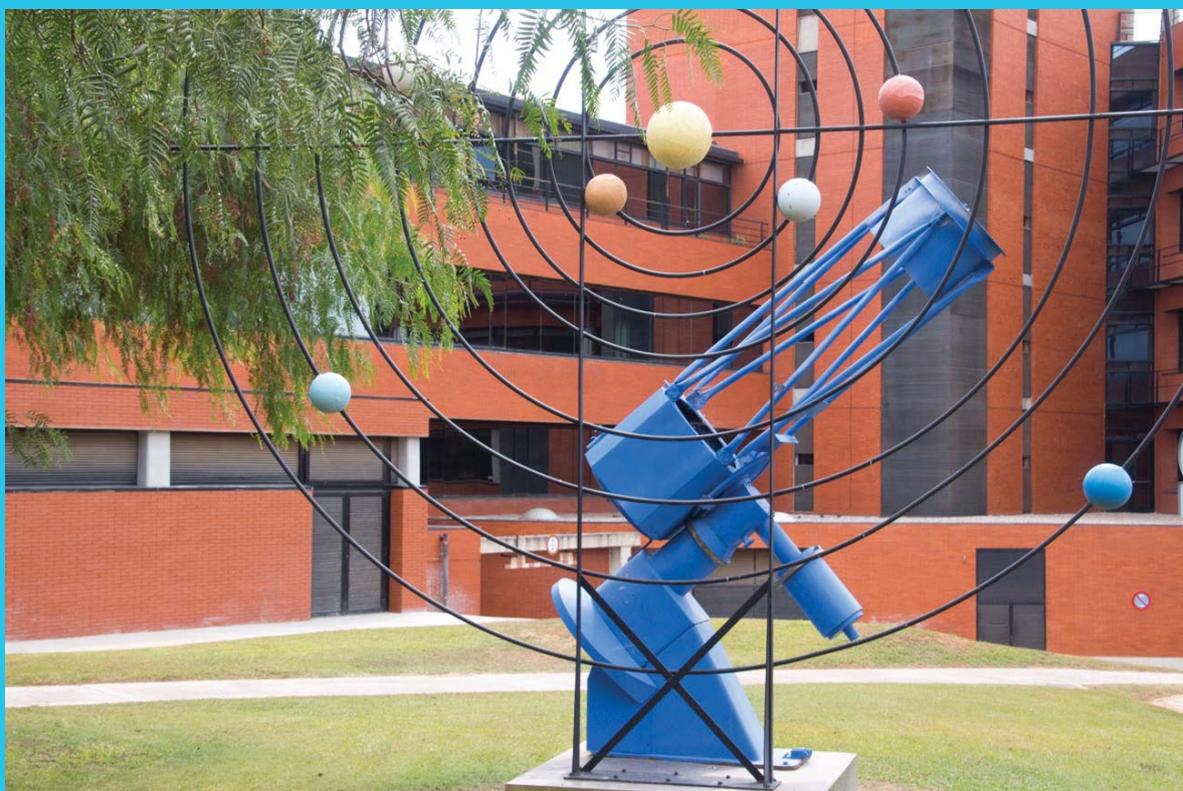




Proceedings of the 30th Meeting

WORKING GROUP
on PROLAMIN ANALYSIS and TOXICITY

Edited by
Peter Koehler
German Research Centre for Food Chemistry



22 - 24 September 2016
Valencia, Spain

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

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April 2017

Impressum

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Cover picture* and picture of participants

Thomas Mothes

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ISBN: 978-3-00-055981-5

* Cover picture: Sculptural set called "Solar system in blue" which is situated in the inner court of the Institute of Agrochemistry and Food Technology (IATA-CSIC). The set is formed by a telescope and a schematic representation of our solar system.

Preface

In October 2014, I asked Cristina Rosell if she would be willing to host the 30th meeting of the Working Group on Prolamin Analysis and Toxicity (PWG) and she accepted with pleasure. She learned about the PWG meeting as a guest at the 2015 meeting in Tulln, Austria and started planning “her own” meeting. Together with her colleague Maria Saneustaquio she organised the PWG meeting 2016 at the Hotel Sercotel Sorolla Palace, Valencia, Spain from 22 to 24 September 2016. Cristina and Maria were present during the entire meeting. As the chairman of the PWG, I assume that going to Valencia at this weekend was like returning into summer. The PWG was hosted by the Institute of Agrochemistry and Food Technology (IATA-CSIC) and the “Asociación de Celíacos de la Comunidad Valenciana”. The PWG, the invited speakers, the participants from industry (cereal starch producers, producers of gluten-free food, producers of kits for gluten analysis) and research institutes as well as the delegates from European coeliac societies came together and had very interesting one-and-a-half days of presentations, discussions and networking.

Analytical and clinical work in the field of coeliac disease and gluten done in the labs of the PWG members as well as results of guests and invited speakers were presented in 22 talks and intensely discussed at the meeting. In addition, one presentation was focussed on regulatory aspects of gluten analysis and labelling. This was the highest number of presentations at the PWG meeting during the last decade. A symposium on “Enzymatic Gluten Degradation” with two presentations of internationally recognised experts highlighted the latest advances in the field of gluten-specific peptidases.

I would like to express my thanks to all participants of the meeting for their active contributions and the discussions that resulted thereof. I am in particular grateful to Maria Saneustaquio and Cristina Rosell from IATA-CSIC for their enthusiasm and hospitality, which made this perfectly organised meeting a great success. Also, very special thanks to Katharina Scherf for her invaluable help in proofreading. Finally, I would like to express my appreciation to all friends, colleagues and sponsors for their ongoing support of the PWG and the meeting.

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1 Executive summary

Among the topics of the meeting were food technological aspects of the production of gluten-free baked goods, the importance of the small intestinal microbiome in the diet of coeliac disease patients, analytical issues of gluten, clinical studies on coeliac disease and non-coeliac gluten sensitivity, serology of coeliac disease, further aspects of the pathomechanism of coeliac disease, as well as legal issues.

Analytical session

Six presentations were given in this session. A novel reference material for barley gluten based on C-hordeins was suggested. It appears that the immunodominant 33-mer peptide is common among wheat cultivars but without being correlated with the gluten content. Data on problems in the quantitation of the gluten content of wheat starches induced lively discussions, which are also related to the future evaluation of ELISA methods for approval with the Codex Alimentarius. This led to a meeting of experts after the end of the conference to discuss about further actions. Finally, breeding activities for wheat without coeliac disease activity and a possible role of non-gluten proteins in coeliac disease were on the agenda.

Clinical session

This session included twelve presentations, which was by far the highest number during the last years. Topics were widespread and included *in vivo* studies with different diets in coeliac disease and non-coeliac gluten sensitivity. Serological studies showed that blood tests are now of major importance in the diagnosis of coeliac disease. The issue of partially hydrolysed gluten for the immune system was highlighted as well as the impact of amylase-trypsin inhibitors on intestinal inflammations. Crystallographic studies on the interaction between HLA-DQ-gluten and gluten-specific T-cell receptors gave insights into the pathomechanism of coeliac disease.

Symposium: Enzymatic gluten degradation

The symposium comprised two presentations on the identification and use of enzymes for degrading gluten and gluten peptides. A very interesting talk described the identification of gluten-specific peptidases of the subtilisin family from dental plaque. Some of these enzymes have a food-grade status and are promising candidates for preparations that could be used for gluten ‘detoxification’ of foods or as oral supplements for gluten degradation in the stomach. The second presentation dealt with the application of peptide libraries to determine the stability of gluten peptides towards gastrointestinal peptidases. Rat enzymes have been used so far, but the approach is promising for the human peptidase system.



30th Meeting of the Working Group on Prolamin Analysis and Toxicity (PWG), Valencia, Spain, 22 - 24 September 2016

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3 Programme

THURSDAY, 22 September 2016

20:00 Arrival of Prolamin Working Group and all participants
Informal get-together with dinner
Welcome by Cristina Rosell
Location: Hotel Sercotel Sorolla Palace, Valencia

FRIDAY, 23 September 2016

08:30 Bus transfer to the institute

09:00 Opening of the meeting (Peter Koehler)

09:15 Alternatives for developing gluten-free bakery foods
Prof. Dr. Cristina M. Rosell, Valencia, Spain

10:00 Analytical research reports

- *Chirido, Ciclitira, Feighery, Gilissen, Koehler, Koning, Lundin, Mothes, Schuppan, Tranquet; guests*

11:00 Coffee break

11:30 Analytical research reports (continuation)

12:10 Clinical research reports

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

12:50 Lunch

14:00 Clinical research reports (continuation)

16:00 Coffee break

16:30 The Prolamin Working Group Executive Meeting (members only)

17:00 Bus transfer to the hotel

18:15 Bus departure from the hotel for all participants
Short reception at the city hall of Valencia
Joint dinner: Restaurante Contrapunto, Palau de les Arts, Valencia

23:00 Bus departure to the hotel

SATURDAY, 24 September 2016

08:30 Bus transfer to the institute

09:00 Interplay between gut microbiota and diet in coeliac disease
Yolanda Sanz, Valencia, Spain

SYMPOSIUM

09:45 Enzymatic Gluten Degradation
Chair: Prof. Dr. Frits Koning, Leiden, The Netherlands

09:50 Identification of Novel and Food-grade Gluten-degrading Enzymes
Eva Helmerhorst, Boston, U.S.A.

10:30 Studying Kinetics of Intestinal Gluten Degradation Using Peptide Libraries
Dr. Andreas Frey, Borstel, Germany

10:50 Coffee break

11:20 Clinical research reports (continuation)

12:20 Discussion of current developments concerning gluten analysis, clinical and legal aspects

Statements by participating organisations, representatives from industry and guests

Outline: Action plan 2017 of the Prolamin Working Group

13:00 Lunch

14:00 Bus transfer to the hotel and farewell

Afternoon

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

Tour of Valencia and joint dinner in a tapas bar

SUNDAY, 25 September 2015

Departure of the Prolamin Working Group

4 Analytical research reports

4.1 Alternatives for developing gluten-free bakery foods

Cristina M. Rosell

Institute of Agrochemistry and Food Technology (IATA-CSIC), Valencia, Spain

Introduction

Cereals, and more precisely wheat, have been at the base of the food pyramid through the human history. Even today, cereals are the main players feeding human population; although their worldwide contribution to nutrient uptake is different. However, there are specific targeted groups with special requirements when consuming cereals, namely gluten-containing grains. Coeliac disease, first considered to be a gastrointestinal disease, is a gluten-sensitive enteropathy with genetic, immunologic, and environmental bases. Great efforts are being made to understand the gluten-related pathologies from the genetic and immunologic point of view and also the implication of diet and gluten-free products on the life quality of the patients [1]. The clearest statement is that the only way to ameliorate the symptoms is keeping a lifelong diet free of gluten products.

In the last decade, gluten-free foods have shifted exponentially from a niche market to become a revolution and to mark a lifestyle. Gluten-free has been described by consumers as: “a mainstream sensation, embraced by both out of necessity and as a personal choice toward achieving a healthier way to live”. However, in this scenario nutritionists must play a fundamental role conducting counselling and closely following the dietary management of coeliac individuals.

The initial challenge when developing gluten-free products as a necessity for solving pathologies was to overcome the technological restrictions that the absence of gluten provoked in the development of fermented cereal-based foods [2]. The main goal was to look for tools to technologically replace the gluten giving sensorially accepted products. However, gluten is not just a great protein matrix, it is a protein with incomparable viscoelastic properties. Because of that its replacement has been an enormous challenge during decades, and it is still a hot topic. Initially, only starches and hydrocolloids were considered but later on, different tools have been developed for defining food recipes resembling the quality of gluten-containing goods.

Nevertheless, in this picture not only the sensorial quality must be considered, it is an essential requirement that those gluten-free foods provide the required nutrients' intake for those gluten-intolerants, contributing also to their wellbeing and healthy status at present and also considering long-term nutrition.

Technological approaches for miming gluten in gluten-free bakery products

Replacement of gluten functionality has been a challenge for food technologists. Its absence leads to less cohesive and elastic doughs that result in bread with a crumbling texture, poor colour and low specific volume. Therefore, in the last years numerous studies have been focused on improving the physical properties of gluten-free foods, particularly fermented and baked foods like bread [3]. Gluten-free recipes are very complex, and gluten-free bread is the result of the interaction of the ingredients.

Generally, bread development without gluten has involved the use of diverse ingredients and additives with the purpose to obtain wheat bread-like properties. Approaches proposed for obtaining gluten-free bread include the use of different naturally gluten-free flours (rice, maize, sorghum, soy, buckwheat) and starches (maize, potato, cassava, rice), dairy ingredients (caseinate, skim milk powder, dry milk, whey), gums and hydrocolloids (guar and xanthan gums, alginate, carrageenan, hydroxypropyl methylcellulose, carboxymethyl cellulose), emulsifiers (DATEM, SSL, lecithins), non-gluten proteins from milk, eggs, legumes and pulses, enzymes (cyclodextrin glycosyltransferases, transglutaminase, proteases, glucose oxidase, laccase), and non-starch polysaccharides (inulin, galactooligosaccharides). Strengthening additives or processing aids have been fundamental for miming gluten

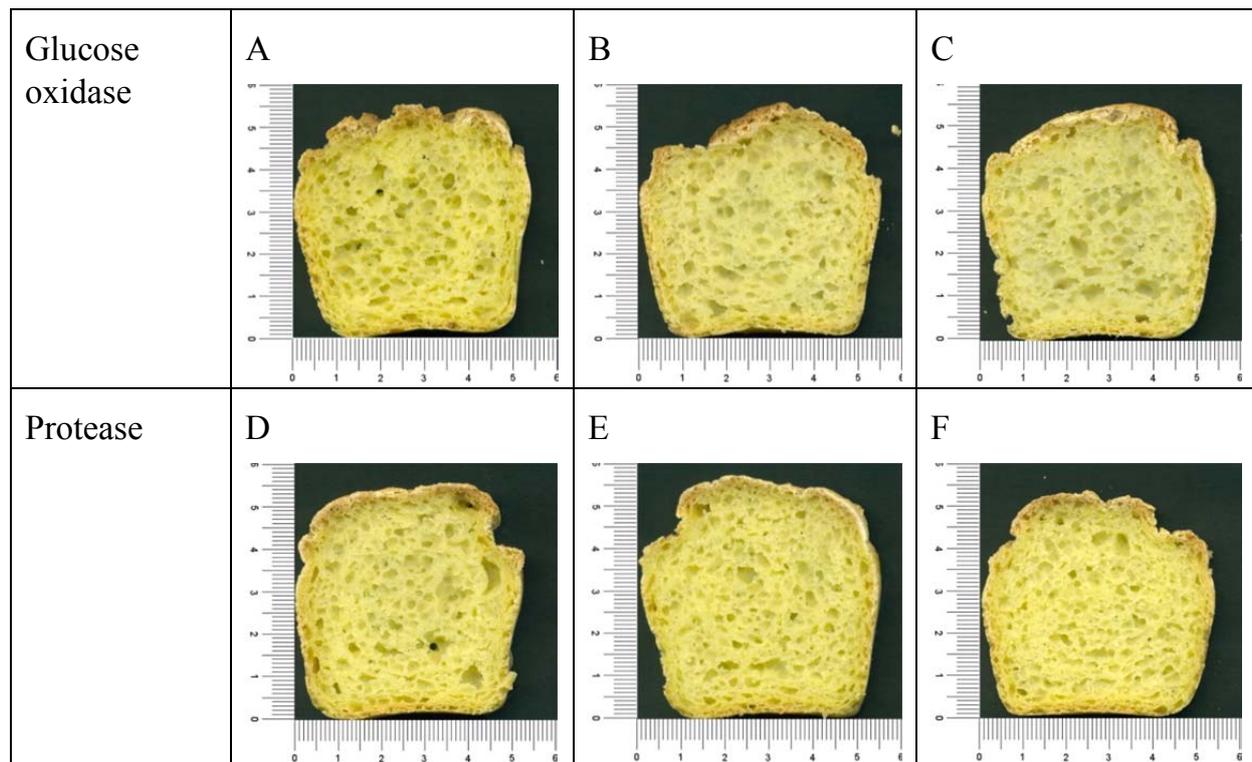


Figure 1. Cross section of corn breads obtained with different enzymes (glucose oxidase and protease) at different levels (expressed as % (w/w) flour basis). Basic recipe contained 1% xanthan gum. A: Glucose oxidase-0%, B: Glucose oxidase-0.01%, C: Glucose oxidase-0.02%, D: Protease-0.05%, E: Protease-0.1%, F: Protease-0.2%

viscoelastic properties [4]. With that purpose, mainly hydrocolloids have been used for building an internal network able to hold the structure of fermented products. With the same purpose, different crosslinking enzymes such as glucose oxidase, transglutaminase and laccase have been used for obtaining a protein network within the flour proteins [5]. Nevertheless, even the disruption of the flour proteins with proteases has been revealed as a good strategy to improve dough performance and in consequence the features of the resulting breads, owing to the decrease of protein hydrophobicity [5]. However, it must be stressed that the effect of the enzymes as gluten-free processing aids is greatly dependent on the type of flour, enzyme source and level, which could lead to improve the bread performance or even to the opposite effect (Fig. 1). Very often the combinations of ingredients and the optimization of the breadmaking process can overcome the technological problems, yielding gluten-free products that meet the consumer's expectations concerning texture and appearance of the fresh bread [6,7].

Nutritional and health aspects of gluten-free products.

Previous reviews showed that much research has been conducted on gluten-free foods from different angles to obtain good quality gluten-free-foods. Nevertheless, the nutritional quality of those products has been of interest only recently. In the last five years, the driving force behind gluten-free research has been the nutritional quality. Very recently, Matos and Rosell [8] reviewed the different available strategies for improving the nutritional quality of gluten-free breads. The absence of gluten in natural and processed foods constitutes the therapy treatment for coeliac disease, which may lead to nutritional consequences linked to the composition of gluten-free products. The exclusion of gluten-containing cereals, important vitamin and mineral sources, from the diet might provoke deficiencies in iron, vitamin B and dietary fibre. In fact, common nutrient deficiencies in coeliac subjects at diagnosis are calory/protein, fibre, iron, calcium, magnesium, vitamin D, zinc, folate, niacin, vitamin B12 and riboflavin [9]. Following a lifelong gluten-free diet requires a parallel nutrition counselling, not only focused on the foods to avoid when sticking to a gluten-free diet, but also the nutritional quality of gluten-free products must enter into the equation to elude deficits and imbalances.

Some concerns have arisen after publishing some reports showing that the nutritional quality of gluten-free products available on the market were poorer than their gluten-containing counterparts. Gluten-free breads are starch based foods low in proteins and high in fat content, with high glycaemic index [10]. Therefore, a lifelong adherence to gluten-free products could lead to undernourishment and also mineral deficiencies that might end in anaemia, osteopenia or osteoporosis. In the particular case of gluten intolerance, it must also be considered of that coeliac disease induces an intestinal lesion that leads to various deficiencies of nutrients, vitamins, and dietary minerals, with ferropenia, vitamin B12, folic acid, and fat-soluble vitamin deficiencies being especially frequent.

Therefore, a careful design of gluten-free bakery goods is needed for obtaining gluten-free baked products resembling the nutritional composition of their gluten counterparts to meet dietary guidelines without changing their dietary pattern and to avoid nutrient deficiencies.

Enrichment or fortification is a strategy commonly applied to mitigate nutritional deficiencies of the population and wheat flour has been a common carrier for minerals and vitamins. In the case of gluten-free products, although this strategy has been less exploited, there are some trends to complement or balance the nutritional composition of those foods. In the case of minerals calcium salts like lactate, citrate, chloride and carbonate have been proposed as sources of elementary calcium for obtaining fortified gluten-free breads [11]. The supplementation of gluten-free bread with proteins has been a technological strategy for improving the protein network and also for increasing the nutritional quality of gluten-free breads. Legume flours have become very useful for protein and fibre enrichment of bakery foodstuff, like gluten-free cakes, although it is necessary to carefully select the legume to avoid any effect on the technological and sensorial quality [12].

Lately, the physical treatment of the raw materials for enhancing the nutritional quality or healthy pattern is gaining popularity. The selection of the particle size distribution in the gluten-free flours has great impact on the technological properties of the products, but it also determines the glycaemic index of the resulting fresh products. For instance, in rice flour, particle size heterogeneity is responsible for different patterns in starch enzymatic hydrolysis, allowing the modulation of their digestibility. Particularly, enzymatic digestibility increases with the reduction of the particle size [13]. With the same purpose, germination, toasting or cooking of the grains have been proposed for increasing the nutritional, functional, and sensory properties of grains such as pulses and cereals [14-15]. For instance germination of rice kernels under controlled conditions of temperature and time allows the degradation of beta-glucans, increases the content of certain essential amino acids and B-group vitamins and improves protein and starch digestibility.

Further research

Currently, research is moving fast and numerous gluten-free foods are launched annually. In spite of scientific advances, there is no date in the near future for having high quality gluten-free food products nutritionally equivalent to gluten-containing products. Lately, consumers' interest in the role of nutrition for health and wellbeing seems a priority. Therefore, today, the main concern of the industry is to innovate, meet and satisfy consumer requirements. In the baking industry that trend has prompted the development of baked goods keeping in mind the healthy concept. Enrichment of formulations, physical treatment of raw materials and the usage of non-common flour sources are alternatives for enhancing the health benefits of gluten-free baked foods. In that scenario, some other approaches like the exploration of the use of

enzymes as “healthy aids” or the use of “smart starch” as vehicle of functional ingredients must be encouraged [16-17].

Acknowledgements

The financial support of the Spanish Ministry of Economy and Competitiveness (Project AGL2014-52928-C2-1-R) and the European Regional Development Fund (FEDER) is acknowledged.

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4.2 Detection of gluten in products containing barley: A proposal for C-hordein as reference material

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² *Fazer Mills, Oy Karl Fazer Ab, Lahti, Finland*

Introduction

When measuring residual barley prolamins (hordein) contamination in gluten-free products by the R5 ELISA method, the concentration of prolamins is overestimated with the gliadin standard [1-3]. The reason for this may be that the composition of the gliadin standard is different from the composition of hordeins. A hordein standard is needed for barley prolamins quantification instead of the gliadin standard. C-hordein, the primary structure of which is almost entirely repeats of PQQPFPQQ, is strongly recognised by the R5 antibody and has 15-20 times more reactivity than the reference gliadin [4]. The aim of this study was to investigate the proportion of C-hordein in whole barley hordein, in order to explain the hordein overestimation with a gliadin reference material in R5 antibody-based ELISA. An additional aim was to determine whether a reference material using C-hordein could be used to quantify hordein, for example, to determine the barley contamination in gluten-free ingredients and products.

Materials and methods

Twenty-nine barley cultivars from Finland for feed and malt purposes were selected for this study (Boreal Plant Breeding Ltd.). The total hordein of these cultivars were extracted by 40% (v/v) aqueous 1-propanol with 5% (v/v) 2-mercaptoethanol, and the hordein composition was determined by reversed-phase-HPLC by the peak area on a C8 column. C-hordein, B-hordein and D-hordein were collected from the C8 column and their protein content was determined with a bovine serum albumin standard. Hordein fractions were analysed in a sandwich gliadin kit (R7006, R-Biopharm, Darmstadt, Germany) to evaluate their immunoreactivities against the R5 antibody.

Barley flour cultivar Elmeri, Einar and Marthe with different C-hordein proportions (33.1%, 25.6% and 17.4%) were selected for spiking in gluten-free oat flour (Provena, Raisio Nutrition Ltd. Finland) to mimic the barley contamination in oat products. The hordein concentration was determined by HPLC, R5 sandwich ELISA with gliadin standard calibration, and with 40% C-hordein standard. The C-hordein was isolated and purified in a preparative ion-exchange column and lyophilised, and 40% C-

hordein standard was prepared by mixing the protein solution of same concentration 4 : 6 (C-hordein : bovine serum albumin, which does not react with R5 antibody).

