General Mills, Inc., Golden Valley, USA

No manuscript provided.

7.2 News from Codex and Regulatory Affairs

Hertha Deutsch

AOECS Codex Delegate, Austrian Coeliac Society, Vienna

Introduction

AOECS, the Association Of European Coeliac Societies, has Observer status in the Codex Alimentarius Commission since 1992. Information about the organisation, the duties, the Codex Standards and Guidelines, the Procedural Manual and all the extensive work of all Codex Committees is published on their website [1].

Regarding gluten-free foods, especial two Codex Committees are very important: Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) and the Codex Committee on Methods of Analysis and Sampling (CCMAS).

Since November 2014, CCNFSDU and CCMAS have been considering the G12 method as a potential additional method for gluten determination.

Considerations in the past years

Details about the discussions in the CCNFSDU and CCMAS sessions were published in the Proceedings of the Working Group on Prolamin Analysis and Toxicity [2,3]. Here are the results:

Conclusion of the CCNFSDU session in November 2014

"CCNFSDU36 agreed to ask CCMAS to examine ELISA G12 as a potential additional method for determination of gluten."

Reply from the CCMAS session in February 2015

"When considering "ELISA G12" as a potential additional method for inclusion in CODEX STAN 118-1979, CCMAS 36 noted that any potential endorsement of G12 would be as a Type I procedure and that it would not be possible to have two Type I methods in the Standard for the same matrices and determination. CCMAS further observed that if the G12 method for detection of the toxic fraction in gluten harmful for individuals were added, the provision in the Standard would need to be differentiated to allow for both methods (R5 and G12) to be included as Type I methods. The Committee noted that G12 had been validated for gluten-free foods, rice matrices, whereas R5 had been validated for gluten-free foods, maize matrices. CCMAS 36 recommended that decision in this regard should be taken by CCNFSDU.

The Committee is invited to consider the above reply."

Conclusion of the CCNFSDU session in November 2015

“The Committee noted the reply from CCMAS in particular with respect to validation of the R5 and G12 methods, based on the two matrices, maize and rice but questioned: which method to adopt for mixed matrices; the comparability of the two methods (if different results emerge) and the implications for “gluten-free” labelling. The Committee decided to seek further clarification from CCMAS with the following request:

- *Taking into account that the thresholds in CODEX STAN 118-1979 were established on the basis of the results given by the ELISA R5 Method, can CCMAS confirm that the results of the two methods (R5 and G12) are fully comparable for all products covered by the standard, in particular:*
  - *products manufactured from ingredients naturally free of gluten (e.g. buckwheat, millet, amaranth, quinoa etc.);*
  - *products manufactured from gluten-containing ingredients (e.g. partially hydrolysed wheat protein, wheat starch, malt extract, glucose syrups etc.);*
  - *products based on oats;*
  - *liquid matrices.”*

Reply from the CCMAS session in February 2016

“The Committee agreed to inform CCNFSDU that the two methods (R5 and G12) for the determination of gluten are not comparable; that comparability data for the two methods were not available; and mixed matrices are not included in the scope of either of the methods obtained during their validation. The developers of these proprietary methods might be able to provide further information on the applicability of the methods.”

Recent CCNFSDU and CCMAS sessions

The 38th session of the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) took place from 5 - 9 December 2016 in Hamburg, Germany. The Committee was attended by delegates from 56 Member Countries, one Member Organisation and 38 International Organisations, one of them was AOECS.

At agenda item 2 „Matters referred to the Committee by the Codex Alimentarius Commission and/or other subsidiary bodies“, sub-item „Matters for action“, the CCNFSDU has to consider the reply from the CCMAS session in February 2016.

During the discussion I informed CCNFSDU that the Working Group on Prolamin Analysis and Toxicity is working on comparability studies and the conclusion of this item is in the CCNFSDU report [4]:

"The Committee agreed not to include the ELISA G12 method in the Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten (CODEX STAN 118-1979) noting that there were no comparability results with R5; and it would be considered at a future date when the results from the ongoing comparability studies by the international Working Group on Prolamin Analysis and Toxicity become available."

The 38th session of the Codex Committee on Methods of Analysis and Sampling (CCMAS) took place on 8 - 12 May 2017 in Budapest, Hungary. The Committee was attended by 47 Member Countries, one Member Organisation and 11 Observer Organisations, one of them was AOECS.