Results and discussion

The C-hordein content of whole hordein of the 29 cultivars ranged 2-fold, from 16.5% to 33.1%. There was slight variation in C-hordein content of the same cultivar Elmeri from 2010, 2014 and 2015 (33.1%, 29.2% and 28.1%). Taken the popularity of the barley cultivars into account, the average C-hordein content of whole hordein in Finland 2012-2015 was 25-26%. The corresponding protein group to the C-hordeins in wheat are the ω 1,2-gliadins, which shows about 70% sequence homology, with a similar repetitive sequence in the central domain of PFPQQPQQ. The ω -gliadin content of total gliadin has been reported to range from 6% to 20% [5], and from 10% to 19% [6], which is in general lower than the content of C-hordein. The gliadin standard contains 11.3% ω -gliadin of total gliadin by HPLC analysis [7].

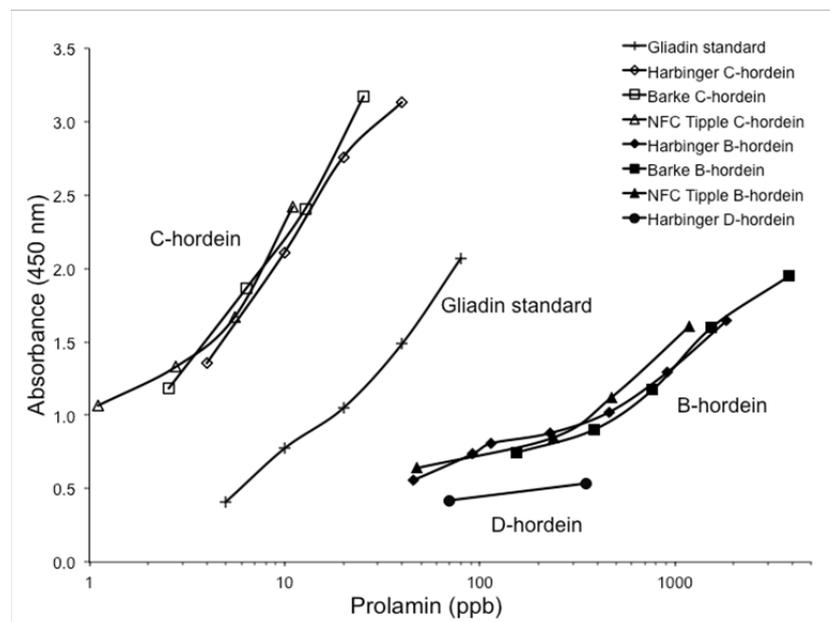


Figure 1. Reaction of isolated hordeins against R5 antibody in sandwich ELISA. Three types of C-hordein and B-hordein were from cultivars Harbinger, Barke and NFC Tipple. D-hordein was from cv. Harbinger

The reactivity of D-, C- and B-hordeins against the R5 antibody varied widely in sandwich ELISA (Fig. 1). C-hordein was 10-20 times more reactive than the gliadin standard, which in turn was 8-25 times more reactive than B-hordein. The slope of the curve indicated that C-hordein and gliadin standard had similar affinity with the R5 antibody, while B-hordein had less, and D-hordein had almost none. The three types of C-hordein reacted similarly with R5 antibody, although their HPLC patterns were different, as well as three types of B-hordein. The varying reactivity of hordein subunits against the R5 antibody is attributable to the number of epitopes. The main

R5 epitope, QQPFP, appeared 13 times in C-hordein (Uniprot Q40055), and minor epitopes QQPYP, QQTFP, PQPFP and QLFPF appeared once each. One main QQPFP epitope and 7 minor epitopes were found in B3 hordein (Uniprot I6TEV5), and 5 QQPFP epitopes in B1 hordein (Uniprot P06470). Only one QQPFP epitope was found in γ 3-hordein (Uniprot P80198) and no R5 epitope was found in D-hordein (Uniprot Q84LE9) [8].

In sandwich ELISA, the affinity (the slope) of C-hordein with R5 antibody behaved similarly to the gliadin standard, and at a ratio of 3 C-hordein : 7 bovine serum albumin (30% C-hordein), the reaction almost matched that of the gliadin standard (Fig. 2). The curves of purified whole hordein of common cultivars, such as cv. Barke and NFC tippel (C-hordein proportions 24.5% and 28.1% respectively), were above that of the gliadin standard and between that of the 30% and 50% C-hordein standard. The curve of cv. KWS Asta, with its low C-hordein proportion (16.5%), was close to the gliadin standard curve and that of 30% C-hordein. The whole hordein of a barley cultivar with low C-hordein content acted like wheat gliadin against R5 antibody, however, the barley cultivars usually have higher C-hordein content than that. C-hordein mixed with inert protein in the right ratio presented the whole hordein in R5 analysis.

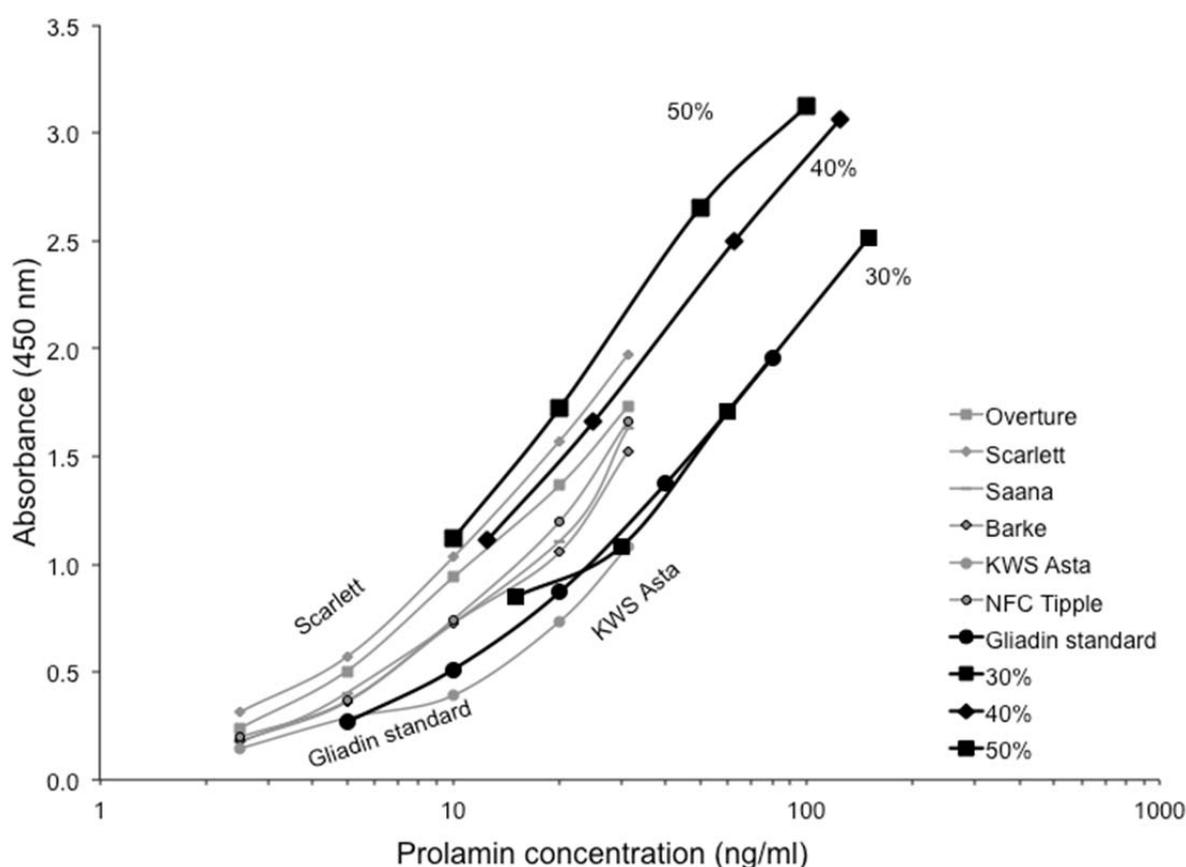


Figure 2. Reaction of purified whole hordein of 6 cultivars in R5 sandwich ELISA compared with 30%, 40% and 50% C-hordein standards and gliadin standard

When measuring the prolamin concentration of prolamin in barley-contaminated oats, with the 40% C-hordein standard, the estimated prolamin concentration was 1.2 times (cv. Elmeri), 0.85 times (cv. Einar) and 0.63 times (cv. Marthe) the HPLC results, however, the concentration calibrated by gliadin standard was 2.5 times (cv. Elmeri), 1.8 times (cv. Einar), and 1.2 times (cv. Marthe) the HPLC results (Fig. 3). For cv. Elmeri and Einar, the estimated value by the 40% C-hordein standard were not significantly different from those determined by HPLC, but for cv. Marthe the estimate was significantly lower, until the standard was changed to 30% C-hordein.

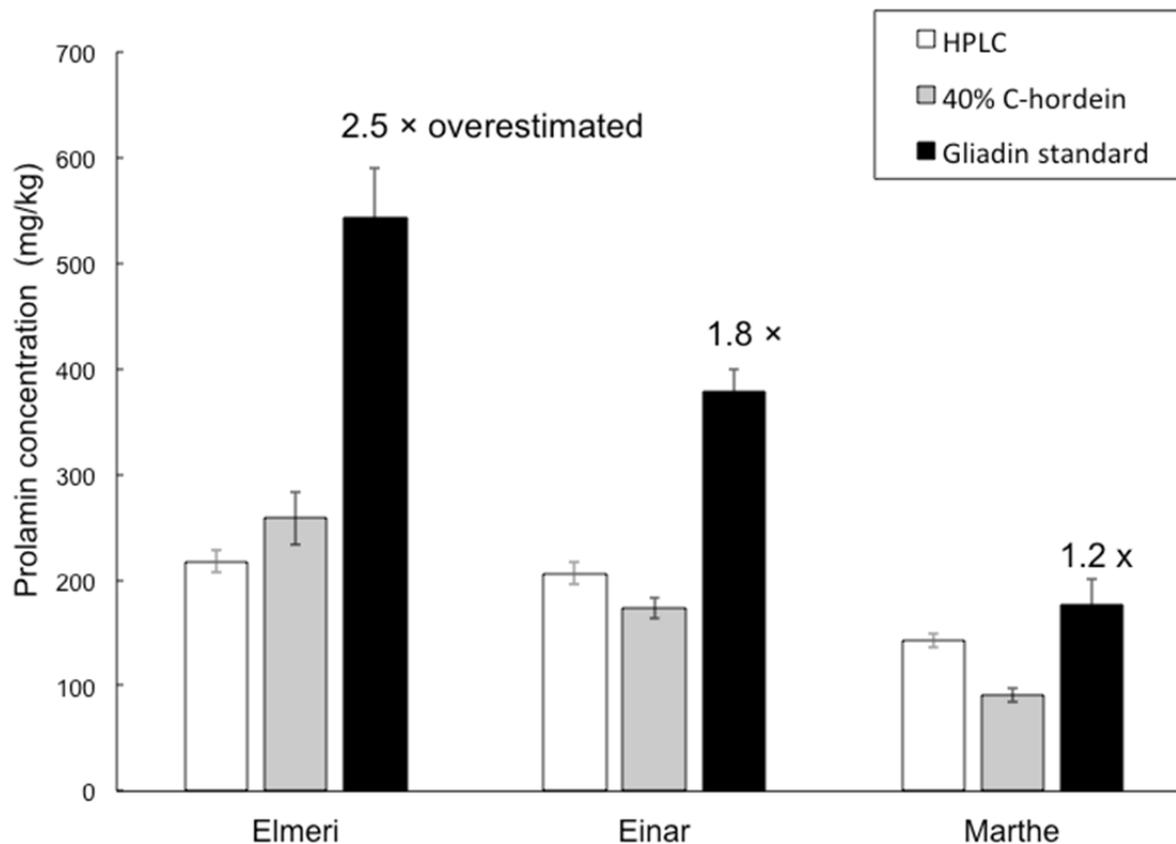


Figure 3. Prolamin concentration of gluten-free oat flour spiked with three barley flours, determined by HPLC, R5 sandwich ELISA with 40% C-hordein standard, and R5 sandwich ELISA with gliadin standard. Error bars show standard error

Conclusion

This study determined that the high proportion of C-hordein in total hordein is the reason for the consistent overestimation of hordein by the R5 ELISA assay which uses gliadin as reference material in gluten-free analysis. We isolated C-hordein and propose it as the reference material for quantifying hordein concentration in gluten-free food originated from barley, including those that may have been contaminated with barley.

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4.3 Quantitation of the 33-mer peptide from α -gliadins in wheat flours by LC-MS/MS

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Introduction

The dietary intake of storage proteins (gluten) from wheat (gliadins, glutenins), rye (secalins), and barley (hordeins) is known to cause coeliac disease (CD) in genetically predisposed individuals. A strict lifelong gluten-free diet is currently the only available therapy. All gluten proteins contain CD-active epitopes [1], which are resistant to cleavage by human gastric, pancreatic, and brushborder enzymes. A 33-mer peptide from α 2-gliadin (LQLQPFQPQLPYPQPQLPYPQPQLPYPQPQPF) was shown to survive gastrointestinal digestion and has frequently been described as most immunodominant gluten peptide [2,3], because it comprises three overlapping DQ2.5/T-cell epitopes, PFPQPQLPY (DQ2.5-glia- α 1a, one copy), PYPQPQLPY (DQ2.5-glia- α 1b, two copies), and PQPQLPYPQ (DQ2.5-glia- α 2, three copies) [1]. Due to its unique structure, the 33-mer peptide plays an important role in the scientific literature with 570 results for a search in the database ScienceDirect with “33 mer” and “coeliac disease” as keywords (as of October 29, 2016). The 33-mer was also used as an antigen to produce two monoclonal antibodies (A1 and G12), which are now used in enzyme-linked immunosorbent assays to determine gluten contents in foods labelled as gluten-free [4].

DNA-sequencing of eleven α -gliadins (α 1 - α 11) from the Norwegian common (bread) wheat (*Triticum aestivum*) cultivar (cv.) Mjølner (MJO) revealed that only α 2-gliadin contained the 33-mer amino acid sequence at positions 56 - 88 [5]. According to a BLAST search in the UniProtKB database within 587 entries for α -gliadins from *Triticum* sp., the 33-mer sequence (100% identity) was found in only 17 protein sequences from *T. aestivum* and in three from *T. spelta* (as of September 13, 2016). Of these 20 sequences, only three have evidence at transcript level (Q9M4L6, Q1WA39 and A5JSA6) inferred from three Chinese wheat cultivars, but only one (P18573) has evidence at protein level based on data of the Norwegian wheat cv. MJO. Despite the high number of papers featuring the 33-mer, there is no information on the presence and quantities of the 33-mer peptide in different wheat species and cultivars.

Therefore, the aim of the present study was to develop a stable isotope dilution assay (SIDA) combined with liquid chromatography tandem mass spectrometry (LC-MS/MS) for the determination of the presence and quantity of the 33-mer. Fifty-seven flours of different wheat species from around the world were investigated, including hexaploid common wheat (*T. aestivum*) and spelt (*T. aestivum* ssp. *spelta*), tetraploid

durum wheat (*T. turgidum durum*) and emmer (*T. turgidum dicoccum*), and diploid einkorn (*T. monococcum*) to assess the importance of this CD-active peptide.

Materials and methods

Preparation and characterization of flour samples

Twenty-three modern and 15 old (year of first registration before 1950) common wheat cultivars from different harvest years grown worldwide, and one rye cultivar (cv. Visello, harvested in 2013) were either obtained as flours or milled on a Quadrumat Junior mill (Brabender, Duisburg, Germany) and sieved (mesh size 0.2 mm). Two spelt, durum wheat, emmer, and einkorn cultivars each were milled on a Laboratory 3100 cross beater mill (Perten Instruments, Hamburg, Germany) to wholemeal flours.

The crude protein content (nitrogen content x 5.7) of the flours was determined by the Dumas combustion method. The contents of albumins/globulins, α -gliadins, gliadins, glutenins, and gluten (sum of gliadins and glutenins) were determined by modified Osborne fractionation of the flours followed by RP-HPLC-UV (210 nm) analysis [6].

Sample preparation

The flours (150 - 200 mg) were defatted with pentane/ethanol (95/5, v/v; 2 x 2.0 mL). After removal of the albumins/globulins, the gliadins were extracted with 60% (v/v) ethanol, dried, and re-suspended in a TRIS-HCl-buffer (pH 7.8). The stable isotope labelled standard (*33-mer, LQLQP*FPQPQLPYPQPQLPYPQPQLPYPQ*PQ*P*F, with *F: L-[¹³C₉][¹⁵N]-phenylalanine and *P: L-[¹³C₅][¹⁵N]-proline) was added (3 μ g) and the gliadin-peptide mixture hydrolysed with α -chymotrypsin (enzyme-to-protein ratio of 1:200) for 24 h at 37 °C. Trifluoroacetic acid (5 μ L) was added to stop the digestion. The peptide mixture was dried, re-dissolved in formic acid (FA) (0.1%, v/v, 500 μ L), filtered (0.45 μ m) and analysed by LC-MS/MS.

LC-MS/MS

A triple-stage quadrupole mass spectrometer (TSQ Vantage, Thermo Fisher Scientific, Dreieich, Germany) was used in the ESI positive mode. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode using the most abundant MRM transition as quantifier and the three MRM transitions following in abundance as qualifiers (Tab. 1). A declustering voltage of -10 V was set for all transitions. The 33-mer and the labelled *33-mer peptides were dissolved in FA (0.1%, v/v, 10 μ g/mL). These two stock solutions were mixed in molar ratios n (*33-mer)/n (33-mer) between 9.2 and 0.02 (1+9, 1+4, 1+3, 1+1, 3+1, 4+1, 9+1, 14+1, 19+1, 29+1, and 39+1) for calibration. An UltiMate 3000 HPLC system (Dionex, Idstein, Germany) was coupled to the mass spectrometer equipped with an XBridge Peptide 3.5 μ m BEH-C₁₈ column (1.0 x 150 mm, 13 nm; Waters, Eschborn, Germany). The LC conditions were set as follows: solvent A, FA (0.1%, v/v) in water, solvent B, FA (0.1%, v/v) in

acetonitrile; gradient 0 - 5 min 5% B, 5 - 22 min 5 - 55% B, 25 - 30 min 90% B; 30 - 35 min 90 - 5% B, 35 - 45 min 5% B, flow rate, 0.1 mL/min; injection volume, 10 μ L, column temperature, 22 $^{\circ}$ C.

Table 1. Multiple reaction monitoring (MRM) parameters of the 33-mer peptide and the stable isotope labelled *33-mer peptide.

Peptide	Precursor ions <i>m/z</i> (charge state)	Product ions ¹ <i>m/z</i>	Collision energy (V)	Retention time (min)
33-mer	979.0 (4+) ²	263.3 (y2) ²	14	19.0
	1305.2 (3+) ³	488.9 (y4) ³	26	
		713.5 (y6) ³	14	
		973.5 (y8) ³	12	
*33-mer	987.0 (4+) ²	279.0 (y2) ²	14	19.0
	1316.0 (3+) ³	510.3 (y4) ³	26	
		735.2 (y6) ³	14	
		996.0 (y8) ³	12	

¹ Charge state: 1+, ² Precursor to product ion transitions were used as quantifier, ³ Precursor to product ion transitions were used as qualifiers

The limits of detection (LOD) and quantitation (LOQ) of the LC-MS/MS method for the 33-mer peptide were determined. Rye flour was used as blank, because of the absence of α -gliadins. The rye prolamin extract was spiked at 7 different concentrations (0.1 - 200 mg/kg) of 33-mer peptide and the samples were prepared and analysed as described above. The LOD was calculated based on a signal-to-noise ratio (S/N) of 3, and the LOQ on an S/N ratio of 10.

Statistics

Linear Pearson's product moment correlations were calculated between contents of 33-mer and α -gliadins, gliadins, gluten or crude protein for all analysed wheat and spelt cultivars. Principal component analysis (PCA) was carried out with XLStat 2016 (Addinsoft, New York, NY, USA) to determine if the contents of 33-mer, α -gliadins, gliadins, gluten, and crude protein could be used to differentiate between spelt, modern and old common wheat cultivars.

Results and discussion

A [¹³C₂₈]- and [¹⁵N₄]-labelled *33-mer peptide (LQLQP*FPQPQLPYPQPQLPYPQPQLPYPQ*PQ*P*F, with *F: L-[¹³C₉][¹⁵N]-phenylalanine and *P: L-[¹³C₅][¹⁵N]-proline, monoisotopic mass 3943.0) was used as stable isotope labelled internal standard. It differed in 32 mass units compared to the unlabelled analyte (33-mer, monoisotopic mass 3911.0). Based on the fragmentation pattern of the 33-mer, the [¹³C]/[¹⁵N]-labelled amino acids were positioned in such a way that the label remained in the product ions (Tab. 1). The response factor was determined using the peak area ratio A (*33-mer)/A (33-mer) at different values of n (*33-mer)/n (33-mer) between

0.02 and 9.2 within the linear range based on the MRM transitions m/z 987.0 \rightarrow 279.2 (*33-mer) and m/z 979.0 \rightarrow 263.3 (33-mer). As expected from SIDA, the response factor was 0.999. The LOD of the LC-MS/MS method to detect the 33-mer peptide was 13.1 $\mu\text{g/g}$ rye flour and the LOQ was 47.0 $\mu\text{g/g}$ rye flour.

The 33-mer was determined in flours of 23 modern and 15 old common wheats from different harvest years and two spelt cultivars. In this context, old common wheat is defined as a cultivar from *T. aestivum* with its year of first registration prior to 1950. All flours were characterised including determination of crude protein contents and quantitation of α -gliadins, gliadins, glutenins, and gluten after modified Osborne fractionation combined with RP-HPLC [6,7].

The 33-mer was present in all common wheat and spelt flours in a range from 90.9 to 602.6 $\mu\text{g/g}$ of flour (Fig. 1A). Overall, the modern wheat cv. Yumai-34 (harvested in 2014, Y14) had the highest (602.6 $\mu\text{g/g}$ flour) and the old wheat cv. Ackermanns Brauner Dickkopf (ABD) the lowest (90.9 $\mu\text{g/g}$ flour) amount of 33-mer. Most of the modern and old wheat flours contained the 33-mer in a range of 200 - 400 $\mu\text{g/g}$ flour with an overall average of 368 ± 109 $\mu\text{g/g}$ flour. Special attention was directed to cv. MJO, because the 33-mer was first identified in this cultivar [5], which had a 33-mer content of 515.0 $\mu\text{g/g}$ flour. A certain trend, e.g., that modern wheat cultivars generally contain higher amounts of 33-mer than old wheat or spelt cultivars could not be derived from the data.

The 33-mer contents of all analysed flours were also calculated based on the amount of α -gliadins (Fig. 1B). MJO had the highest content of 33-mer in α -gliadins (23.2 mg/g α -gliadins) caused by the high 33-mer content and the low amount of α -gliadins (2.2%) in flour. ABD had the lowest amount of 33-mer in α -gliadins (4.1 mg/g α -gliadins). The overall average content was 11.7 ± 3.1 mg/g α -gliadins. Because there is virtually no data in the literature, it was difficult to compare these values with earlier studies, but one paper by van den Broeck *et al.* on the quantitation of the 33-mer using LC-MS with external calibration found comparable values for two wheat cultivars [8].

Correlations and PCA

The 33-mer contents of the 51 modern and old common wheat and spelt cultivars (based on flour) were correlated to the contents of α -gliadins, gliadins, gluten, and crude protein. A weak correlation ($r = 0.568$) was observed between 33-mer and α -gliadin contents, but there was no correlation to gliadin contents ($r = 0.469$), gluten contents ($r = 0.526$) or crude protein contents ($r = 0.466$).

PCA with 33-mer, α -gliadins, gliadins, gluten, and crude protein contents of the 51 flours was performed to assess whether these variables could be used to differentiate between spelt, modern common wheat, and old common wheat cultivars (Fig. 2). However, PCA revealed that these five variables were unsuitable to differentiate between spelt, modern common wheat, and old common wheat cultivars. Five old common wheat cultivars were placed on the far left, but the other ten old cultivars

were located right in the middle at similar coordinates as the modern common wheat cultivars. The two spelt cultivars were also situated in between the common wheat cultivars.

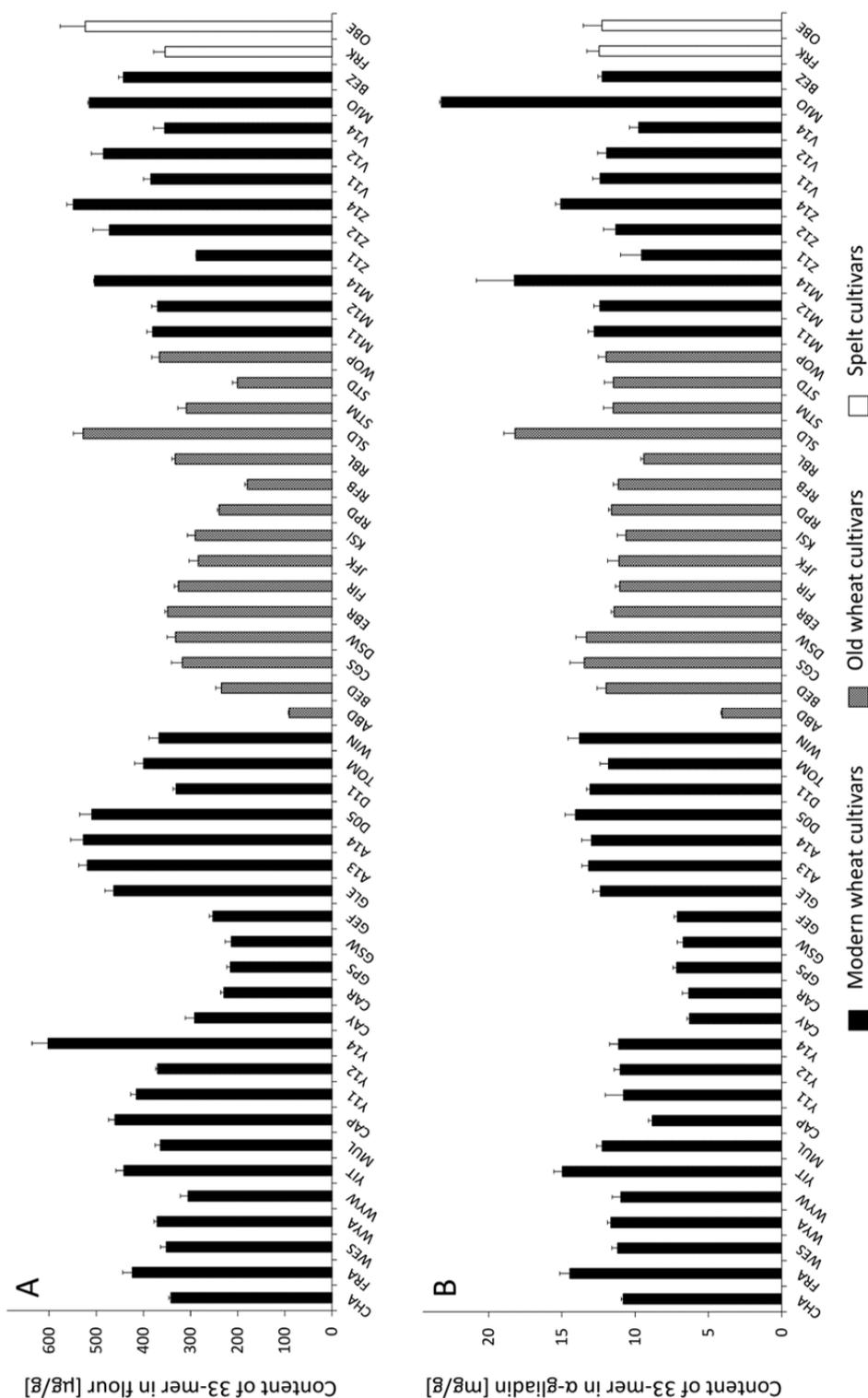


Figure 1. Contents of 33-mer based on flour [$\mu\text{g/g}$] (A) and based on α -gliadins [mg/g] (B). 23 modern and 15 old common wheat cultivars (49 samples in total due to multiple harvest years) and two spelt cultivars were analysed. Wheat cultivars registered prior to 1950 were designated as old

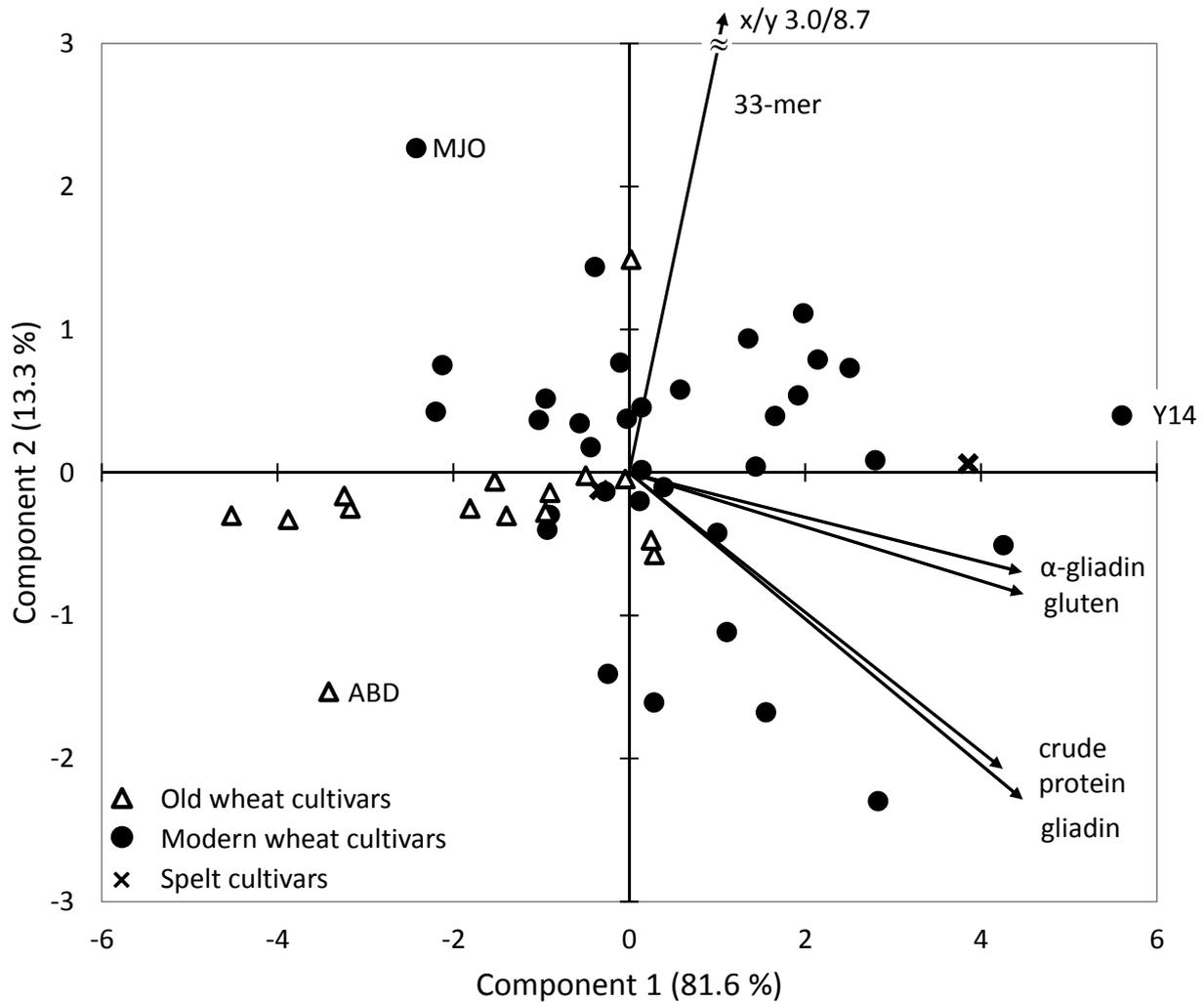


Figure 2. Principal component analysis biplot of data for 33-mer, α -gliadin, gliadin, gluten, and crude protein contents. 23 modern and 15 old common wheat (49 samples in total due to multiple harvest years) and two spelt cultivars were analysed. Wheat cultivars registered prior to 1950 were designated as old. ABD, wheat cv. Ackermanns Brauner Dickkopf, MJO, wheat cv. Mjølner, Y14, wheat cv. Yumai-34, harvest year 2014)

The 33-mer peptide was also analysed in two durum wheat and two emmer cultivars (genome AABB) as well as in two diploid einkorn cultivars (genome AA), but it was not detected in each of these wheat species ($< \text{LOD}$). In comparison to hexaploid common wheat, durum wheat, emmer, and einkorn do not contain the D-genome, which originated from hybridisation of *T. turgidum dicoccum* (genome AABB) with *Aegilops tauschii* (genome DD). The absence of the 33-mer peptide can be explained by the fact that this peptide is encoded by genes located in the Gli-2 locus on chromosome 6D, which is missing in durum wheat, emmer, and einkorn [5].

Conclusion

This is the first study to establish a SIDA combined with LC-MS/MS to quantitate the immunodominant 33-mer peptide from α 2-gliadin in wheat flours. All 40 modern and old common wheat and spelt cultivars analysed contained the 33-mer peptide (51 flour samples in total, because several flours were available from different harvest years). The special attention paid to this peptide in the scientific literature seems to be legitimated not only because of its unique structure containing six copies of three overlapping coeliac-active epitopes, but also because of its presence in all hexaploid wheat cultivars analysed in this study. Further work will focus on correlating the 33-mer content analysed by LC-MS/MS with the gluten content determined by ELISA using the G12 antibody, which was raised against the 33-mer.

Acknowledgement

The authors would like to thank Andreas Börner (Leibniz Institute of Plant Genetics and Crop Plant Research, Resources Genetics and Reproduction, Gatersleben, Germany), Friedrich Longin (University of Hohenheim, LSA - Research Group Wheat, Stuttgart, Germany), Anette Moldestad (Nofima, Ås, Norway), Roland Poms (Imprint Analytics, Neutal, Austria), Sándor Tömösközi (Budapest University of Technology and Economics, Department of Applied Biotechnology and Food Science, Budapest, Hungary), and Bin Xiao Fu (Canadian Grain Commission, Grain Research Laboratory, Winnipeg, Canada) for providing wheat grains and flours.

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4.4 The gluten content of wheat starches

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Introduction

The only generally accepted treatment for coeliac disease (CD) is a lifelong strict gluten-free diet that involves avoidance of wheat, rye and barley. Wheat gluten contains gliadin, low (LMWG) and high (HMWG) molecular weight glutenin proteins, all three of which have been shown to be CD-toxic [1-3]. Many gluten-free foods are available. This includes those that are commercially marketed, 80% of which in the UK are based on purified wheat starch. Foods that are supplied as gluten-free are required to contain <20 mg/kg gluten and those that are labelled "very low gluten" 21-100 mg/kg gluten. The only FAO certified assay to quantify the gluten content of foods for individuals with CD is based on the R5 monoclonal antibody (mAb) that recognises gliadin but not glutenin [4]. The value for the gluten content of a given food for this assay is determined by quantifying the gliadin content and multiplying the value by two to yield the gluten content of a given food. This extrapolation, based on the gliadin content may be invalid due to the differing solubility of gluten proteins, that is gliadin and glutenins, when food is processed.

Aims

We wished to improve the extent and accuracy of quantification of CD-triggering peptides in purified wheat starch that is a common ingredient of many commercially available processed gluten-free foods for individuals with CD.

Materials and methods

We have generated three mAbs to wheat gluten proteins. This includes PN3 to wheat gliadin that was raised against and detects coeliac-toxic A-gliadin AA31-49 [5,6], CDC5 to the CD-toxic immunodominant epitope in wheat gliadin that was raised against and detects α 2-gliadin AA57-75 [7] and CDC7 to wheat glutenin generated to the protein 1Dy10 HMWG glutenin subunits (HMWG) [8]. We developed three separate competitive ELISAs employing the three separate mAbs, PN3, CDC5 and CDC7. We assessed the gluten content of three wheat starches termed A, B & C that are supplied as standards for the Transia kit that is marketed to quantify the gluten content of foods based on the use of a mAb raised against ω -gliadin [9].

Results and discussion

Gliadin contents of wheat starches A, B and C were 34.2, 52.9 and 234.6 mg/kg as determined by PN3 mAb. Gliadin contents of the wheat starches measured using CDC5 mAbs followed the same trend of increasing gliadin content from starch A to C, but the values were higher. Gliadin contents were 106.9, 370.8 and 1033.1 mg/kg. Starches A, B and C contained 114.1, 431.1 and 1481.4 mg/kg glutenin as assessed with CDC7 mAbs (Tab. 1).

Table 1. Gliadin and glutenin content (in mg/kg) of wheat starches A, B and C as determined with PN3, CDC5 and CDC7 mAb.

Antigen measurement	Starch A	Starch B	Starch C
Gliadin (mg/kg) with PN3	34.2	52.9	234.6
Gliadin (mg/kg) with CDC5	106.9	370.8	1033.1
Glutenin (mg/kg) with CDC7	114.1	431.1	1481.4

Gluten contents were then calculated based on measurements of the three mAbs. Two approaches were undertaken: a) by following the standard method of extrapolating gliadin content to total gluten by multiplying the gliadin content by factor 2 ; b) by summing up gliadin and glutenin content to obtain the gluten content. Two different results were obtained, depending on whether gliadin content was measured with PN3 or CDC5 mAb (Tab. 2).

When PN3 mAb measurement was used to extrapolate the gliadin content of starch A to total gluten, the obtained value was 68.4 mg/kg gluten which is within the limit for “very low gluten” labelling of foodstuffs. When another anti-gliadin mAb (CDC5) was used for the same starch, the gluten content was more than 3 times higher (213.8 mg/kg), exceeding the 100 mg/kg cut-off value for “very low gluten”.

Summing up the values of gliadin and glutenin measurements to obtain total gluten led to two different results: 148.3 and 221 mg/kg depending on whether values of PN3 or CDC5 measurements were taken to be summed up with CDC7 measurements (Tab. 2). Interestingly, the calculation for total gluten based on the approach $\text{gluten} = 2 \times \text{gliadin}$ (PN3) was more than 2-fold lower than when gluten was calculated by summing up gliadin (PN3) plus glutenin (CDC7) which equalled 148.3 mg/kg for wheat starch A. On the contrary, for CDC5 mAb these two approaches resulted in very similar final gluten contents (213.8 and 221 mg/kg respectively) (Tab. 2).

Similarly, when the gliadin content of wheat starch B was extrapolated to total gluten (by multiplying the gliadin content by 2), the obtained value was 105.8 mg/kg for PN3 measurement and 7-fold higher gluten content (741.6 mg/kg) was seen for CDC5 measurement. When total gluten of starch B was obtained by the other approach, i.e. summing up the values of gliadin and glutenin (CDC7) measurements, they resulted in 484 and 801.9 mg/kg gluten for PN3 and CDC5 measurements, respectively. Gluten content calculated by $\text{gluten} = 2 \times \text{gliadin}$ as opposed to $\text{gluten} = \text{gliadin} + \text{glutenin}$

(CDC7) differed 4.6-fold for PN3 mAb measurements and 1.1-fold for CDC5, the higher values obtained by the gluten = gliadin + glutenin approach (Tab. 2).

The results for wheat starch C had a similar trend. Gluten content obtained by multiplying gliadin measurements by factor 2 resulted in 4.4-fold higher total gluten content for CDC5 measurement (2066.2 mg/kg) than PN3 measurement (469.2 mg/kg). The other approach whereby glutenin content (obtained with CDC7 measurement) was summed up with gliadin content resulted in 3.6-fold increase of gluten content for PN3 mAb measurements (from 469.2 to 1716 mg/kg) and 1.2-fold increase of gluten content for CDC5 mAb measurements (from 2066.2 to 2514.5 mg/kg) (Tab. 2).

Table 2. *Gluten content (in mg/kg) of wheat starches A, B and C as determined by multiplying gliadin content by 2 versus summing up the measurements of gliadin and glutenin content.*

Gluten content (in mg/kg)	Starch A	Starch B	Starch C
a) gluten = 2 x gliadin (PN3)	68.4	105.8	469.2
a) gluten = 2 x gliadin (CDC5)	213.8	741.6	2066.2
b) gluten = gliadin (PN3) + glutenin (CDC7)	148.3	484.0	1716.0
b) gluten = gliadin (CDC5) + glutenin (CDC7)	221.0	801.9	2514.5

Further, ratios of glutenin to gliadin content of the wheat starches were determined by dividing the glutenin values obtained with CDC7 mAb by gliadin values assessed either by PN3 or CDC5 mAb (Tab. 3).

Table 3. *Ratios of glutenin to gliadin contents in wheat starches A, B and C depending on which gliadin monoclonal antibody (PN3 or CDC5) is used for comparison with CDC7 mAb measurements.*

Glutenin : gliadin ratio	Starch A	Starch B	Starch C
CDC7 : PN3	3.3	8.1	6.3
CDC7 : CDC5	1.1	1.2	1.4

Gliadin content of starches A, B and C depended on whether PN3 or CDC5 was chosen for the measurement (Tab. 1). Ratios of gliadin contents in the three wheat starches were determined by dividing the gliadin content obtained with CDC5 by gliadin content obtained by PN3 mAb. The scale of difference in gliadin content varied amongst starches (3.1- to 7.0-fold) (Tab. 4).

Table 4. *Ratios of gliadin contents in wheat starches A, B and C as obtained with the two gliadin antibodies (CDC5 and PN3).*

Gliadin : gliadin ratio	Starch A	Starch B	Starch C
CDC5 : PN3	3.1	7.0	4.4

Discussion

We demonstrated that a broadened repertoire of mAbs specific for CD-triggering peptides enabled improved measurement of gluten in foods, by allowing a more realistic measurement of the CD-triggering epitopes within the glutenins. This applied particularly to the measurements where PN3 mAb was used for measuring gliadin content and then obtaining total gluten either by the “standard method“ or by summing up gliadin and glutenin measurements. This observation was less applicable to CDC5 measurements as multiplying gliadin content obtained by CDC5 mAb by a factor 2 differed very little to obtaining gluten content by summing up its gliadin content with glutenin. The total gluten based on CDC5 and CDC7 mAb measurements indicate that the epitopes that these two mAbs detect were more equally distributed as opposed to those detected by PN3 and CDC7 mAbs in the three starches.

None of the three wheat starches were “gluten-free”, as they all contained more than 20 mg/kg gluten. When anti-gliadin mAb PN3 was used for the measurements of gluten contamination of starch A it resulted in values that would classify it as “very low gluten” foodstuff as it contained less than 100 mg/kg gluten. This was applicable for the “standard” method of determining the total gluten by multiplying the gliadin value by factor 2. However, when total gluten content was assessed by summing up the values obtained with PN3 mAb and anti-glutenin mAb CDC7, the gluten values were well above the cut-off value for “very low gluten”. This data clearly demonstrates the importance of measuring both groups of proteins in gluten responsible for CD toxicity (gliadins and glutenins). This is particularly important for processed foodstuffs like wheat starches where gliadin : glutenin ratios have been shown to vary greatly [10,11].

Glutenin to gliadin ratios of the wheat starches varied between 1.1-8.1. Our results demonstrate that multiplying gliadin content by factor 2 to estimate for the glutenin may be invalid for processed foodstuffs, which is in agreement of Wieser and Koehler’s [10] observations. Measurement of gliadin alone therefore cannot predict total gluten content in foods. The standard method of multiplying gliadin content by 2 would lead to gross underestimation of gluten content in our wheat starches if the antibody used for detection of gliadins was PN3 mAb.

The lower glutenin to gliadin ratios were obtained when comparing the glutenin contents with gliadin determined with CDC5 mAbs. The higher ratio of glutenin to gliadin obtained with PN3 as anti-gliadin antibody can be explained by the lower amounts of the gliadin peptide that PN3 detected in the wheat starches. This was further confirmed by calculating the gliadin to gliadin ratios determined by PN3 and CDC5 antibodies which showed that the amount of detected gliadin in a foodstuff depends greatly on which antibody is used for quantification. This concept was demonstrated previously in a study of van Eckert et al. [12] who showed that two different anti-gliadin antibodies (PN3 and R5) reacted with different individual proteins in different protein sub-fractions of the reference gliadin separated by two dimensional electrophoresis.

Further, our results differed from the reference values for gluten contamination of the three wheat starches A, B and C. The reference values were provided in the manufacturer's information sheet and had been obtained using a monoclonal antibody which detects ω -gliadins and were as follows: <100 mg/kg gluten for starch A, 300 - 600 mg/kg gluten for starch B and 1000 - 2500 mg/kg for starch C [9]. The value of <100 mg/kg complied with the previous regulations for labelling foods to be gluten-free but not with the current regulations of <20 mg/kg. Our results of gluten contamination did follow the same trend of increasing gluten contamination from starch A to C, but the values were not the same. Gluten contamination assessment with antibodies of different specificity can therefore result in different gluten amounts, which is consistent with Allred and Ritter's observations [13]. Of note, the manufacturer of the three starches A, B and C did not provide information as to which wheat cultivars the starches were obtained from. In this respect we do not know whether the flour from the same cultivar was used and resultant starches subjected further to three different washing processes or whether three different cultivars were used.

There is a dilemma in the field of gluten measurement of what should be quantified in order to assess the overall toxicity of foods for CD sufferers [14]. There are several CD-triggering epitopes [15]. It is probably unrealistic to detect all of them. The gliadin fraction of wheat gluten has long been established as CD-triggering. However, the glutenins have only recently been shown to exacerbate the disease. Our monoclonal antibodies detect CD-triggering epitopes distributed amongst both groups of proteins, for which there is substantial clinical data confirming their role in CD pathogenesis. It is interesting, although not surprising, that contamination of wheat starches with glutenins was notably higher than with gliadins. This is likely due to different solubility characteristics of the gluten protein fractions as a result of food processing [13,16,17]. The glutenins are less water-soluble and therefore more likely to stay adsorbed to starch granules after washing [16]. It is therefore crucial to detect glutenin contamination of processed foodstuffs and thereby improve the extent of measured gluten components. Our findings are consistent with Allred [13] who demonstrated that all processed foodstuffs (n = 40) tested in their study contained 4- to 10-fold higher gluten values when assessed with the mAb that has high affinity to glutenins as opposed to R5 mAb with high affinity for gliadins.

Conclusion

We suggest that a broadened repertoire of mAbs specific for CD-triggering peptides enables improved measurement of gluten in foods for individuals with CD, by allowing a more realistic measurement of the CD-triggering epitopes within the glutenins. The total gluten content depended on the specificity of the mAb(s) used for quantitation. In addition, the glutenin to gliadin ratios varied greatly between wheat starches. We therefore suggest that multiplying the gliadin content by a factor of 2 to

estimate the total gluten content of a given nominally gluten-free food, particularly those that are based on purified wheat starch may be invalid for processed foodstuffs.

Acknowledgement

The authors wish to thank the Rosetrees Trust and Clinical Research Trust for support.

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4.5 Comparison of immunomethods for the characterisation of gluten immunogenic peptides in a commercial beer

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Introduction

Gluten is present in the most commonly consumed cereals (wheat, barley, rye and oats) and serves as ingredient in many processed foods. Manufacturing of processed foodstuffs digests gluten to different degrees, especially by hydrolysis and fermentation. This digestion of total gluten gives rise to peptides and, ultimately, to amino acids. In the small intestine, some of these peptides are resistant to gastrointestinal digestion and trigger the immune response that causes the symptoms of the disease. This pool of peptides is termed gluten immunogenic peptides (GIP). However, the characterization of these peptides is still incomplete. The great heterogeneity of gluten proteins makes this task complicated and tedious. In the last years, it has been shown that a few highly immunogenic peptides could account for more than 90% of the coeliac-specific response [1-3]. The dominant immunogenic peptide in wheat is the α -gliadin 33-mer [2].

Beer is the most widely consumed alcoholic beverage, both among coeliac and non-coeliac individuals. Its production involves the fermentation of starches, mostly from cereal grains (barley, wheat, maize, rice...). This fermentation hydrolyses gluten proteins contained in the cereal grains and produces GIP which remain in the final product. The differential hydrolysis of prolamins in brewing processes may generate peptide pools with uncertain immunogenicity. Current methods based on the R5 antibody to officially analyse gluten content in beer and grant the “gluten-free” label may overlook these immunogenic peptides. However, the new generation of monoclonal antibodies (mAbs) like A1 and G12 with a sensitivity and specificity for the 33-mer several orders of magnitude higher compared to R5 antibody may result in differences in immunogenicity estimation for hydrolytic prolamins [7]. Beers, due to their diversity, are some of the samples in which the immunomethods may show the highest differences in gluten content measurement. Here, we evaluated the reliability of the methods based on R5 and G12 to estimate the potential toxicity by GIP contained in a commercial beer, which was previously characterised by HPLC-MS and peripheral blood mononuclear cell (PBMC) reactivity from coeliac patients.