At agenda item 2 "Matters referred to the Committee by the Codex Alimentarius Commission and/or other subsidiary bodies", sub-item "Matters for information", the secretary informed CCMAS about the decision of CCNFSDU - see above. The Committee noted this information without any comments.

Since a few years an important item is on the agenda of CCMAS:

Development of Procedures/Guidelines for determining equivalency to Type I Methods

In the past years, an electronic Working Group considered in general the subject of equivalency of Type 1 Methods and finally CCMAS agreed to the following conclusion for the CCMAS report [5]:

"The Committee could not reach consensus on the use and scope of the equivalency approach and agreed to reconsider this matter in the future when more information became available. The Committee noted that most of the work in determining equivalence falls on the Standards Development Organisations (SDOs), and noted the offer of the SDOs, through the Inter-Agency Meeting (IAM), to look into this matter and provide recommendations to a future session of CCMAS."

Codex Methods of Analysis

The Codex Procedural Manual [1] defines 4 types of methods. If any new method should become a Codex Method, it is mandatory to fit in one of the following types:

"(a) Defining Methods (Type I)

Definition: A method which determines a value that can only be arrived at in terms of the method per se and serves by definition as the only method for establishing the accepted value of the item measured.

(b) Reference Methods (Type II)

Definition: A Type II method is the one designated Reference Method where Type I methods do not apply. It should be selected from Type III methods (as defined below). It should be recommended for use in cases of dispute and for calibration purposes.

(c) Alternative Approved Methods (Type III)

Definition: A Type III Method is one which meets the criteria required by the Committee on Methods of Analysis and Sampling for methods that may be used for control, inspection or regulatory purposes.

(d) Tentative Method (Type IV)

Definition: A Type IV Method is a method which has been used traditionally or else has been recently introduced but for which the criteria required for acceptance by the Committee on Methods of Analysis and Sampling have not yet been determined."

Conclusion

Gluten-free foods are imported and exported all over the globe. It is essential for coeliacs, food manufacturers and national food control authorities that all approved gluten detection methods should show comparable results.

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8 Perspectives and action plan of the PWG

Peter Koehler

Biotask AG, Esslingen, Germany

The Prolamin Working Group executive meeting and joint discussion held on 29 September 2017, led to the decisions and statements outlined below.

Action plan

I. Analytical

- Due to a job change of the chairman, the distribution of the PWG gliadin reference material is no longer possible by Peter Koehler.
- Efforts to find alternative distributors (R-Biopharm AG, Leibniz-Institute for Food Systems Biology) failed.
- The distribution of PWG-gliadin has been discontinued from October 2017 until a new distributor has been identified.
- New reference material: The MoniQA initiative is currently identifying cultivars of wheat, rye and barley and aims at providing flours as gluten reference.
- However, the PWG thinks that flour is not a suitable reference material and supports a protein sample as reference material.

II. Clinical

- For the symposium of the 2018 meeting the topic “Wheat Genomics” has been selected. In addition, a speaker on “Gluten Epitopes and Interaction with Antibodies” will be invited

III. Members, Policy

- Katharina Scherf is a new member of the group.
- Rudolf Valenta and Martin Stern left the group.
- New group members will be identified by the group and two potential members will be invited to the next meeting.
- Fernando Chirdo is responsible for the website.
- This printed, citable book (print run: 250 copies with ISBN number) was made possible by funding of Dr. SCHÄR GmbH/Srl, (Burgstall, BZ, Italy). It will be distributed among leaders of opinion in gluten analysis and clinical medicine. An electronic version can be downloaded free of charge from the PWG website.

Next meeting: 2018

We are very pleased to announce the venue for our meeting in 2018:

Ayr, Scotland, United Kingdom

Host:

Ms. Pauline Titchener
Neogen Europe Ltd.
E-mail: p.titchener@neogeneurope.com

Time: 27 - 29 September 2018

Focus of the meeting:

- Wheat genomics
- Gluten epitopes and interaction with antibodies

The meeting will be limited to 55 participants and attendance is by invitation only. Invitations will be sent by March 2018. Registration deadline will be June 15, 2018.

For registration please contact:

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Very special thanks to the hosts for this kind invitation!

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