Materials and methods

Beer samples, negative control (rice prolamins), peptide synthesis, and synthetic peptides were used. Patients with active coeliac disease and healthy subjects were included in this study. T-cell isolation from coeliac patients, cell proliferation assays, interferon (IFN)- γ , lateral flow immunoassay (LFIA) A1/G12 (GlutenTox® Sticks, Biomedal), competitive ELISA G12 (GlutenTox® Competitive G12 Biomedal) and R5 (Ridascreen, R-Biopharm), and immunoprecipitation assays were made as described in [4,5] and are not reproduced here for space reasons.

Results and discussion

In a previous study, we characterised about 100 Belgian beers by LFIA and ELISA based on G12/A1 mAbs (some examples showed in Table 1 and [6]). Although the sandwich ELISA configuration may underestimate the presence of some gluten peptides with only one epitope, LFIA and G12 competitive ELISA provided a similar estimation of gluten content. However, the underestimation appeared to be higher in a R5 sandwich than in the A1/G12 LFIA (Table 1). This observation may indicate that the abundance of tandem epitopes for A1 and G12 is more frequent than that of R5 epitopes.

To analyse the differential epitope recognition present in beers, we selected a beer based on the difference in gluten estimation by R5 and G12 ELISA (Table 1, in bold). The Strong Ale 5 was fractionated and characterised with HPLC-MS and the gluten content of each fraction was further analysed using LFIA A1/G12. All immunoreactive fractions contained peptides recognised by A1, G12 and R5. Five peptides were selected according to the presence of epitopes with potential immunogenicity (i.e. reactive to R5, A1 and G12). These peptides were synthesised ([4] and Fig. 1). The R5 competitive ELISA showed 5- to 9-fold less reactivity for the barley beer epitopes compared to the G12 competitive ELISA. A1 competitive ELISA showed an intermediate affinity for the immunogenic peptides compared to R5. The biggest differences in reactivity were found in peptide QP 22.2, which contains two tandem epitopes for R5 and one for A1 (QP 22.2 in Fig.1). QP22.2 reactivity for R5 was six-fold larger than to A1 and a hundred-fold larger than for G12. Interestingly, despite its great reactivity to R5, this peptide induced a very weak reactivity to PBMCs from coeliac patients, slightly superior to the negative control (rice prolamins) (Fig. 2 and [4]). In contrast, the most reactive peptide for G12 (PP 24.1) also confirmed the highest immunogenicity by PBMC activation and IFN- γ production. These results were consistent with those obtained by G12/A1 competitive ELISA, but not according to R5. Therefore, there is no correlation between the reactivity for the R5 mAb and the immunogenicity of peptides. Moreover, the highest sensitivity of G12 for such GIP could be an indication of the presence of immunogenicity risks in many cases.

Table 1. *Gluten levels of the 30 Belgian beer samples analysed with mAbs G12/A1 and mAb R5 ELISA.*

Beer	Cereal	Gluten Content (mg/kg)		
		GlutenTox® sticks	GlutenTox® Competitive ELISA G12	ELISA Sandwich R5
Abbey tripel 1	Barley+wheat	>100	112.84	23.35
Abbey tripel 2	Barley	20-100	77.01	5.99
Abbey tripel 3	Barley	3-20	17.85	<3
Abbey dubbel 1	Barley	>100	113.15	11.48
Abbey dubbel 2	Barley	20-100	81.18	<3
Abbey dubbel 3	Barley	20-100	66.38	7.23
Abbey dubbel 4	Barley	<3	<3	<3
Abbey dubbel 5	Barley+wheat	20-100	72.11	24.24
Abbey dubbel 6	Barley+wheat+oat	20-100	99.86	6.59
Strong ale 1	Barley+wheat	20-100	9.75	31.57
Strong ale 2	Barley	>100	100.01	8.85
Strong ale 3	Barley	>100	137.74	24.40
Strong ale 4	Barley	<3	<3	<3
Strong ale 5	Barley	3-20	9.75	<3 *
Fruit lambic 1	Barley+wheat	>100	273.96	19.24
Fruit lambic 2	Barley+wheat	>100	1,660.31	98.61
Fruit lambic 3	Barley+wheat	20-100	57.88	4.59
Fruit lambic 4	Barley+wheat	>100	101.85	16.73
Fruit lambic 5	Barley	<3	<3	<3
Specialty 1	Millet	<3	<3	<3
Specialty 2	Barley+corn	<3	<3	<3
Specialty 3	Barley+corn	<3	<3	<3
Season 1	Barley+wheat	>100	194.57	23.67
Season 2	Barley	>100	148.35	18.55
White 1	Barley+wheat	>100	937.23	198.62
White 2	Barley+wheat	>100	608.07	300.58
Sour ale 1	Barley	>100	113.03	26.27
Sour ale 2	Barley	<3	<3	<3
Pale lager 2	Barley	>100	260.06	18.55
Pale lager 1	Barley	20-100	98.23	6.15

* The gluten content of Strong Ale 5 was also estimated by Competitive ELISA R5: 9.27 ppm

Peptide	Amino acid sequence	mAb G12	mAb A1	mAb R5
		IC50 (µg/ml)	IC50 (µg/ml)	IC50 (µg/ml)
PP 24.1	PQP Q QPQLPFP Q QP <u>Q</u> Q Q FP Q QP Q Q	0.35	1.45	1.50
PP 24.2	P Q Q Q LPFP Q QP <u>Q</u> Q Q FP Q QP Q Q	0.41	2.60	1.82
PP 24.3	PQP Q QPQLPFP <u>Q</u> Q Q FP Q QP Q Q	0.85	1.90	5.90
PP 22.1	P Q Q Q LPFP <u>Q</u> Q Q FP Q QP Q Q	0.48	2.66	4.16
QP 22.2	Q Q Q Q FP Q LP <u>Q</u> Q Q FP Q QP Q Q	450.01	25.01	4.23

R5 epitopes are in *italics*. A1 epitopes are underlined. G12 epitopes are in **bold**

Figure 1. Relative affinity of G12, A1 and R5 mAbs for different immunoreactive peptides from the barley beer previously characterised by HPLC-MS and PBMC activation.

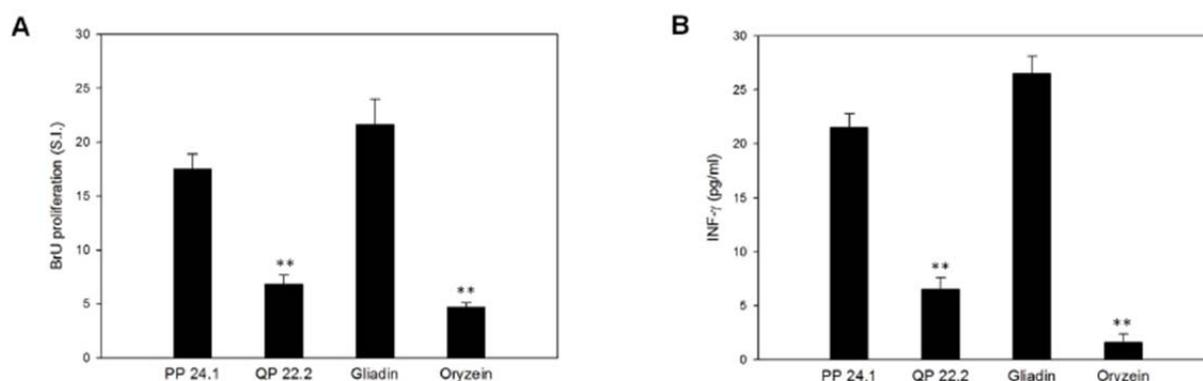


Figure 2. Potential immunogenicity of PP 24.1 and QP 22.2. (A). Proliferative responses of PBMCs to different peptides. (B). IFN-γ production by PBMCs with different peptides. Results are expressed as mean ± SD of duplicated cultures (n = 14). Gliadin and oryzein were used as the positive and negative control, respectively, and significant differences with respect to gliadin at **p < 0.005 are shown

Next, we wanted to assess the similarities between the pool of GIP detectable by G12 and those detectable by R5. To do so, we fractionated the Strong Ale 5 beer with immunochromatography using agarose beads conjugated with G12 mAb (Fig. 3). Three fractions were obtained: Input (barley beer), Flow Through (barley beer minus the G12 reactive peptides) and Output (peptides bound to the G12 columns that are released by heat denaturation). All three fractions were characterised and we

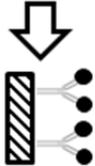
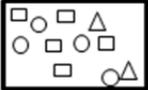
Experimental design	Beer fractions	Protein concentration determination (%)	Relative affinity of beer fractions for the moAbs (%)		Celiac immunogenic pattern (T-cell activation) (%)	
			Competitive ELISA G12	Competitive ELISA R5	Proliferative response of T-cells	IFN- γ release
	Input	100	100	100	100	100
	Output	5	100	22	91	90
	Flow Through	95	0	78	9	10

Figure 3. Relative content of proteins, G12 and R5 reactive peptides and coeliac immunogenic pattern of the different barley beer fractions. Fractions were obtained in the process of separation of peptides by G12 mAb immunodepletion

quantified the relative content of proteins, G12 and R5 reactive species, and T-cell activation.

As expected, material immunocaptured by G12 (peptides and proteins) comprised no more than 5% of the total protein content in the beer. However, these peptides were responsible for about 90% of the immunogenicity of the total beer. Strikingly, almost 80% of the reactivity of the R5 mAb is located in the pool of peptides and proteins with poor immunogenic activity, as corroborated by the proliferative responses of T-cells and IFN- γ production by PBMCs of coeliac patients.

Conclusion

We have shown that analysing a beer with standard methods like ELISA R5 to grant it the “gluten-free” label might not fully guarantee the absence of potential damage to coeliac patients. There is experimental evidence that a better indicator of the potential immunogenicity is the reactivity to the α -gliadin 33-mer A1 or G12, even if the 33-mer canonical sequence is not supposed to be present in barley. Other epitopes of the 33-mer are enough to sensitively detect GIP by G12/A1 in barley beers.

In contrast, the presence of the preferred R5 epitope QQPFPP in certain peptides of the Strong Ale 5, was not sufficient to detect those GIP that appeared detectable by HPLC-MS analysis. In general, the G12 immunomethods appear to be the most

specific practical techniques described so far to assess the potential immunogenicity of barley beers.

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4.6 Pathogenesis of coeliac disease: complexes between transglutaminase and gluten peptides

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Introduction

Coeliac disease can be characterised by three features: (A) triggered by the ingestion of gluten, (B) presence of the genetic factor (HLA-DQ2 or DQ8), and (C) the generation of autoantibodies against tissue transglutaminase (TG2) [1]. After the ingestion of gluten, these proteins (gliadins, glutenins, hordeins, and secalins) are not sufficiently digested by human gastrointestinal enzymes due to their high proline and glutamine contents. These long peptides pass through the epithelial layer and first trigger the innate immune response. Intraepithelial lymphocytes activate defence mechanisms, which initiate apoptosis and are increase of epithelial permeability. Secondly, the gluten peptides are modified by TG2 that catalyses deamidation and transamidation. The modified peptides stimulate gluten-specific T-lymphocytes, which finally lead to the damage of the villi of the small intestine. Furthermore, antibodies are formed against gluten peptides, TG2 and gluten peptide-TG2-complexes [1-3].

TG2 plays a key role in the pathogenesis of coeliac disease. Firstly, it causes deamidation of specific glutamine residues to glutamic acid, which increases the immune response. It also initiates transamidation and formation of gluten peptide-TG2-complexes that lead to the formation of antibodies against them. TG2 is a Ca^{2+} -dependent protein-glutamine γ -glutamyltransferase (EC 2.3.2.13), which catalyses the formation of inter- and intramolecular $\text{N}^{\epsilon}(\gamma\text{-glutamyl})\text{lysine}$ bonds. The transfer of the acyl residue between the γ -carboxyamine group of glutamine as acyl donor and primary amines as acyl acceptors involves a two-step reaction mechanism. The three amino acids cysteine-277, histidine-335, and aspartic acid-358 of the active site of the enzyme are involved in this mechanism. According to the hypothetical model of “hapten-carrier-like complexes” these covalently bound gluten peptide-TG2-complexes should be responsible for the formation of anti-TG2 antibodies.

However, the investigation of the structures of these gluten peptide-TG2-complexes is still at the beginning. Therefore, the aim of this study was to identify the binding sites between TG2 and peptides derived from all CD-active gluten protein types of wheat, rye, and barley.

Materials and methods

Characterisation of microbial transglutaminase

The microbial transglutaminase from *Streptomyces mobaraensis* (ABEnzymes, Darmstadt, Germany) was dissolved in formic acid (0.1%), filtered (0.45 µm) and measured by liquid chromatography with mass spectrometric detection (LC-MS (QTOF)). Furthermore, the microbial transglutaminase was characterised by the analysis of the tryptic peptides by LC-MS/MS (iontrap). For this purpose, the enzyme was incubated with trypsin in TRIS-HCl-buffer (0.1 mol/l; pH 7.8) for 24 h at 37 °C. After purification with solid phase extraction, the hydrolysates were dried, dissolved again in formic acid (0.1%) and analysed by LC-MS/MS (iontrap).

Identification of isopeptides

For the reaction of microbial transglutaminase and a defined model peptide gli 56-75 (LQLQPFPPQPQ₆₅LPYPQPQLPY) to peptide-enzyme-complexes, both were dissolved in TRIS-HCl-buffer (0.1 mol/l; pH 7.8; 2 mmol/l CaCl₂) and incubated for 2 h at 37 °C. The peptide-enzyme-complexes were incubated with trypsin for 24 h at 37 °C. After purification with solid phase extraction the hydrolysates were analysed by LC-MS/MS (iontrap).

Results and discussion

Characterisation of microbial transglutaminase

Initially, the microbial transglutaminase (mTG) had to be characterised by molecular weight and by sequence analysis. The characterisation by molecular weight was carried out by LC-MS/MS (QTOF) with a high intensity. The identified molecular weight of the microbial transglutaminase was determined as $37,863.6 \pm 0.5$ (Fig. 1), which is comparable to the data (P81453) of the UniProt KB database. Also Kanaji *et al.* could identify the same molecular weight for microbial transglutaminase and showed the separation of signal- and propeptide during the MS measurement [4].

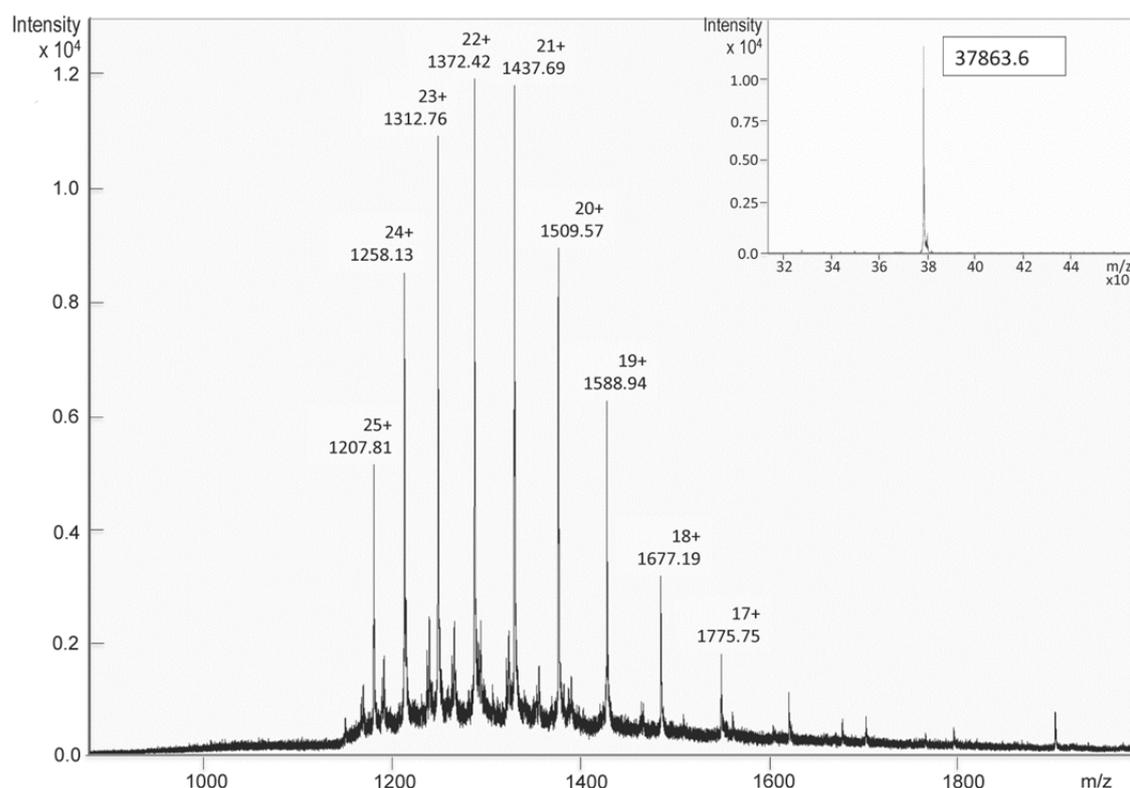


Figure 1. Mass spectrum of microbial transglutaminase. The spectrum corresponds to the average of scans of the base peak-chromatogram at 6.7-7.1 min. The simulated maximum entropy peak is shown in the upper right corner

Furthermore, the analysis of the tryptic peptides of mTG confirmed the characterisation and comparability with P81453. Thirteen tryptic peptides were evenly distributed over the whole sequence, without the signal- and propeptide.

Table 1. Tryptic peptides of microbial transglutaminase, their m/z ratio with charge state, the position in the UniProt database sequence and the score of the search with MASCOT-software (≥ 30).

Tryptic peptide	m/z (charge state)	Position	Score
DSDDRVTPPAEPLDR	841.75 (2 ⁺)	76-91	49
AETVVNNYIR	589.69 (2 ⁺)	103-112	49
LAFASFDEDRFKNELK	644.06 (3 ⁺)	156-171	41
ESFDEEKGFQR	686.20 (2 ⁺)	191-201	42
ALENAHDESAYLDNLKK	965.79 (2 ⁺)	212-227	48
ELANGNDALRNEDAR	553.49 (3 ⁺)	229-234	60
SPFYALR	470.71 (2 ⁺)	244-251	30
YGDPAFRPAPGTGLVDMSR	707.92 (3 ⁺)	293-312	80
NWSEGYSDFDR	688.23 (2 ⁺)	373-383	50
SWNTAPDKVK	573.25 (2 ⁺)	394-401	34

Identification of isopeptides

To identify the binding sites between mTG and the model peptide, the transglutaminase was first allowed to react with the peptide, the complexes were hydrolysed and these tryptic peptides were analysed with LC-MS/MS. In the second part, an analysis strategy for the identification of the isopeptides had to be developed. For this purpose, all theoretically possible combinations between a lysine residue (K) of the enzyme and a glutamine residue (Q) of the model peptide, their masses and their precursors (m/z) in the different charge states were calculated and theoretically fragmented. In the next step, the measured MS- and MS/MS-spectra were searched for the calculated masses of the precursors and fragments.

For the identification of the binding Q, a data analysis strategy had to be developed to detect fragments which confirm the binding site. Initially, the spectra were searched for isopeptide bonds at Q₆₅, because Fleckenstein *et al.* (2004) already showed that this position is a binding site for TG2 [5]. Fig. 2 presents the isopeptide of the model peptide with the possible binding site (Q₆₅) and the tryptic peptide KWQQVYSHR of the transglutaminase. To confirm the position of the isopeptide bond at Q₆₅ the specified fragments b_{10 α} or b_{11 α} as well as b_{9 α} , b_{8 α} , y_{11 α} or y_{12 α} and y_{10 α} had to be identified. At last, to confirm the identification of the whole isopeptide, 5 other fragments, e.g. b_{5 β} or y_{6 β} had to be identified.

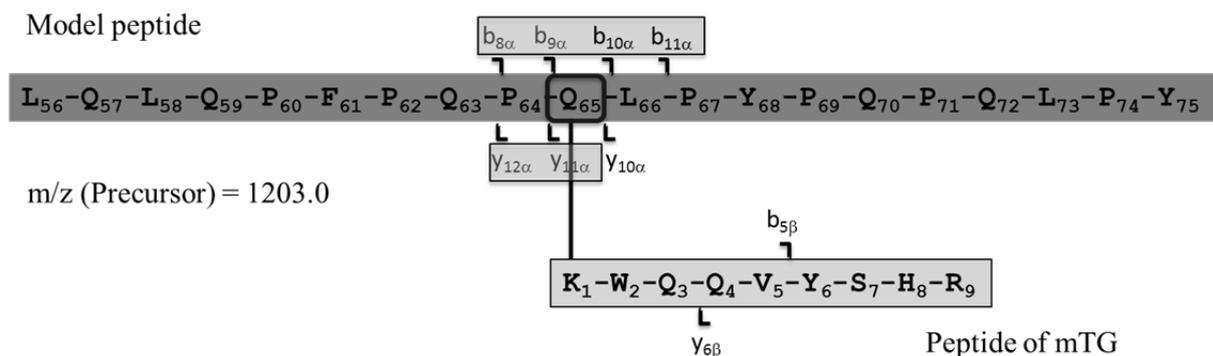


Figure 2. Strategy for identification of the binding Q in isopeptide bonds. Isopeptide (precursor $m/z = 1203.0$ (3^+)) of the model peptide and the tryptic peptide KWQQVYSHR of microbial transglutaminase (mTG) with the specified fragments for confirmation of the binding site

The mass spectrum of the isopeptide of the model peptide gli 56-75 and the tryptic peptide KWQQVYSHR of the microbial transglutaminase is presented in Fig. 3. Twenty-three fragments of the b- and y-series could be identified in the entire isopeptide and all signals had an adequate intensity. Another condition for the certain identification is to identify at least three consecutive fragments. With the detected fragments b_{10 α} to b_{14 α} and b_{5 β} to b_{8 β} as well as y_{8 α} to y_{10 α} and y_{12 α} to y_{14 α} this requirement was fulfilled. Also the conditions for the affirmation of the binding Q

were fulfilled by the identification of the specified fragments $b_{10\alpha}$ and $b_{11\alpha}$ as well as $y_{12\alpha}$ and $y_{10\alpha}$.

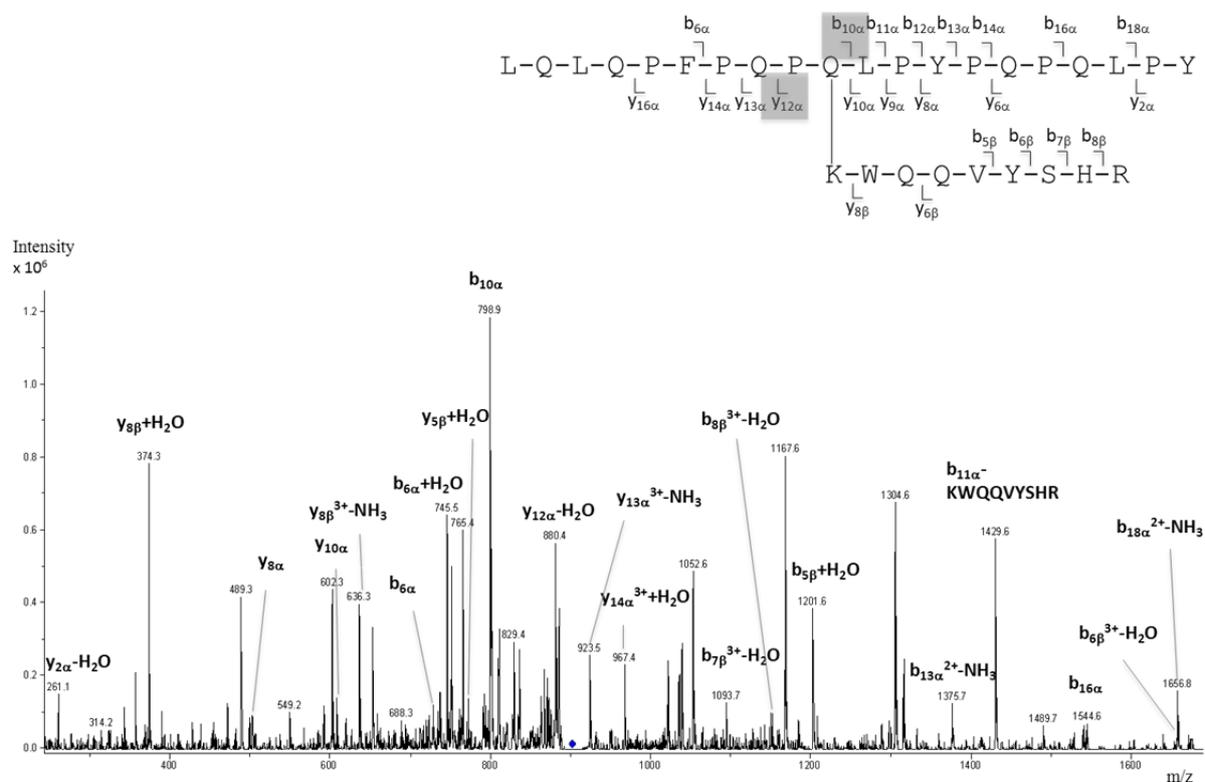


Figure 3. Mass spectrum of the isopeptide ($m/z = 1203.0$ (3^+)) of the model peptide gli 56-75 and a tryptic peptide of the transglutaminase

Until now, five tryptic peptides of mTG were identified, which form isopeptide bonds with the Q_{65} of the model peptide gli 56-75. Tab. 2 shows these tryptic peptides and their position in the transglutaminase sequence.

Table 2. Identified tryptic peptides of the microbial transglutaminase, which bind to the model peptide gli 56-75 at Q_{65} , their position in the sequence and m/z ratio and the charges state of the formed isopeptides.

Tryptic peptide	Position	m/z (charge state)
KWQQVYSHR	113-121	1203.0 (3^+)
SWNTAPDK	394-401	1098.9 (3^+)
VAKESFDEEKGFQR	188-201	1012.0 (4^+)
NTPSFK	252-257	1023.5 (3^+)
NWSEGYSDFDRGAYVITFIPK	373-393	1210.9 (4^+)

Conclusion

First, the characterisation of the microbial transglutaminase by molecular weight and sequence analysis was performed by two different types of LC-MS/MS. The results of the microbial transglutaminase used here were comparable with the data of the UniProt KB database (P81453).

The preliminary experiments on the identification of isopeptides were focused on the development of a strategy, whereby first results were achieved. Until now, this strategy allowed the identification of five isopeptides with the binding site at Q₆₅ of the model peptide gli 56-75.

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4.7 Potential of non-prolamin storage proteins in coeliac disease

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Introduction

Brachypodium distachyon is a small annual grass that belongs to the *Pooideae* subfamily of the grasses, and based on the recent phylogenetic analyses is the closest wild relative of wheat and barley. This wild grass with special biological properties (small size, rapid generation time and self-fertility) and genomic attributes (small genome (272Mbp), diploid accessions) is suitable for use as a model system of cereals. *B. distachyon* accession Bd21 offers many advantages, such as self-fertility, simple nutrient requirements and short lifecycle. Sequencing and annotation of the Bd21 genome were recently completed, making further functional proteomic studies feasible [1-3]. The main storage proteins of Bd21 are the 11S, 12S and 7S globulin type proteins similar to oat and rice. The prolamins, including the avenin-like proteins and the gliadin-like prolamins, represent less than 12% of the total protein content which is significantly lower compared to wheat or barley. Due to specific proteomic features this annual grass is a good model plant to investigate the toxic nature of non-prolamin seed storage proteins. 11S-12S globulins account for 70-80% of total seed protein content [4]. In our previous study, the published chromosome specific *B. distachyon* genome sequences and a seed specific cDNA library data were used for sequence-based identification of proteins with regions identical to known coeliac disease-specific epitopes [5]. These results have highlighted the presence of possible cross-reactive epitope homologues to coeliac disease-related trigger molecules. Although *Brachypodium* is not considered for human nutrition, we took advantage of its use as a model species for the understanding whether abundant non-prolamin cereal seed proteins with linear epitope homologues play a role in the development of the humoral immune response and to help select other food sources suitable for a gluten-free diet.

Materials and methods

Serum samples from coeliac patients with known HLA-DQ haplotypes and positive for coeliac disease antibodies on gluten intake (n = 13, 8 females, 5 males), median age 5.7 years, range 1.4 - 13.5 years), serum samples from coeliac patients adhering to

a strict gluten-free diet (GFD) resulting in normalised antibodies (anti-transglutaminase IgA < 10 U/l) and mucosal healing (n=3), from ten newly diagnosed Crohn's disease patients with ileocolon manifestation (median age 6.4) and from eight healthy control subjects were used for immunoblotting studies.

In case of Bd21 total protein extracts, proteins were extracted with SDS buffer following the protocol of Dupont and co-workers [6] which extracted a greater percentage of protein from wheat flour than other methods and facilitated removal of starch.

After the 2D gel electrophoresis (GE) the proteins were transferred to an ImmobilonP PVDF membrane and IgA-based immunoblots were carried out. Rice glutelin antibody coupled with anti-rabbit IgA as a secondary antibody was used in 2D Western blot analysis to confirm the presence of seed storage globulins [7].

Protein sequences identified based on the online nanoLC-MS/MS analysis were retrieved from the UniProt database and were used for detailed epitope mapping analyses and protein characterisations. p-BLAST was used to find protein homologues in Poaceae. Coeliac disease-specific linear T-cell and B-cell epitopes were collected from the ProPepper database [8]. Epitope mapping was carried out using the motif search algorithm of the CLC Genomic Workbench (8.5.1).

Results and discussion

Brachypodium distachyon, a model plant of monocot species with low prolamin content was investigated to characterise immune reactivity against non-prolamin proteins in the seed. Altogether, 28 immune-reactive protein spots were analysed by online nanoLC-MS/MS (Fig 1.). Antibody reactivity against *Brachypodium* proteins was detected in all coeliac disease patients and two of Crohn's disease patients. While positive IgA reactions of coeliac serum samples were detected against proteins from a wide range of molecular weight (approximately 15,000 to 65,000) and variable isoelectric points, the protein spots showing immune reactivity with Crohn's disease serum samples possessed an approximate molecular weight of 24,000. No proteins reacted when sera of healthy controls and sera of patients on a strict gluten-free diet were applied. Most of the spots were identified as 7S or 11S-12S type seed storage globulins. A few prolamin-type storage protein sequence hits were also identified as secondary or tertiary protein hits with similar sequence coverage values: gamma gliadin-like proteins, HMW glutenin-like proteins, and LMW type respectively. Some enzymes and proteins with non-storage function were identified in the 35,000 region, like glucose/ribitol dehydrogenase, aldo-keto reductase, xyloglucan endo-transglucosylase/hydrolase and aspartic peptidase.

The most significant globulin hits (I1GPS5, I1GMC8, I1HMK7, I1IPF2 I1HNN9) as well as the most frequently identified prolamin protein (I1HRM6) were subjected to *in silico* sequence analyses. Two adjacent cupin-1 domains characteristic for both 7S and 11S-12S seed storage globulins were found in all of the *Brachypodium* globulin hits

(Fig. 2). The identified epitope homologues represented peptides with polyQ stretches and were positioned in two glutamine-rich regions of the protein. Additionally, a peptide with six residues (QPEQPF) was identified in the 11S seed storage globulin I1H9NH9. This peptide was the deamidated version of a known immune reactive AGA-specific B-cell epitope QPQQPF (IEDB Epitope ID 147232) that gets deamidated during coeliac disease pathogenesis. This deamidated version represents one of the primary targets of serum deamidated gliadin peptide (DGP) antibodies in coeliac disease. Interestingly, none of the I1H9NH9 *Poaceae* homologues contained this deamidated peptide. No epitope homologues were found in the metabolism-related proteins.

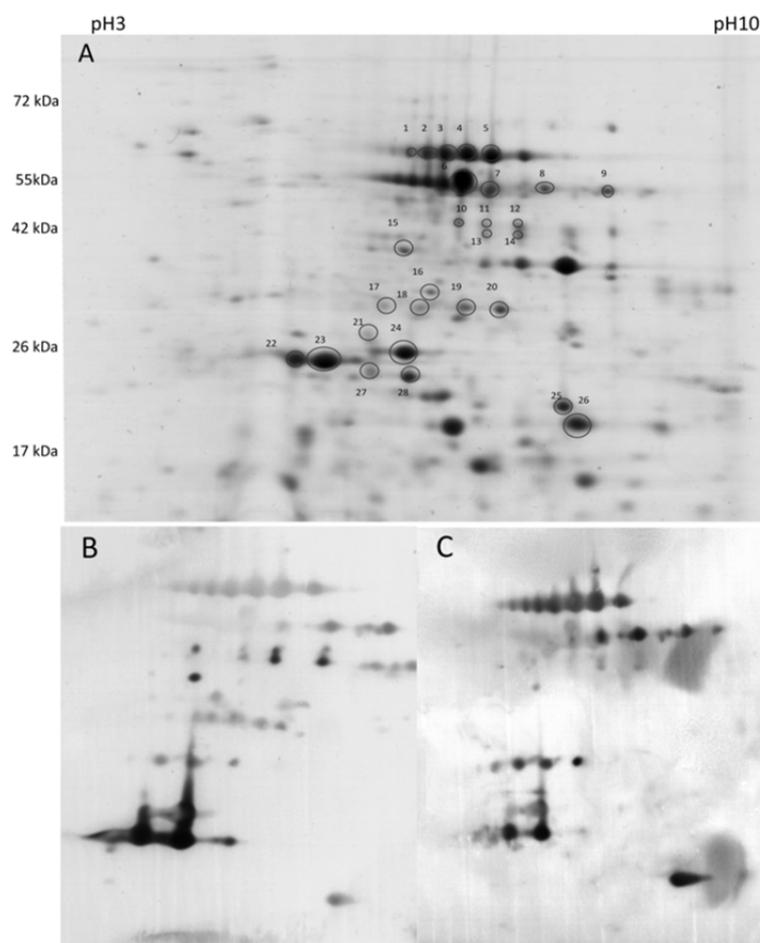


Figure 1. Identification of coeliac disease-related proteins of *Brachypodium distachyon* 'Bd21' using anti-IgA detection and patients' blood sera. (A) 2D gel electrophoresis of total protein extract of inbred line Bd21. Proteins were separated on 3-10 pH IPG strips followed by separation on 12% acrylamide gels. Labelled protein spots represent immune-reactive proteins and were sent for online nanoLC-MS/MS analyses. Molecular weight range is marked on the left-hand side. (B) and (C) Representative immunoblots using sera with IgA reactivity of therapy naïve coeliac disease patients, (B) HLA-DQ2, (C) HLA-DQ8

When gluten-related known T-cell epitopes were mapped to the *Brachypodium* proteins, no known epitopes were found. However, a type-I diabetes-specific T-cell epitope, EEQLRELRRQ [9] was identified from I1GPS5 with 100% sequence identity at the position 281. To check for novel T-cell epitopes, MHC-II binding predictions were carried out from the main 7S, and 11S-12S globulin hits using HLA-DR3-DQ2 and HLA-DR4-DQ8 MHC-II haplotypes (Fig. 2.) [10]. Some previous studies have focused on the investigation of immune reactivity and toxic behaviour of non-gluten proteins of wheat related to coeliac disease [11-14]. Recently, Huebener and colleagues have analysed the possible involvement of non-gluten proteins as target antigens in coeliac disease-related humoral response [15]. Serine-protease inhibitors, alpha-amylase inhibitors, farinins and seed globulins have demonstrated a significant immune response. Additionally, 35% of coeliac disease patients' sera showed reactivity against protein spots identified as seed globulins using the protein extract of 'Butte86' wheat [14]. Increased coeliac serum antibody reaction was also measured against cereal globulin extracts by Troncone et al. [13]. Although 7S and 11S-12S seed storage globulins both represent strongly conserved protein families with cupin-1 domains in cereals, our epitope analyses highlighted some remarkable differences between the protein families [10]. These differences indicate the presence of possible sub-classes with various immune-reactive potential. The amount of these unique globulin sub-classes can also be different in the grains of the different species, with a

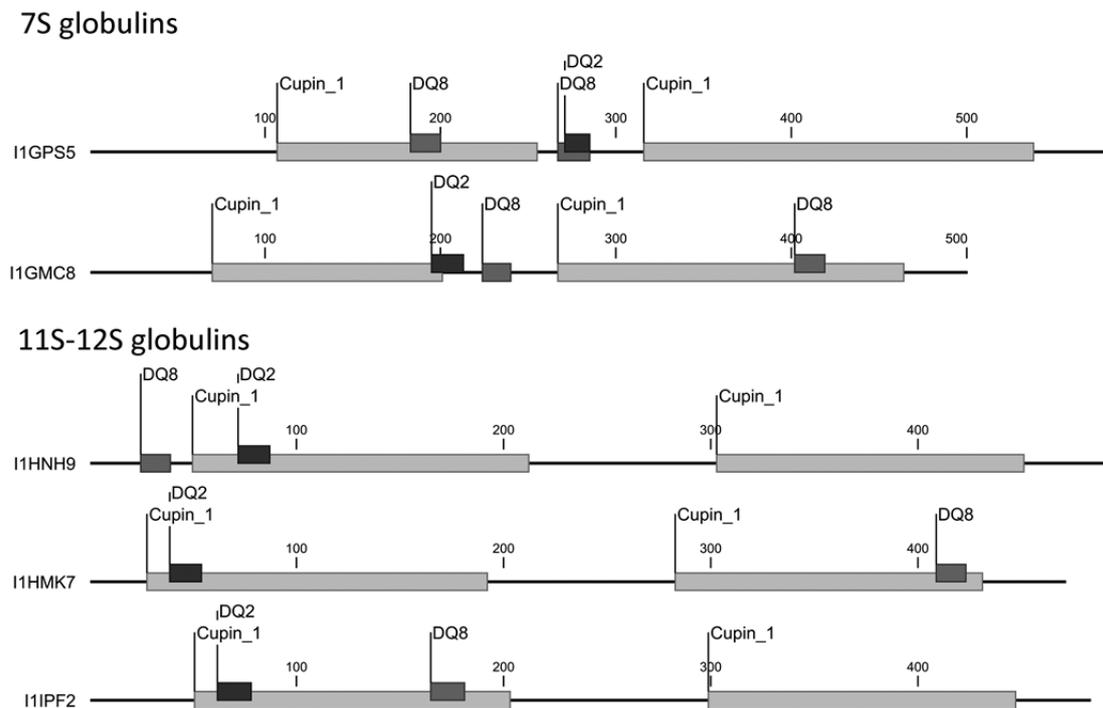


Figure 2. MHC II class T-cell epitope prediction of *Brachypodium* 7S and 11S-12S globulin proteins using HLA-DQ2 and -DQ8 alleles and IEDB analysis resource Consensus tool. Selection of predicted binders was carried out using the top 1% binders based on consensus percentile rank values. Predictions were calculated for each allele separately. Predicted epitopes are mapped to the protein sequences

significantly lower amount expressed in wheat and in cereals, where prolamins serve as major storage protein components. This fact partially explains why these proteins were overlooked compared to the most abundant prolamins in wheat, rye or barley.

Our study confirmed that globulin-type cereal seed storage proteins are specifically related to coeliac disease, as patients suffering from other immunological inflammatory diseases, like Crohn's disease did not recognise these globulin-type cereal storage proteins. Adverse results of the immunoblot analyses with sera of coeliac patients on a gluten-free diet had also strengthened the assumption that seed storage globulins may act as secondary B-cell stimulants due their strong sequence homology to epitopes originated from the primary gluten triggers. In progressive stages of the disease, villous atrophy and increased gut permeability contribute to why these proteins can serve as cross-antigens. The recovered intestinal mucosa of the coeliac patient on a strict gluten-free diet better prevents the passage of ingested proteins and probably, in this way, the strong immune reactivity can be controlled.

Conclusion

In summary, our results indicate that both in coeliac disease and type-I diabetes MHC-II-presentation and B-cell response may be developed not only for prolamins, but also for seed storage globulins even in distant relatives of wheat, such as *Brachypodium distachyon*, having seed storage globulins similar to oat and rice. However, its seed storage proteins, especially globulins, belong to quite conserved proteins in plants, which when eaten, may cause some problems due to the presence of some B-cell epitope homologues and possible T-cell reactive peptides present in the globulin fraction. Therefore, such cereals would not be harmless food alternatives for coeliac patients. High-resolution 2D gel electrophoresis followed by immunoblotting and protein identification have proven that 7S and 11S-12S seed storage globulins may act as antigens for coeliac disease specific IgA antibodies. Storage globulins are only present as contaminants in wheat gluten; therefore they play a less significant role. Contrary to this, seed storage globulins are the main source of nutrient storage in cereals like rice, oat or *Brachypodium*. Despite the strongly conserved structure of 7S globulins, proteins like Glo3A in wheat and I1GPS5 in *Brachypodium* and some of the 11S-12S globulins represent a special class of seed globulins with an epitope-dense region between the two cupin-1 domains and therefore might represent a higher risk for coeliac disease patients. Our study draws attention on the presence of conserved seed storage protein families in various cereal species, such as wheat, oat, rice and *Brachypodium*. Although 7S and 11S-12S seed globulins are present in low amounts in the wheat grain, they represent major storage protein groups in species like oat or rice. Therefore the presence of some epitopes in highly conserved regions may be also characteristic on orthologues in other species. The level of the response to globulins may depend on the type of seed globulins and amount of proteins with immune responsive peptide content. It is also suggested to perform further investigations whether diets enriched in seed storage globulins (like rice or oat) inhibit sufficient

healing, especially in patients with combined high risk to type-I diabetes and proven susceptibility to these proteins.

Acknowledgement

This project was supported by the European Union and co-financed by the European Social Fund (grant agreement no. TÁMOP-4.2.2.A-11/1/KONV-2012-0008), OTKA 101788, OTKA K10088 and OTKA PD 115641.

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4.8 Preview of the ‘Well on Wheat?’ (WoW) project

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Abstract

The ‘Well on Wheat’ (WoW) project aims to generate robust data on the effects of wheat-based food products on gastrointestinal function and metabolism. The first objective of the project is to obtain in-depth analytical data of the composition of whole meal obtained from bread wheat, spelt wheat and emmer wheat as well as the dough and the finally baked bread made thereof. The second objective is to study the effects of two alternative fermentation processes: yeast and sourdough fermentation, on compositional changes. Objective 1 and 2 will give insight in the overall effects of food processing on the (bio)chemical composition as defined by proteomics, carbohydrate analysis (carbohydrates, fibers, FODMAPs), phytate, selected micronutrients and pesticide residues. The third objective is to study the effects of consuming the various bread types (according to grain type and fermentation type) in individuals with irritable bowel syndrome (IBS), which will be monitored for effects on intestinal function and physiology, including e.g. faecal microbiota/metabolism, and using markers for gut permeability and inflammation as well as measuring subjective perceptions. The project will generate new scientific insights that will be translated into recommendations to food industries, health professionals and patient/consumer organisations. This project will be carried out in the framework of the Health Grain Forum and supported by the ICC (International Association for Cereal Science and Technology) and funded by private and public organisations.

Introduction

During the last decade, a significant movement to the adoption of gluten-free and wheat-free foods has developed in Western societies. The prevalence of wheat intolerance (coeliac disease) and wheat allergy are well known, being $\approx 1\%$ and 0.2% of the general population, respectively. However, in the US, nearly 30% of the adult population has expressed a desire to reduce or eliminate wheat and/or gluten from their daily diet [1] while a recent questionnaire-based study in the Netherlands [2] showed that 6.2% of a cohort of 785 adults reported adverse symptoms after the ingestion of gluten-containing foods. The most widely reported intestinal symptoms were bloating, abdominal discomfort and flatulence, but extraintestinal symptoms were also

mentioned such as fatigue and headache. Symptoms were generally experienced several days a week, starting mostly between one and six hours after consumption and lasting several hours. These self-reporting 'gluten sensitive' individuals were mainly younger females ($\approx 80\%$) living in urban regions with a trend of higher education levels (which confirms previous data of a UK study on self-reported gluten sensitivity [3]). Over one third of the reported symptoms met the consensus criteria for a positive diagnosis, the 'Rome III criteria for IBS', which have been established due to the absence of reliable biomarkers and specific laboratory tests [4].

The reasons why so many people feel more comfortable on a gluten-free diet may extend beyond the food itself. Several popular books [5-7] and many statements on social media have promoted gluten-free ('Palaeolithic') diets, suggesting that wheat consumption has adverse health effects leading to various chronic diseases. Furthermore, it is often claimed that products made from modern bread wheat varieties have negative health effects, but not foods made from so-called 'ancient' wheats such as spelt (which is closely related to modern bread wheat) and emmer (which is more closely related to modern pasta wheat), which are generally cultivated under organic conditions. These messages are, however, in contradiction to ample scientific data that have demonstrated significant health-promoting effects of whole grain consumption [8-12]. Despite these proven health benefits, the negative messages have resulted in a significant decline in the consumption of breads and other wheat products in Western countries.

In this context, IBS has often been considered by the patients themselves to be associated with food and especially wheat consumption. IBS is the most commonly diagnosed functional gastrointestinal (GI) disorder with a prevalence of 10-20% worldwide, predominantly among women [13]. Structural abnormalities and tissue damage are generally absent, but psychiatric co-morbidity is often reported, indicating a psychosomatic component in a subgroup of these patients. Although several factors have been associated with IBS, including e.g. microbial perturbations, altered permeability, motility and visceroperception, the exact pathophysiology is not yet clear. Also markers for mucosal immune activation and inflammatory responses have been reported in a subset of IBS patients that may disappear after elimination of wheat/gluten from the diet [14,15]. This condition is often referred to as 'non-celiac wheat sensitivity' (NCWS) or 'non-celiac gluten sensitivity' (NCGS) [16].

Dietary factors such as FODMAPs (fermentable, oligo-, di-, monosaccharides and polyols) have been recognised as triggers for symptoms in some subjects, by providing substrates for colonic fermentation [16 and refs therein, 17]. It has also been reported that replacing a bread wheat-based diet by whole grain products from 'ancient' wheats such as spelt, has benefits for IBS patients [18,19]. In addition to gluten and FODMAPs, the presence of relatively high quantities of amylase-trypsin inhibitors (ATIs) in bread wheat has also been suggested as a potential IBS causing factor [20,21]. Direct comparative data about the effects of foods obtained from different

wheat types and their possible contribution to the pathophysiology of NCWS are, however, still lacking. Here we propose a research strategy to address this issue.

Project design

The WoW project will study the effect of different grains in IBS patients to provide information on the wheat- and disease-related issues at three levels: (1) The biochemical composition of wheat grains and changes during processing steps (milling, fermentation, baking) into consumable food products (bread); (2) The impact of bread consumption on well-being and GI symptoms, gut permeability, immune function and the microbiome; and (3) The impact of the opinions and perception of consumers/patients on wheat consumption or avoidance regarding gastrointestinal symptoms and well-being. The project will be managed by the academic and funding partners in a contractually agreed pre-competitive manner.

Materials and methods

Grains. Grains from bread wheat (*Triticum aestivum*) (representing current bread products), spelt wheat (*T. aestivum ssp spelta*) and emmer wheat (*T. dicoccum*) (both representing ‘ancient’ wheat species) obtained commercially will be analysed for biochemically for proteins using proteomics (detection of gluten, globulins, albumins, ATIs, lectins, indigestible peptides), fibre (including fructans and other FODMAPs), phytate, phenolics, minerals (such as zinc and magnesium), at the level of flours, fermented (yeast and sourdough) doughs, and breads.

Cohort and intervention groups. We aim at measuring the effects of wheat consumption in IBS patients, recruited from a large cohort of well-characterised IBS patients [22] that has been established at Maastricht University Medical Centre. Three groups will be used in the intervention study, including successively: a running-in period (1 week), a free-from diet (2 weeks), a yeast or sourdough bread food challenge (2 weeks), a free-from wash-out diet (2 weeks) and a sourdough or yeast bread food challenge (2 weeks). It should be noted that the first and the second challenge are reversed regarding the yeast and the sourdough breads. The three groups will differ in their challenge: the groups 1, 2 and 3 will be challenged blinded with yeast and sourdough bread from either bread wheat, spelt wheat or emmer wheat.

Sampling human materials. At each step in the challenge sequence, patient samples will be taken from (1) the stool to analyse microbiota composition and metabolites from bacterial protein and carbohydrate fermentation ((i.e. short chain fatty acids, branched chain fatty acids, etc.); (2) breath metabolome to identify volatile organic compounds reflecting host and microbial metabolism; (3) blood to determine alkylresorcinols, inflammation markers (C-reactive protein and cytokines); zonulin; and (4) urine sugar ratios as proxy for gut permeability. Furthermore, validated scores will be applied to measure wellbeing and GI symptoms.

Yeast versus sourdough fermentation. Significant differences are expected in the biochemical composition after yeast fermentation as compared to sourdough fermentation [23]. If the results are not significant, the intervention schedule will be adapted accordingly.

Nocebo effects. In healthy consumers, nocebo effects related to wheat/gluten avoidance will be determined through a food challenge with a single bread type that will be differently labelled and offered in four categories of emotional perceptivity and acceptability.

Ethics. Before starting, the project will be evaluated, commented and approved by a Medical-Ethical Committee.

Wheat cultivation. In an extension to the WoW project, the various wheat types (see Grains above) will be compared after growth under different conditions (organic vs standard) to determine effects of environment on composition. This data will may help to explain possible differences, between the grain types obtained from different European countries and cultivation practices.

Partners and Sponsors

The project proposal has been initiated by Maastricht University and further elaborated together with Wageningen University & Research, the Dutch Bakery Centre (all from The Netherlands) and Rothamsted Research (UK). Most research partner organizations are members of Health Grain Forum (HGF) and the project fits into the activities of the HGF working group on 'Cereals and health' and has been included in the general HGF programme. The International Association for Cereal Science and Technology (ICC; Austria) will serve as financial administrative partner for the following sponsoring entities:

- AB-Mauri Bakery Ingredients, Made, Netherlands
- CSM Innovation Bakery Center, Bingen, Germany
- CYMMIT, Texcoco, Mexico
- DSM Food Specialties, Delft, Netherlands
- Fazer Bakeries, Helsinki, Finland
- ICC- Intl. Association for Cereal Science and Technology, Vienna, Austria
- IWGA- Intl. Wheat Gluten Association, Kansas, USA
- Lantmännen EK, Stockholm, Sweden
- Mondelez, Saclay, France
- Dutch Bakery Center, Wageningen, Netherlands
- Nutrition et Sante, Revel, France
- Puratos BV, Groot Bijgaarden, Belgium

- Sonneveld Group BV, Papendrecht, Netherlands,
- Zeelandia Zierikzee, Netherlands
- Baking Industry Research Trust Howick, Auckland, New Zealand
- Health Grain Forum, Vienna
- The project is in part publicly funded by the Dutch Topsector Agri&Food.

The sponsoring organisations have neither a role in the design and execution of the project, nor in the collection, analyses, interpretation and publication of the data.

Expected outcomes

The project will generate comparative data on the biochemical composition of grains of bread wheat, spelt and emmer, with a major focus on those compounds expected to have positive or negative effects on health, such as carbohydrates (starch, fibre, FODMAPs), proteins (gluten, CD-immunogenic gluten peptides, albumins, globulins, lectins, ATIs), phenolic compounds, phytate, and minerals (Zn; Mg). In an extension of the project, biochemical analyses will be carried out on grains grown under different conditions.

After milling, breads will be made using yeast fermentation and sourdough fermentation and the compositions of the doughs and flours compared. A great challenge is the production of breads from flours of the different grain types that are visually and organoleptically similar, so that they can be used in the double-blind food challenges.

Administration of the baked products to patients with IBS (i.c. NCWS) will reveal any effects on the aetiology of non-celiac wheat sensitivity.

The project will also provide insight into the occurrence of nocebo effects by collecting data obtained about post-consumption gastrointestinal symptoms from a group of healthy volunteers that prefer to avoid gluten.

The results will be published in international scientific journals. New scientific insights will be translated into recommendations to wheat-processing industries, governmental regulatory bodies, health professionals, patients and consumers, and will underpin innovation in the production of wheat-based foods.

Acknowledgements

Here, we would like to mention the names and affiliation of the other scientific co-operators: Koen Venema, Frederik-Jan van Schooten, John Penders and Rob Markus (Maastricht University), Petra Kuiper and Zsuzsan Proos (NBC), Hetty Busink-van den Broeck, Ingrid van der Meer, Ruud Timmer (Wageningen University & Research). Thanks are due to the sponsors for their financial support. The project will be partly financially supported by the Dutch Topsector AgriFood (TKI 1601P01). Rothamsted

Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC) of the UK.

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5 Clinical research reports

5.1 Oats in the diet of children with coeliac disease: a double-blind, randomised, placebo-controlled multicenter study

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Introduction

Coeliac disease (CD) is an inherited autoimmune condition triggered in genetically susceptible individuals by amino acid sequences within the prolamin fraction of ingested wheat (gliadins), barley (hordeins) and rye (secaline).

At present, the only treatment for this condition is the lifelong complete withdrawal of gluten from the diet [1].

Strict adherence to a gluten-free diet (GFD) is required to control the symptoms of CD and to prevent the autoimmune and neoplastic complications associated with this condition [2].

However, full compliance with a GFD heavily affects dietary choice and the quality of life. Although the quality of gluten-free food has significantly improved in the last decades, some problems still remain partially unresolved, in particular the lower technological performance of gluten-free cereals [3].

On this basis, the inclusion of oats in the GFD could be of great value. Oats are a good source of fibres and, in particular, of vitamins and minerals, and beta-glucans, which are healthy compounds that reduce LDL-cholesterol and the glycemic index of foodstuff [4,5]. Moreover, the inclusion of oats unquestionably improves the

nutritional value and increases the palatability of gluten-free products, while expanding food choices and ultimately improving the quality of life for people with CD [6].

Although oats are included among the gluten-free ingredients (gluten content does not exceed 20 parts per million -ppm-) by European Commission Regulation No. 41/2009 [7] the safety of oats in CD is still a matter of debate.

Some clinical trials have concluded that oats are well tolerated by CD patients on a GFD, but early studies found that some patients consuming oats as part of a GFD suffered an intestinal inflammation similar to that in untreated coeliac patients [8].

Previous studies were limited by small sample sizes or short follow-up periods and to the best of our knowledge there has been only one randomised placebo-controlled clinical trial.

Moreover, oats is not a staple food in the diet of Mediterranean populations. This is probably the main reason why an oats “resurrection” in the GFD has not raised immediate interest in Southern European countries.

Therefore, we aimed to evaluate in a large randomised, double-blind, cross sectional placebo-controlled multicenter clinical trial the clinical, serological and mucosal safety and the acceptance of gluten-free oat-based products from selected oat varieties in the diet of Italian children with CD.

Clinical monitoring during the study was based on: a) score of intestinal symptoms; b) serological CD markers (TG-IgA, AGA-IgA, antiavenin) c) mucosal parameters integrity monitored by double sugars intestinal permeability test (IPT).

Material and methods

306 children aged 4-14 years with biopsy-proven diagnosis of CD, on a GFD for at least 2 years, were recruited at 8 Pediatric Gastroenterology centres in Italy (Ancona, Bari, Bolzano, Catania, Monza, Palermo, Roma, Cava de’Tirreni) between 2008 and 2012. Patients who (1) have chronic conditions, including type 1 diabetes or inflammatory bowel disease, or (2) did not adhere to the GFD as demonstrated by elevation of serological markers at enrollment, were excluded.

Children were assigned on the basis of a stratified randomization to one of two groups: those assigned to group A received 6 months of diet A, 3 months of standard GFD as a wash out period, and 6 months of diet B; those assigned to group B received 6 months of diet B, 3 months of standard GFD as a wash out period, and 6 months of diet A (Fig. 1).

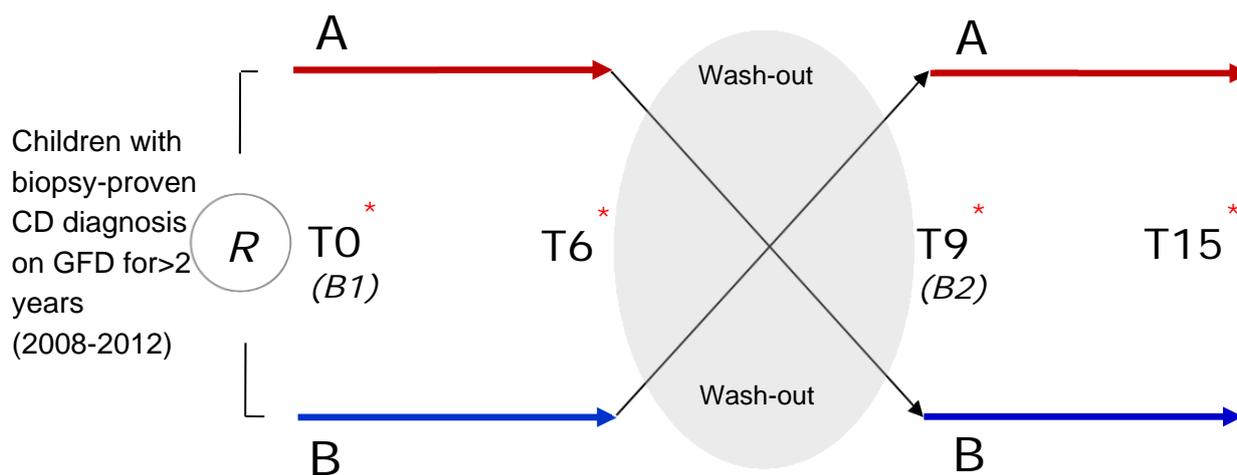


Figure 1. Study cross-over design

A and B diets included gluten-free products (flour, pasta, biscuits, cakes and crispy toast) with either purified oats or placebo, that were identical in form, and colour, and were both provided by a company leader in gluten-free production in Italy.

The minimum oat intake required (calculated as 1 g/kg/day) was 15 g/day for children aged 3-6 years, 25 g/day for children aged 7-10 years and 40 g/day for children aged 11-16 years. The oat varieties used were specially grown, milled and packaged to avoid any cross-contamination with gluten-containing cereals or food.

Gluten contamination of used oats was measured by Ridascreen® ELISA test.

Clinical [Body Mass Index (BMI), class of BMI, Gastrointestinal Symptoms Rating Scale (GSRS) score], serological [IgA anti-transglutaminase antibodies, IgG anti-deamidated gliadin peptides (AGA) and IgA anti-avenin] and intestinal permeability test (IPT) data were measured at basal (B1, recruitment) and after six months of diet A or B in the first period (T6), after three months of wash-out at the beginning of the second period as a second basal point (B2) and after six months of diet A or B (T15).

At each time point of follow-up the daily intake of oat was assessed by means of a three-day diary and symptoms and/or side effects related to the ingestion of the products under investigation was promptly recorded.

Statistical analysis

Sample size was estimated using intestinal permeability test (IPT) as primary response variable and considering a clinical difference between the two diets of 0.01 as minimum.

Since the resulting data was not normally distributed, a non parametric approach was used for all statistical analyses. For descriptive purposes, absolute variations in clinical and anthropometric variables between T6 and B1 and T15 and B2, respectively in the first and in the second period of treatment, were calculated and graphically represented by boxplots.

Ninety-five percent confidence intervals (95% CI) for median values were calculated and comparisons between diets groups in each treatment period were performed using Wilcoxon rank sum test. A positive variation indicated an increase in the variable, that was considered statistically significant when 95% CI did not contain zero value.

First and second carry-over effect (θ , λ) and direct treatment effect (τ) were evaluated by a non-parametric statistical approach using medians as summary statistic. Confidence intervals for each effect were estimated using a probability of 0.90 for the first two (θ , λ) and 0.95 for τ . A probability of 0.05 was chosen to assess the statistical significance.

Results and discussion

After the exclusion of 129 patients who dropped out, the analysed cohort included 177 children (79 in group A and 98 in group B). There were 124 girls (70%) and the median age of the cohort was 8.9 years (range 6.9 to 11.2). Table 1 shows the patients' main anthropometric and clinical characteristics at the first basal. No significant

Table 1. Subjects' anthropometric and clinical characteristics at basal according to treatment groups. M=males, F=females, IQR=interquartile range.

[n (%)]	Groups		p
	AB (n=79)	BA (n=98)	
Gender			
Males	22 (27.8)	31 (31.6)	0.703
Female	57 (72.2)	67 (68.4)	
Biopsy [n (%)]			
1	1 (1.4)	2 (2.5)	0.647*
2	2 (2.9)	1 (1.2)	
3A	9 (13)	7 (8.6)	
3B	27 (39.1)	27 (33.3)	
3C	30 (43.5)	44 (54.3)	
Chi-square test; *Fisher exact test			
Median [IQR]	AB	BA	p
Age (years)	8.9 (6.9 ; 11.25)	9.3 (6.925 ; 12)	0.435
Age at diagnosis (years)	3.877 (2.145 ; 6.522)	4.219 (2.927 ; 6.49)	0.246
BMI (kg/m ²)	16.7 (15.35 ; 19.55)	18.2 (15.85 ; 20.48)	0.054
BMI percentile	60 (35.5 ; 87)	69.5 (42.5 ; 91.75)	0.058
GRSRS Score	2 (0 ; 4.5)	2 (0 ; 4)	0.894
Agal _g	4.6 (2.3 ; 8.13)	5.6 (2.55 ; 8.9)	0.347
TTGI _g A	4.31 (2.1 ; 7.5)	5.1 (2.55 ; 7.8)	0.520
TPI	0.056 (0.029 ; 0.082)	0.0465 (0.0275 ; 0.0795)	0.316
Anti-avenin	0.0595 (0.048 ; 0.07875)	0.0575 (0.05 ; 0.07)	0.775

p-values refer to Wilcoxon rank sum test; IQR: 1st-3rd quartile

differences were found between the two groups, so it can be confirmed that randomization worked well.

No significant differences were found between the two groups in the two treatment periods both for clinical parameters and serological parameters, neither for the mucosal parameters as reported in Fig. 2, 3 and 4, respectively.

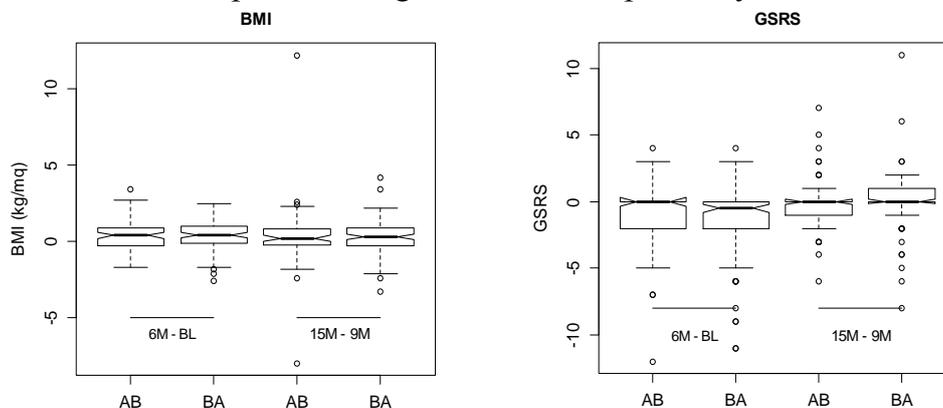


Figure 2. Absolute variation in the two treatment periods according to groups in the clinical parameters BMI, BMI class and GSRs

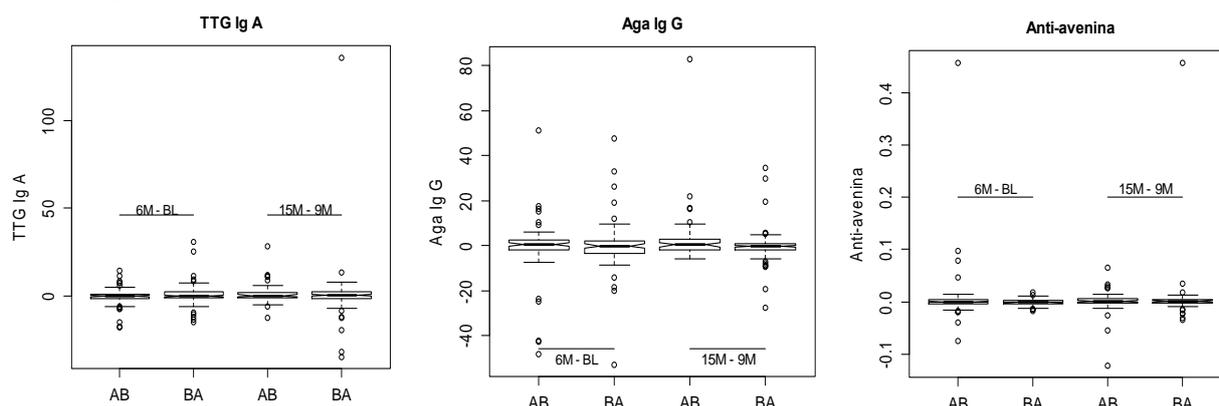


Figure 3. Absolute variation in the two treatment periods according to groups in the serological parameters TTG, IgA, Aga IgG, anti-avenin)

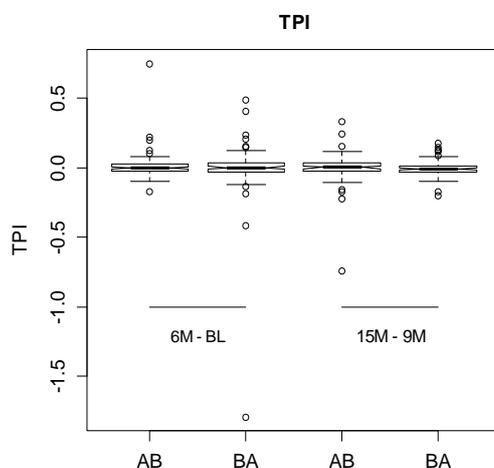


Figure 4. Absolute variation in the two treatment periods according to groups in the mucosal parameters (intestinal permeability, TPI)

Table 2. Differences in clinical, serological and mucosal parameters during the two diet periods.

Median (95%CI) 1 st -3 rd quartiles	1° period (T6-B1)			2° period (T15-B2)		
	AB	BA	p	AB	BA	p
BMI	0.4 (0.19; 0.61)	0.4 (0.22; 0.58)	0.78	0.2 (0.01; 0.39)	0.3 (0.11; 0.49)	0.50
BMI Class	1 (-0.87; 2.87)	0.5 (-0.46; 1.46)	0.87	0 (-1.6; 1.6)	0 (-1.12; 1.12)	0.40
GRSR Score	0 (-0.36; 0.36)	-0.5 (-0.82; -0.18)	0.31	0 (-0.18; 0.18)	0 (-0.16; 0.16)	0.10
Aga Ig A	0.15 (-0.48; 0.78)	0.3 (-0.35; 0.95)	0.98	0.8 (0.17; 1.43)	-0.1 (-0.99; 0.79)	0.50
Aga Ig G	0.5 (-0.27; 1.27)	-0.2 (-1.12; 0.72)	0.16	0.3 (-0.62; 1.22)	-0.39 (-0.87; 0.09)	0.00
TTG Ig A	-0.04 (-0.54; 0.46)	0.1 (-0.47; 0.67)	0.10	0.2 (-0.32; 0.72)	0.45 (-0.23; 1.13)	0.60
TPI	0 (-0.0091; 0.0091)	0 (-0.0101; 0.0121)	0.64	0 (-0.01; 0.02)	-0.01 (-0.02; 0)	0.00
Anti- avenin	0 (-0.0015; 0.0015)	-0.001 (-0.002; 0)	0.24	0.001 (-0.0005; 0.0025)	0.001 (0; 0.002)	0.40

Table 3. Results of crossover analysis between the two study groups.

	θ	λ	τ
Median (1-α/2 %CI)	1st-order carry-over effect	Direct-by-period interaction	Direct treatment effect
BMI	0.084 (-0.05; 0.20)	0.05 (-0.15; 0.20)	-0.5 (-0.12; 0)
BMI Class	0.50 (-1.0; 1.50)	0.50 (-1.0; 2.0)	-0.25 (-1.0; 0.25)
GRSR Score	0 (0; 0)	0 (-0.5; 0)	0 (-2.5; 0)
Aga Ig A	0.29 (-0.35; 0.90)	0.14 (-0.70; 1.05)	-0.15 (-0.50; 0.25)
Aga Ig G	0.29 (-0.35; 0.90)	0.15 (-0.70; 1.05)	-0.15 (-0.50; 0.25)
TTG Ig A	0.4 (-0.05; 0.95)	0.30 (-0.25; 0.80)	-0.02 (-0.25; 0.23)
IPT	0.001 (-0.01; 0.01)	-0.003 (-0.014; 0.007)	0.004 (-0.0002; 0.0089)

Table 3 shows the results of crossover analysis; differences between the two groups in the variables of interest were summarised using medians and the first order carry-over effect, direct-by-period interaction, and a direct treatment effect according to the diet sequences AB, BA was evaluated by means of confidence intervals. A positive sign in

the direct treatment effect estimates indicated that treatment B was associated with the higher average of the variable.

Differences in treatment carry-over at the time of the second baseline measurements (θ) and differences in treatment carry-over at the time of the second treatment measurement (λ) and direct treatment effect (τ) were found not statistically significant for all clinical, serological and mucosal variables studied. The upper limit of the 95% confidence interval of IPT direct treatment effect was found lower than the highest difference considered clinically relevant (0.01).

These data confirm that this clinical trial is a study of non-inferiority, so an oats-enriched diet did not cause any modifications in coeliac children. There is no difference in treatment A with respect to treatment B.

Conclusion

In a large group of CD children, we found that the prolonged daily intake of a considerable amount of pure oats did not cause any significant change in terms of clinical symptoms, serological parameters and intestinal permeability.

Coeliac disease children can safely add pure oats to their GFD. The inclusion of oats in the GFD would be beneficial, as they provide a good source of fibres, have a higher satiety value than other cereals, add texture and flavor to baked goods and could increase compliance with a GFD by providing patients with more alternatives.

Disclosure of interest: All authors have conflict of interest with Heinz Company s.r.l.

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5.2 A study of morphological and immunological responses to a 14 day gluten challenge in adults with treated coeliac disease

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Introduction

Coeliac disease (CD) is a gluten-induced enteropathy [1]. The treatment is to exclude gluten completely from the diet, whereupon the derangement of the gut and the serum antibodies normalise. The clear association to distinct HLA-types has also shown the central role of T cells in the pathogenesis of CD [2].

Non-coeliac gluten sensitivity is defined as a gluten-induced disease with similar symptoms as CD, but without the typical findings in the gut and in the blood [3]. The prevalence of this condition may be higher than CD in many countries [4], leading to an increased awareness of gluten-induced disease in the population and a significant number of people eating a self-prescribed gluten-free diet without a proper diagnosis.

A gluten challenge is performed when the patient has started on a gluten-free diet without proper diagnosis. According to guidelines [5,6], the recommended dose of gluten should be at least 3 grams daily, and the duration should be at least 8 weeks, or 2 weeks in the case of strong gluten-related symptoms.

In this study we seek to evaluate different response parameters to a 14 day gluten challenge in adults with treated coeliac disease.

Materials and methods

We included twenty adults (Fig. 1) with treated coeliac disease for a two week gluten challenge. The participants were on a gluten-free diet, had normal duodenal biopsies and normal anti-transglutaminase IgA titers prior to challenge. Challenge was performed with a low-FODMAP (fermentable oligo-, di-, monosaccharides and polyols) muesli bar containing 5.7 grams of gluten, taken once daily (Fig. 2). We took duodenal biopsies at the end of challenge. Blood was drawn at several time points. We

used HLA-gliadin-complexes (tetramers) linked to a fluorochrome, along with several monoclonal antibodies to characterise gluten-specific T cells in blood in a flow cytometer. Symptoms and quality of life parameters were scored by the use of GSRS-IBS, visual analogue scales and SF-36. Serum samples from the first day of challenge was collected at baseline, and then every second hour until 6 hours after the first dose of gluten to look for cytokine changes during the initial exposure. Fecal samples were collected at several time points during the study to characterise the microbiota and gluten associated changes herein.

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

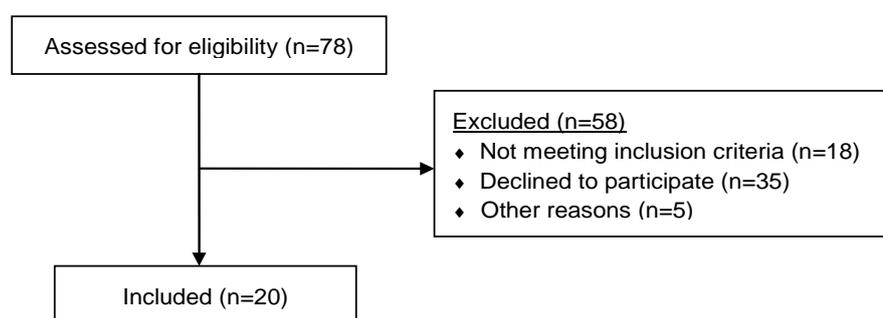


Figure 1. Flowchart showing the number of individuals assessed for eligibility, excluded and included in the study

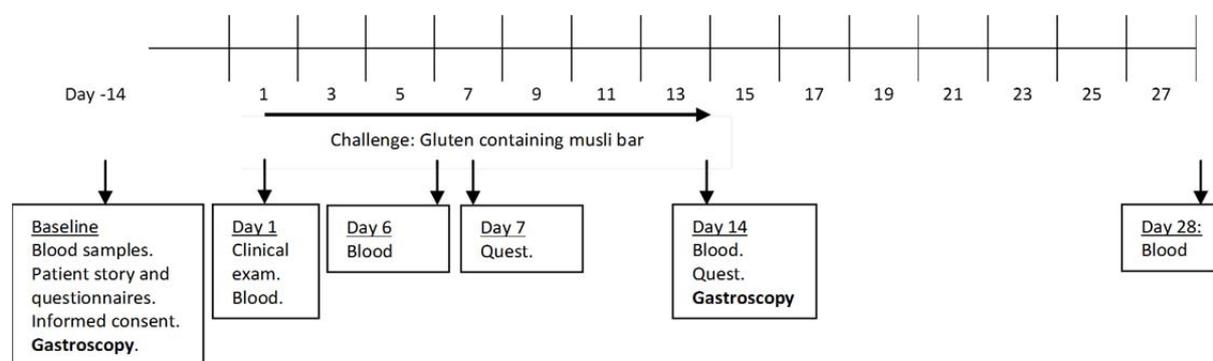


Figure 2. Timeline showing the course of the study

Results and discussion

The manuscript is in preparation and the data are still unpublished. We therefore choose not to present the results here.

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5.3 A double-blind placebo-controlled cross-over challenge with gluten and fructans in individuals with self-reported gluten sensitivity

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Introduction

Non-coeliac gluten sensitivity (NCGS) is a term within gluten-related disorders that has been applied for the condition where persons report symptom relief on a gluten-free diet in absence of coeliac disease and wheat allergy [1]. An important characteristic for the group is the experience of symptoms after intake of gluten-containing cereals, but the role of gluten as symptom inducer is questioned. There are no reliable diagnostic biomarkers for NCGS, but a standardised blinded placebo-controlled gluten challenge is proposed to confirm the condition [2].

Results from two earlier double-blinded placebo-controlled studies have given conflicting results. The first study showed that subjects who received gluten reported more gastrointestinal symptoms than those who received placebo [3]. The second study showed no specific or dose-dependent effect of gluten as compared to placebo after dietary reduction of fermentable, poorly absorbed short-chained carbohydrates (FODMAP) in subjects who were believed to have NCGS [4]. Subjects in these trials had coeliac disease excluded by negative HLA-DQ2/8 or normal duodenal biopsy while on a gluten-containing diet. Several double-blinded placebo-controlled gluten challenge trials have followed [5-7]. Recent trials suggest that gluten challenge induces symptom recurrence in only a minority of subjects who meet clinical criteria for NCGS [6]. Two other Italian trials aimed to discover true NCGS and found increased symptom score on gluten intake as compared to placebo, but only 5% and 14% were classified as NCGS according to predefined criteria [5,7]. In both studies gluten was administered as capsules.

All these trials have studied the role of gluten and used carbohydrate depleted gluten flour as the active challenge vehicle. However, people do not eat pure gluten flour. They report wheat as the symptom inducer. Considering the composition of wheat, where gluten co-exists with a substantial amount of FODMAP, in specific fructans, this component has not been taken into account in any challenge trial of NCGS [8]. The aim of the present study was therefore to investigate the effect of gluten and fructans separately in self-reported gluten-sensitive persons with muesli bars as challenge vehicle.

Materials and methods

Study design

We describe a double-blind, placebo-controlled challenge study, where subjects were randomised to gluten, fructan and placebo challenges in a cross-over design (ClinicalTrials.gov, NCT02464150). The study took place at Oslo University Hospital, Rikshospitalet from October 2014 to May 2016.

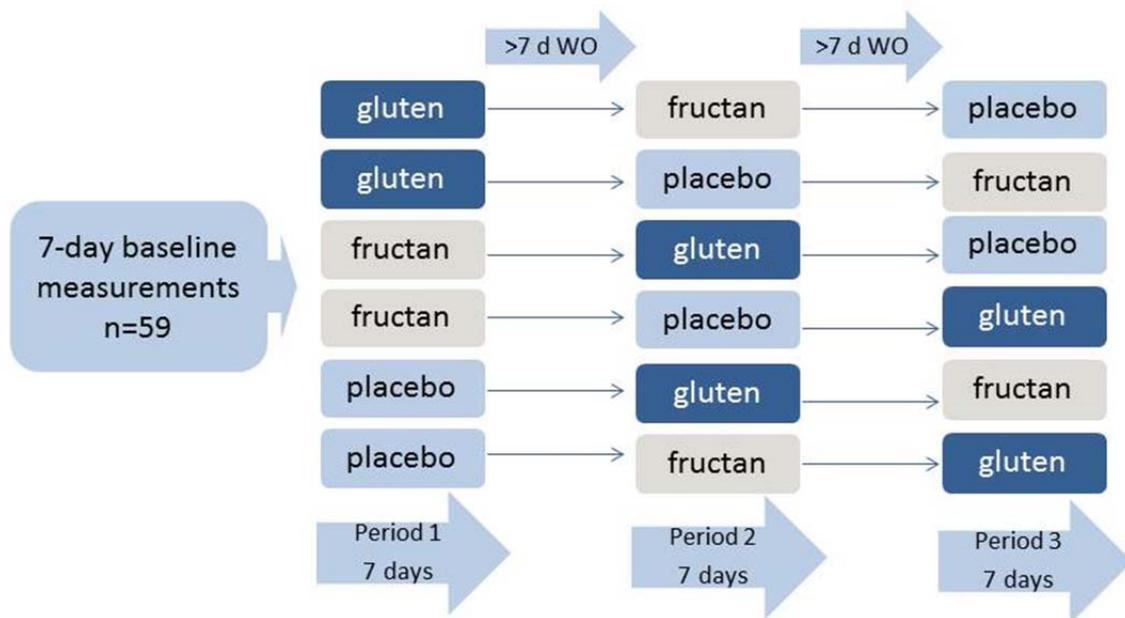


Figure 1. Study design. WO, wash out

Subjects

The study subjects were 59 adults (6 males) aged 18-80 who self-reported gluten sensitivity, had been strictly adherent to a gluten-free diet (GFD) for at least six months followed by symptom relief and had no coeliac disease or wheat allergy. Coeliac disease was considered adequately excluded by negative duodenal biopsy while on gluten-containing diet or negative genotype HLA-DQ2/8. Wheat allergy was considered eliminated in case of negative wheat-specific IgE. Exclusion criteria were pregnancy or lactation, use of immunosuppressive agents, inflammatory bowel disease

or other comorbidity, substantial infection, long travel distance or allergy to nuts or sesame seeds.

Subjects were recruited by advertisements on the Oslo University Hospital's web page, at the University of Oslo, at the web page of the Norwegian Coeliac Association and their social media, and by referrals from general practitioners and other hospitals in the area.

Food challenge protocol

The subjects were randomised to one of three challenges (gluten, fructan or placebo) for one week, followed by a minimum of one week washout period. They continued their GFD and were told to keep their diet otherwise consistent with the baseline diet throughout the study.

The challenge vehicle was a 50 g, 220 kcal low-FODMAP gluten-free muesli bar that was eaten once a day. The muesli bars were developed and produced by the Monash University, Melbourne. The fructan bar additionally contained 2.1 g of fructo-oligosaccharides and the gluten bar 5.7 g of gluten. The gluten used was commercially available, carbohydrate depleted wheat gluten (Vital Wheat Gluten, Manildra Group, Gladesville, New South Wales, Australia) with nutritional content of 75% protein, 9% carbohydrate of which 4% sugar, 6% fat, 9% water and 1% ash (Cargill analysis). The muesli bars were balanced in carbohydrates, fiber, fat and protein and had similar appearance, texture and taste, confirmed by pre-testing in 12 healthy adults where the gluten bar could not be differentiated from the fructan or placebo bar.

Aim and outcome

The aim was to investigate the effect of gluten and fructans separately in a double-blind placebo controlled challenge in individuals with self-reported gluten sensitivity in absence of coeliac disease. The primary outcome was overall gastrointestinal symptoms measured by the Gastrointestinal Symptom Rating Scale – irritable bowel syndrome version (GSRS-IBS). The secondary outcome was daily overall gastrointestinal symptom score by visual analogue scale (VAS) for abdominal pain, bloating, passage of wind, nausea, stool dissatisfaction and overall gastrointestinal symptoms.

Results and discussion

The manuscript is in preparation. Results will therefore not be presented here.

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5.4 Natural history and management of potential coeliac disease

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Introduction

According to the most recent European Society of Gastroenterology, Hepatology and Nutrition (ESPGHAN) guidelines, coeliac disease (CD) is considered an immune-mediated systemic disorder elicited by gluten and related prolamins in genetically susceptible individuals [1]. Even if enteropathy remains the prominent feature of the disease, it is now widely accepted, that, from a histological point of view, it can range from complete villous atrophy to minimal mucosal abnormalities. In this context, the term potential coeliac disease (PCD) is used to define patients with normal or slightly altered intestinal mucosa (Marsh 0-1), but a positive CD serology. Patients with PCD may or may not have symptoms and may or may not develop an overt form of CD over time [2]. The number of patients with a diagnosis of PCD is increased so far because of the screening of general population and it is now estimated to be at the considerable number of 1/5-1/10 of the total CD diagnosis [3].

Clinical management of patients with potential coeliac disease

Even if PCD patients do not show clear signs of enteropathy, some of them may present clinical symptoms. In our experience, abdominal pains and failure to thrive are the most frequent ones and are found in around 1/3 of symptomatic patients. Other symptoms are diarrhea (approximately 16% of patients), lack of appetite (13%), low blood ferritin (8%), vomiting and constipation (5%), anemia and hypertransaminasemia (3%). Biagi et al. have hypothesised that in PCD the intestinal mucosa is maintained architecturally normal thanks to an increased enterocytic proliferation, which, however, will end up in a reduced enterocytic maturity and thus in a reduced absorptive capacity of the small bowel [4]. There is a general consensus for this kind of patients to adopt a gluten-free diet (GFD) and to control, during the follow up, the remission of symptoms.

On the contrary, the management of asymptomatic patients with PCD remains a major clinical problem. Some have suggested that, because this condition could be the first step of the disease, all patients should adopt a lifelong GFD. This was supported by the fact that an undiagnosed case of CD has a four-fold increased risk of mortality for all causes [5] and that the “metabolic identikit” of PCD patient is similar to the one of CD patients and differs from controls [6]. Moreover, despite per definition there is no clear

villous atrophy in potential coeliac disease, in the last decade an increasing number of studies have suggested that mild signs of inflammation are often present. In fact, from an immunohistochemical point of view, 70.8% of PCD patients show increased numbers of lamina propria CD25+ and/or enhanced expression of ICAM-1 and crypt HLA-DR [7]. On the other hand, preliminary observations from our group demonstrated that most of the asymptomatic children with PCD remained healthy on a gluten-containing diet. During three years of follow up, CD-associated antibodies fluctuated (32.6%) or even disappeared (14.6%) and, after three years, only 30.8% of the patients developed villous atrophy [8]. Therefore, it would be an overtreatment to consider all PCD patients as coeliac. Unfortunately, we still have no good way to identify which subsets of seropositive patients will develop mucosal damage.

Natural history of potential coeliac disease: a 9 years prospective longitudinal study

The main challenge remains to find criteria that allow to differentiate, among PCD patients, those who will develop villous atrophy from those who will not. In order to study the long-term natural history of PCD disease and to explore risk factors associated with the development of mucosal atrophy, our group performed a 9 year prospective longitudinal study [9]. 175 asymptomatic children were left on a gluten-containing diet. Antibodies and clinical symptoms were checked every 6 months, and a small bowel biopsy was taken every 2 years to evaluate histological, immunohistochemical, and anti-TG2 deposits. Patients were genotyped for HLA and a set of non-HLA CD-associated genes. At the end of the follow up, 67% of the patients still had a normal duodenal architecture (Fig. 1). Monitoring the individual profile of anti-TG2 antibodies, 43% of patients showed persistently elevated anti-TG2 level, 20% became negative during follow-up, and 37% showed a fluctuant anti-TG2 course with transiently negative values. Analysing the cumulative incidence of CD in relation to individual risk factors, we noticed that the anti-TG2 titer at the entry was not statistically different between those who remained potential and those who progressed (become atrophic or developed symptoms), but the variation of anti-TG2 correlated to the final outcome. In fact, none of the negative anti-TG2 patients developed full-blown disease, whereas among those who developed severe damage, 78.8% had persistently positive anti-TG2 compared with 43% of those who did not develop the intestinal damage. Up to date, the detection of intestinal deposits of immunoglobulin A (IgA) anti-TG2 by immunofluorescence was reported to be the best marker to identify, among patients with potential CD, those who will eventually develop a gluten-dependent enteropathy [8-10]. Moreover, to further explore which variable or risk factor is likely to differentiate the patients who progress to flat mucosa from those who do not, a multivariate analysis was performed: male sex, slight mucosal inflammation at time 0 (estimated by the numbers of CD25+ cells and $\gamma\delta$ + lymphocytes) and a certain genetic profile (genes HLA-DQ2 and polymorphism of *IL12A*, *OLIG3*, and *IL2/IL21*) may well start to delineate a cohort of individuals who are more prone to develop the disease.

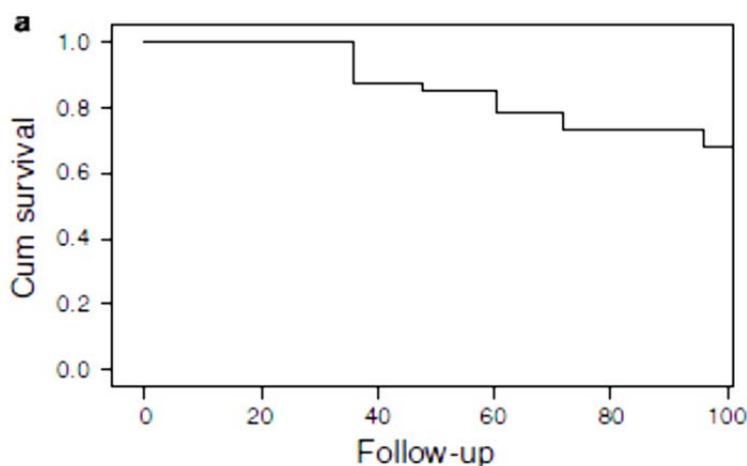


Figure 1. Cumulative survival function in the cohort study. Percentage of cases who remained potential during follow-up [9]

Conclusion

Potential coeliac disease is a condition more and more frequently diagnosed because of the screening of the general population, however the clinical management of the disease remains debated. The presence of symptoms should always induce to prescribe a gluten-free diet. On the contrary, prescribing a gluten-free diet to all asymptomatic patients could be an overtreatment, as we demonstrated that only 33% of the patients will develop mucosal atrophy within 9 years. Unfortunately, there are still no biomarkers that can allow to differentiate with confidence patients that will develop an intestinal damage from those who will not. However, preliminary studies suggest that male sex, the number of CD25+ and $\gamma\delta$ + lymphocytes in the intestinal mucosa at the time of diagnosis, as well as HLA-DQ dose and some non-HLA polymorphisms may help to identify a cohort of individuals more prone to develop overt disease.

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5.5 Association between IL-33/ST2 axis and active coeliac disease

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Introduction

IL-33, a member of the IL-1 family, is mainly expressed by epithelial, endothelial and mesenchymal cells [1]. In resting cells, it is located principally in the nucleus, where it can regulate the expression of certain genes by its interaction with different transcriptional factors. It has been proven that this protein can be released from the nucleus into the cytoplasm and then to the extracellular space by different kinds of stimuli inducing a strong inflammatory response through its receptor, ST2. The IL-33 receptor is a heterodimeric complex made up of two different proteins ST2L, which actually binds IL-33 and an accessory protein known as IL-1RAc. This receptor is expressed by different cells: lymphocytes, mast cells, NK cells, ILC2, and endothelial, epithelial and mesenchymal cells. The ST2L protein has a splice variant, named as soluble ST2 (sST2) which can be released into the extracellular space where it works as decoy factor for IL-33 [2-3].

Since the discovery of IL-33 many different functions have been linked to this cytokine. Firstly, it was recognised for its capacity to promote Th2 responses [4]. More recently, it has been shown that IL-33 may also promote Th1 functions [5]. It is clear that IL-33 acts not only as a cytokine, but also as alarm signal, stimulating many pro-inflammatory responses [6]. Particularly, necrotic cells, but not apoptotic ones, release an active form of IL-33 with potent biological effects [2]. Based on the functional properties of this molecule, we aimed to investigate whether IL-33 may play a role in coeliac disease (CD) pathogenesis. It has been recently observed that CD patients present increased levels of IL-33 [2]. In the present work, we evaluate the expression of IL-33 and its receptor in human intestinal mucosa. These are the initial studies in order to link the biology of IL-33 and the mechanisms of CD pathogenesis.

Materials and methods

Blood samples from a total of 40 untreated CD patients and 39 healthy controls were evaluated in this study. Duodenal biopsies from 9 untreated CD patients and 9 healthy controls were used to perform immunofluorescence assays. All the samples were taken during the routine protocol for CD diagnosis in the Gastroenterology Units of Hospital Sor María Ludovica (paediatric patients) and Hospital San Martín (adult patients). The study was approved by the ethics committee of both institutions.

For immunofluorescence analysis, 4 µm sections of duodenal paraffin-embedded tissues were used. Antigen retrieval was performed by heat treatment in citrate buffer, and stained with commercial antibodies from R&D systems (AF3625 for IL-33 and AF523 for ST2). Nuclei were stained with DAPI.

Images were obtained using a SP5 Leica confocal microscope. The cellular density was calculated as the number of positive cells for each marker in a predefined zone.

Levels of ST2 and IL-33 in serum samples were determined by commercial ELISA kits (R&D System, cat. DY523 for ST2, and ab119547 for IL-33).

Comparison of the expression levels for IL-33 and ST2 in serum and positive cell numbers in *lamina propria* between control subjects and active CD patients was performed using unpaired T-tests with 95% of confidence.

Results and discussion

First, we determined the concentration of circulating IL-33 and the soluble form of ST2 (sST2) in untreated CD patients and healthy controls by commercial ELISA. Increased levels of sST2 in sera of untreated CD patients were observed. The mean

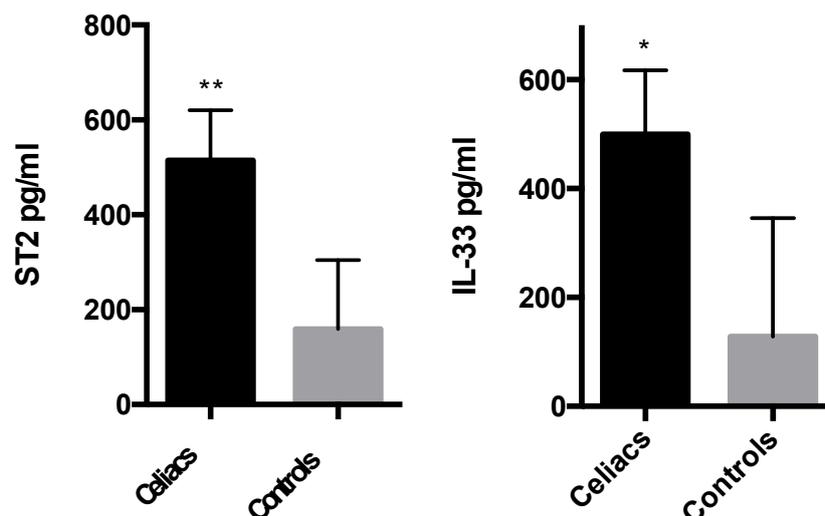


Figure 1. Concentration of IL-33 and its decoy receptor (sST2) in serum samples of untreated CD patients ($n=37$ for sST2 and 21 for IL-33) and healthy controls ($n=39$ for sST2 and 9 for IL-33). Plots show the mean value plus SEM bars of the concentration of IL-33 and sST2 (pg/ml). (IL-33, $*p<0.01$, sST2: $**p<0.005$)

value of sST2 was 264,1 pg/ml for untreated CD patients while 96.5 pg/ml was observed for healthy controls (Fig. 1). Untreated CD patients presented also increased levels of IL-33. The mean value for CD patients was 277.1 pg/ml and 15.13 pg/ml for healthy controls.

Next, we aimed to evaluate the expression of IL-33 and ST2 in the intestinal mucosa. To this end, we evaluated sections of duodenal biopsies from untreated CD patients and healthy controls by immunofluorescence. The immunofluorescence images showed a higher number of IL-33 and ST2 expressing cells in the *lamina propria* of active CD patients than in controls. The epithelium showed a faint staining. However, in some CD patients some cells from the epithelial compartment showed a strong staining for both proteins.

The ST2⁺ cells were located close to the epithelium. IL-33⁺ cells did not appear randomly distributed. On the contrary, they seem to be organised or associated with some structures resembling blood vessels.

In order to quantify the number of IL-33⁺ or ST2⁺ cells located in the *lamina propria*, we performed manual counting of each group of cells. Cells were counted per μm^2 of *lamina propria* to obtain the density of positive cells. Higher numbers of IL-33⁺ and ST2⁺ cells were observed in duodenal *lamina propria* from untreated CD patients (Fig. 2).

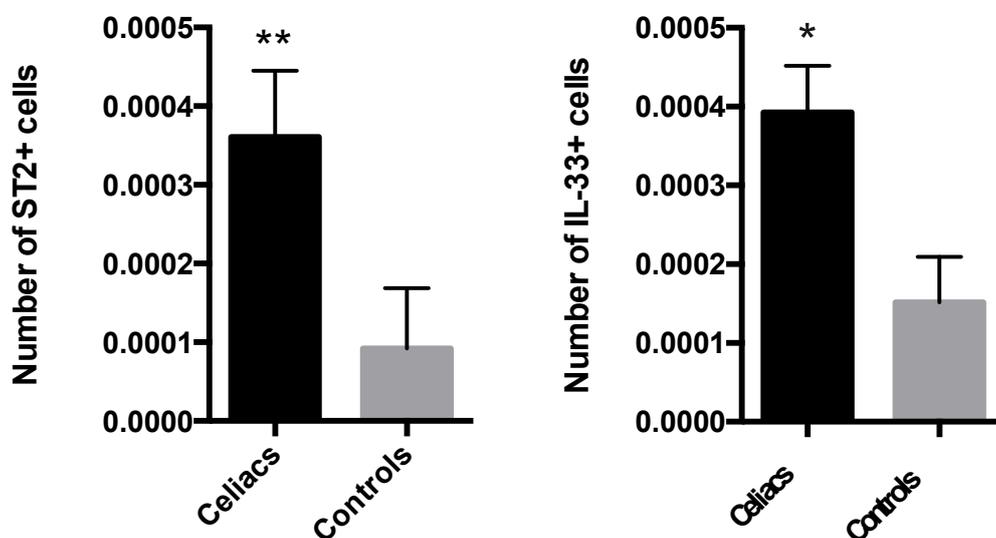


Figure 2. Increased numbers of IL-33⁺ cells and ST2⁺ cells in duodenal lamina propria from untreated CD patients. Counting of IL-33⁺ cells and ST2⁺ cells in immunofluorescence images of predefined areas of duodenal lamina propria of untreated CD patients (n=9) and healthy controls (n=9). Plots show the mean value plus SEM bars of the number of IL-33⁺ cells and ST2⁺ cells per μm^2 of lamina propria, (ST2 ** $p < 0.05$, IL-33 * $p < 0.1$)

Since CD is a very well-known Th1 driven enteropathy [8], the high expression of IL-33 in duodenal mucosa and in peripheral blood of untreated CD patients is intriguing.

Recent works have demonstrated the broad spectrum of effects of IL-33 in different pathologies. Yang et al. proved that only cytotoxic T cells in a context Th1 or Th17 express ST2 [9]. These authors also confirmed that IL-33 synergises with IL-12 to induce effector cells. In addition, other studies confirmed that IL-33 and ST2 expression was necessary to mount a cytotoxic response against some virus infections and tumours, highlighting the role of the IL-33/ST2 axis in the induction of potent cytotoxic T cells [10-11]. Bourgeois et al. showed that IL-33 directly interacts with iNKT and NK cells to induce IFN- γ production [12]. On the other hand, IL-33 enhanced Th1/Th17 responses in some mouse models [13-14]. Therefore, we hypothesise that the IL-33/ST2 axis may have a role in CD pathogenesis, probably expanding the inflammatory process, and promoting a Th1 and cytotoxic response.

Further work is in progress to investigate the signal involved in the up-regulation of this factor and also the consequences of its systemic release.

Conclusion

In this study, we found higher levels of IL-33 and sST2 in serum of untreated CD patients compared with healthy controls, together with an increase in the number of IL-33⁺ and ST2⁺ cells in duodenal *lamina propria* of untreated CD patients.

Since IL-33 is mainly located in the nucleus of different cells, upregulation of IL-33 expression points to its role as alarm signal.

Though the test used to evaluate serum IL-33 levels does not discriminate active and inactive form, it is likely that part of the circulating IL-33, released from the intestinal mucosa, may reach distant tissues initiating a tissue damage process. IL-33 together with other cytokines, such as IFN- γ and IL-15 may also be part of the inflammatory mediators which link CD to other inflammatory/autoimmune diseases such as type I diabetes and rheumatoid arthritis.

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5.6 Abrogation of coeliac immunogenicity of gluten peptides by amino acid point substitutions

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Introduction

The only generally accepted treatment of coeliac disease (CD) is a life-long gluten-free diet. Wheat gluten proteins include gliadin, low- (LMWG) and high-molecular-weight glutenins (HMWG), all three of which have shown to be CD-toxic [1,2,3]. A gluten-free diet significantly reduces the quality of life for affected individuals such that new approaches are sought. We have identified naturally existing variants of gliadins and glutenins that might be less immunotoxic in CD [4].

Aims

We sought to test selected variants of α -gliadin peptides in CD T-cell proliferation assays with gluten-sensitive T-cell lines that had been generated from duodenal biopsies from individuals with CD. This would allow us to evaluate their CD-toxic immunogenicity. We sought to identify peptides with lower CD-toxic immunogenicity as a prelude to providing the basis of new dietary strategies as part of a gluten-free diet for individuals with CD.

Materials and methods

We generated gluten-specific polyclonal T-cell lines from duodenal biopsies taken from individuals with CD (n=11) as previously described [5] although not all the T-cell lines were used in every study. The candidate peptides, as shown in Fig. 1, were tested in proliferation assays using radioactive labelled thymidine to measure T-cell proliferation. A stimulation index >2 was considered positive. We tested five α -gliadin peptides synthesised as 16mers. The results of proliferation T-cell assays with medium alone, PT digested gluten, peptides 1, 3, 5, 6, and 9 are presented in Tab. 1. The set of tested peptides harbored the overlapping T-cell epitopes DQ2.5-glia- α -1a and DQ2.5-glia- α -2, and naturally occurring variants that differed in a few amino acids (shown in Fig. 1).

Sample	Medium only	Gluten	Peptide 1	Peptide 3	Peptide 5	Peptide 6	Peptide 9
PATIENT A	1.00 (435 ± 55.8)	8.88 (3868 ± 259.5)	2.36 (1028 ± 141.9)	1.90 (826 ± 67.9)	1.85 (808 ± 16.3)	1.44 (627 ± 87.0)	1.67 (729 ± 181.7)
PATIENT B	1.00 (1594 ± 176.8)	6.17 (9836 ± 1573.1)	2.82 (4492 ± 338.0)	14.76 (23533 ± 144.6)	2.61 (4168 ± 117.6)	0.83 (1326 ± 263.7)	1.04 (1663 ± 258.1)
PATIENT C	1.00 (133 ± 19.9)	3.63 (484 ± 63.3)		1.96 (261 ± 54.6)			1.29 (172 ± 30.8)
PATIENT D	1.00 (235 ± 23.7)	2.41 (566 ± 96.1)	1.63 (383 ± 65.0)	1.55 (364 ± 49.5)	1.24 (292 ± 29.0)		
PATIENT E	1.00 (80 ± 16.1)	4.17 (331 ± 20.7)	3.45 (274 ± 36.7)	6.58 (523 ± 154.1)	8.54 (679 ± 36.6)	1.16 (92 ± 22.6)	
PATIENT F	1.00 (629 ± 0)	7.41 (4660 ± 191.6)			1.63 (1026 ± 78.5)		
PATIENT G	1.00 (535 ± 66.8)	5.58 (2989 ± 285.8)		5.26 (2816 ± 65.8)		0.8 (431 ± 58.0)	1.16 (623 ± 84.5)
PATIENT H	1.00 (283 ± 23.5)	53.94 (15248 ± 1536.0)		0.70 (196 ± 30.1)		0.68 (193 ± 34.8)	
PATIENT I	1.00 (367 ± 45.2)	3.65 (1340 ± 194.9)	1.33 (487 ± 31.2)	2.57 (944 ± 66.5)	0.91 (335 ± 21.7)	0.9 (330 ± 61.7)	0.82 (301 ± 37)
PATIENT J	1.00 (52 ± 0)	13.00 (676 ± 121.2)		1.99 (104 ± 14.8)		1.23 (64 ± 5.2)	
PATIENT K	1.00 (99 ± 8.5)	3.47 (344 ± 63.7)		1.53 (152 ± 3.5)		1.34 (133 ± 14.8)	

Table 1: Results of 11 proliferation assays: 11 gluten-sensitive T-cell lines were tested with medium only, peptic tryptic digested gluten and peptides 1, 3, 5, 6 and 9. Stimulation indices for individual antigens are marked in bold and corresponding arithmetic mean ± standard deviation in brackets. Note that SI greater than 2 were considered as positive.

Results and discussion

Approximately half of gluten-specific cell lines (5:10) recognise immune-dominant peptide 1 (QLQFPQPQLPYQPQ) or its deamidated counterpart (QLQFPQPELPYQPQ, peptide 3). Stimulation indices vary from 0.7-14.76. That confirms different sensitivity of T cells obtained from different patients to particular epitopes. Notably, indices increase when peptide 1 is deamidated (Fig. 2).

Peptide 3's point substituted variant (QLQFPQPELSYPQPE, Peptide 5) triggered positive T-cell responses in 2:6 CD gluten-sensitive T-cell lines, the results of which are shown in Fig. 3. Peptides with two substitutions (QLQPFQPKLSYPQPE, Peptide 6) or three amino acid substitutions (QLQFPKPKPKLPYPKPKPQ, Peptide 9) did not stimulate any tested gluten-sensitive T-cell lines (n=8 and n=5, respectively). The above results indicate the importance of both deamidated glutamic acid (at position 65, Fig. 1) and proline (at position 67, Fig. 1) in triggering coeliac-specific reactions.

Conclusion

We have shown that introduction of two selected amino acid substitutions in α -gliadin peptides abrogates responses of CD gluten-sensitive T cells to these peptides. We suggest that these peptides will need additional assessment using CD small intestinal biopsy organ culture and in vivo testing to confirm their lack of CD toxicity.

Peptide	Sequence																Substitutions
	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	
Peptide 1	Q	L	Q	P	F	P	Q	P	Q	L	P	Y	P	Q	P	Q	
Peptide 3	Q	L	Q	P	F	P	Q	P	E	L	P	Y	P	Q	P	E	deamidated
Peptide 5	Q	L	Q	P	F	P	Q	P	E	L	S	Y	P	Q	P	E	S67
Peptide 6	Q	L	Q	P	F	P	Q	P	K	L	S	Y	P	Q	P	E	K65, S67
Peptide 9	Q	L	Q	P	F	P	K	P	K	L	P	Y	P	K	P	E	K63, K65, K70
<i>Epitope DQ2.5-glia-α1a positions</i>				<i>p1</i>	<i>p2</i>	<i>p3</i>	<i>p4</i>	<i>p5</i>	<i>p6</i>	<i>p7</i>	<i>p8</i>	<i>p9</i>					
<i>Epitope DQ2.5-glia-α2 positions</i>					<i>p1</i>	<i>p2</i>	<i>p3</i>	<i>p4</i>	<i>p5</i>	<i>p6</i>	<i>p7</i>	<i>p8</i>	<i>p9</i>				

Figure 1. Amino acid sequences for α -gliadin peptides and substituted variants used in the study. Letters in bold indicate amino acid substitutions within the immunodominant p57-72 α -gliadin peptide. The bottom part of the table shows epitopes within this peptide and the position of their binding into the HLA-DQ2 groove (p1-9)

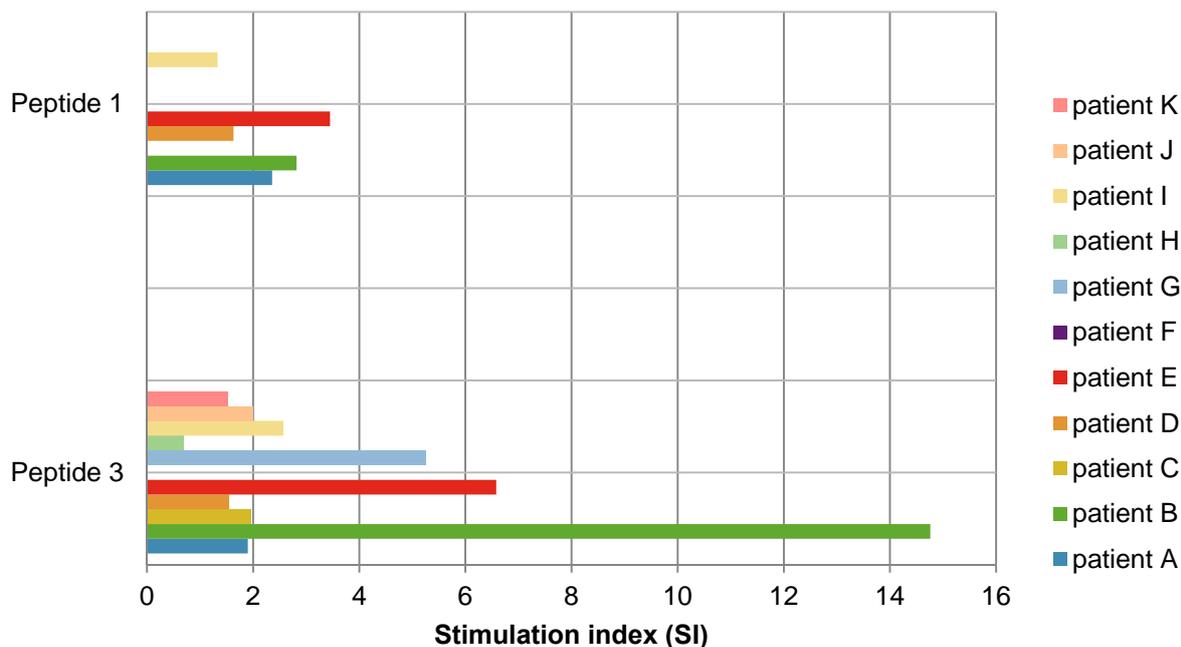


Figure 2. Proliferative response of T-cell lines to immunodominant peptide 1 (epitope DQ2.5-glia- α 1a positions and DQ2.5-glia- α 2) and to deamidated counterpart

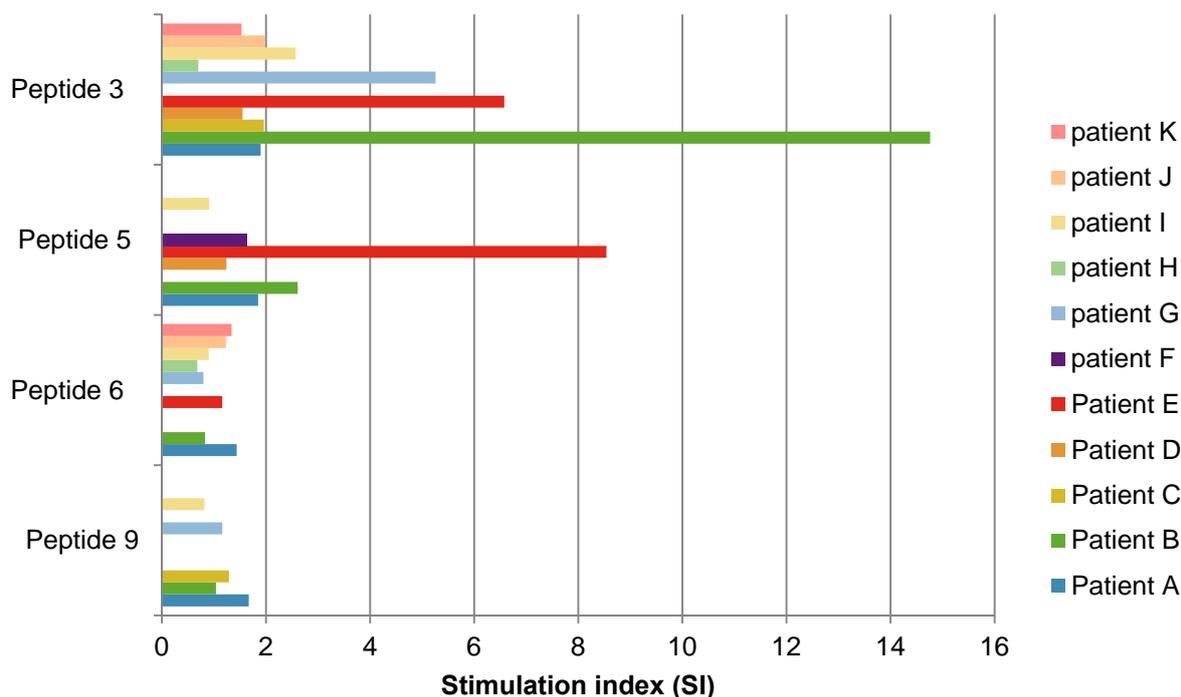


Figure 3. Proliferative response of T-cell lines to different α -gliadin peptides; peptide 3, peptide 5, peptide 6 and peptide 9. With every further peptide, one amino acid substitution is introduced. With 2 amino acid substitutions, T-cell response is completely abrogated

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5.7 Estimation of (sero) prevalence of coeliac disease in children and adolescents in the LIFE Child study cohort

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Introduction

The prevalence of coeliac disease (CD) in population-representative cohorts is reported between 0.18 and 2.38% [1] depending on the size and mean age of the screened population as well as on the applied serological tests.

Currently, four studies are available estimating the frequency of CD in Germany [1-4] which screened in different cohorts (paediatric and/or adult cohorts) and applied varying screening strategies. The prevalence of biopsy-proven CD was found between 0.18 and 0.37% [1,2]. This seems to be underestimated considering the frequency of CD in other European countries [5]. Contrary, seroprevalences in randomly selected cohorts were found between 0.8 and 1.35% [3,4]. This suggests a higher frequency of CD in Germany but might be due to a certain low degree of unspecificity of the applied antibody tests. Otherwise, a recent screening [6] demonstrated that 98% of the participants with IgA anti-tissue transglutaminase (IgA-aTTG) values ≥ 10 times of upper limit (ULN) exhibit mucosal impairment compatible with CD (\geq Marsh 3A).

The primary objective of our ongoing study was to estimate (sero)prevalences in a paediatric randomly selected German cohort by applying additional confirmatory antibody tests and HLA genotyping. Furthermore, we asked the question if antibody test results alone can predict the actual prevalence of CD in a paediatric cohort.

Materials and methods

Study population

The Leipzig Research Centre for Civilization Diseases (LIFE) Child study has been designed to understand how and through which mechanisms and mediators genetic,

metabolic and environmental factors influence health and development in children and adolescents [7]. LIFE Child is a prospective, longitudinal population-based cohort study of urban children from fetal life until adulthood. The study focuses on monitoring of normal growth, development and health non-communicable diseases such as childhood obesity, atopy and mental health problems. Families were randomly selected and invited by the residents' registration office. Between 2011 and 2015, 3080 children and adolescents were included, of whom 2363 participants were enrolled for our CD screening due to the inclusion criteria (age between 1 and 18 years, at least two serum aliquots of the first visit with blood withdrawal available).

Study procedure

The study procedure is shown in Fig. 1. We performed a four-step CD screening. The first step comprised determination of IgA-aTTG and IgG-antibodies against deamidated gliadins (IgG-aDGL) in sera (T0) using tests of EUROIMMUN AG (Lübeck, Germany). Afterwards, samples with positive results for IgA-aTTG (including also samples with IgA-aTTG values between 0.5 and 1 x ULN) and for

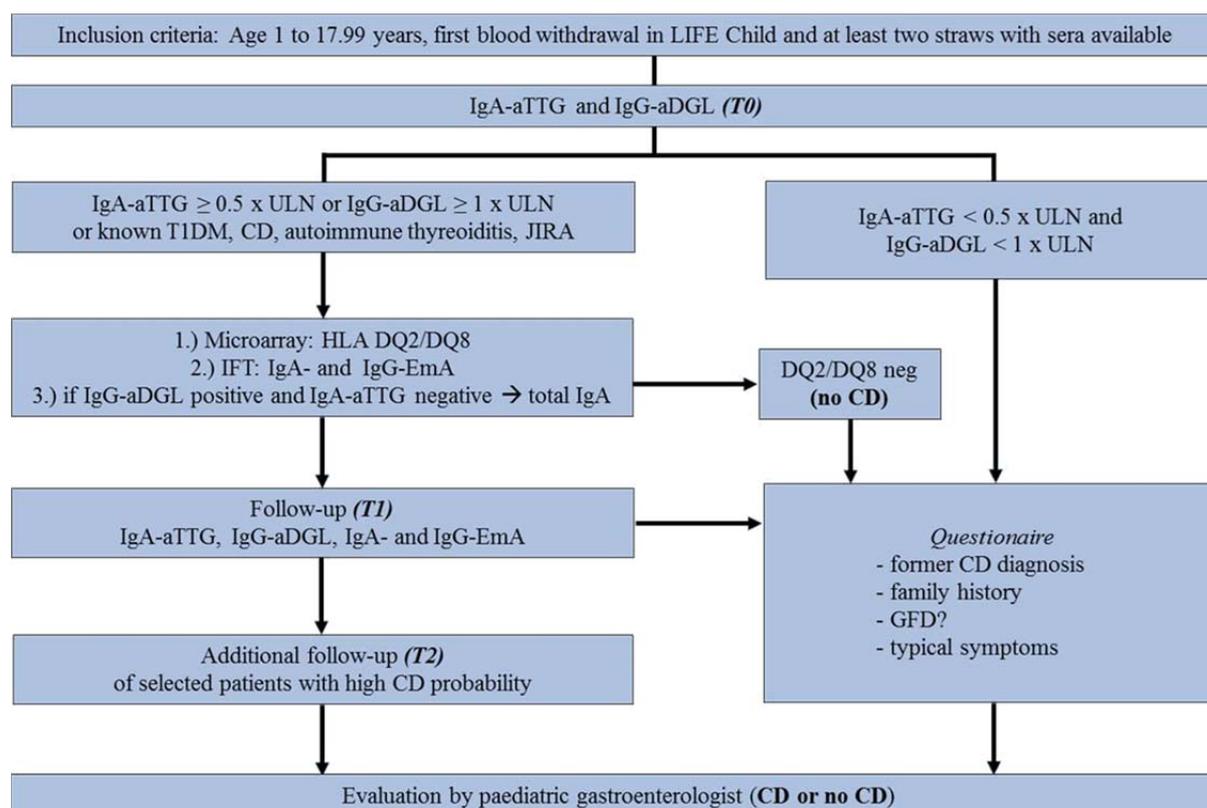


Figure 1. Study procedure. CD coeliac disease, EmA endomysium antibodies, GFD gluten-free diet, IgA-aTTG IgA anti-tissue transglutaminase, IgG-aDGL IgG-antibodies against deamidated gliadins, JIRA juvenile idiopathic rheumatoid arthritis, T1 follow-up in LIFE Child, T1DM diabetes mellitus type 1, T2 personal interview, ULN upper limit of normal

IgG-aDGL or samples of patients with CD-associated disorders as well as known CD were tested for endomysium antibodies (EmA) and HLA-DQ2 (HLA-DQ2.5 and/or DQ2.2) and -DQ8 using tests of EUROIMMUN AG (Lübeck, Germany). In sera only positive for IgG-aDGL, total IgA was nephelometrically measured (Roche, Mannheim, Germany). IgA values of ≤ 0.05 g/l were considered as sign of selective IgA deficiency (sIgAD).

In a third step, available follow-up sera (T1) of patients with conspicuous results for IgA-aTTG and/or IgG-aDGL (HLA-DQ2 and/or DQ8 positive or HLA status not known) were analysed for IgA-aTTG, IgG-aDGL and EmA. Finally, we invited participants (T2) who showed positivity for either IgA-aTTG and/or IgG-aDGL at T0 and T1. Cases with negative HLA results were not pursued.

The final step included a personal interview which comprises questions concerning gastrointestinal complaints and further CD-relevant symptoms, associated diseases, family history and gluten uptake as well as the decision of the participant for further clarification. The interview was performed by an experienced paediatric gastroenterologist. The latter is still ongoing.

Interim analysis

To compare our data with other screening studies, the following groups were considered:

- Known CD (group 1): known CD with HLA-DQ2/DQ8 positivity.
- Seropositivity (group 2): IgA-aTTG >1 x ULN **or** (IgG-aDGL >1 x ULN and sIgAD) + group 1.
- Potential CD (group 3): (IgA-aTTG >1 x ULN and IgA-EmA-positive) **or** (IgG-aDGL >1 x ULN and sIgAD and HLA-positive) + group 1.
- Probable CD (group 4): IgA-aTTG ≥ 10 x ULN **and** IgA-EmA-positive + group 1.

Results and discussion

Characteristics of our screening cohort are shown in Table 1. Results regarding the first two steps of our screening procedure are depicted in Fig. 2. Of 2363 children and adolescents, 29 were only positive for IgA-aTTG (1.23%) and 23 only for IgG-aDGL (0.87%). The results for IgA-aTTG are comparable with a previous observation in an adult cohort [3]. Double positivity for the indicated antibodies was observed for 11 patients (0.47%, not shown). Of seven participants who noted that CD was previously diagnosed, one was positive for IgA-aTTG (probable under GFD) but only four (including the indicated patient) were HLA-DQ2 and/or DQ8 positive. We conclude that inclusion of probands indicating to have CD into the calculation of prevalence without HLA-typing [4] leads to an overestimation of frequency.

Table 1. Baseline demographics and clinical characteristics.

Probands included for analysis	2363
Gender (female in %)	49.0
Age in years (mean)	8.5
Probands with known CD (according to LIFE interview)	7
Cases of suspected CD	2
Children with diabetes mellitus type 1	6
Children with autoimmune thyroiditis	4
Children with juvenile idiopathic arthritis	4

Data were obtained by LIFE child questionnaire

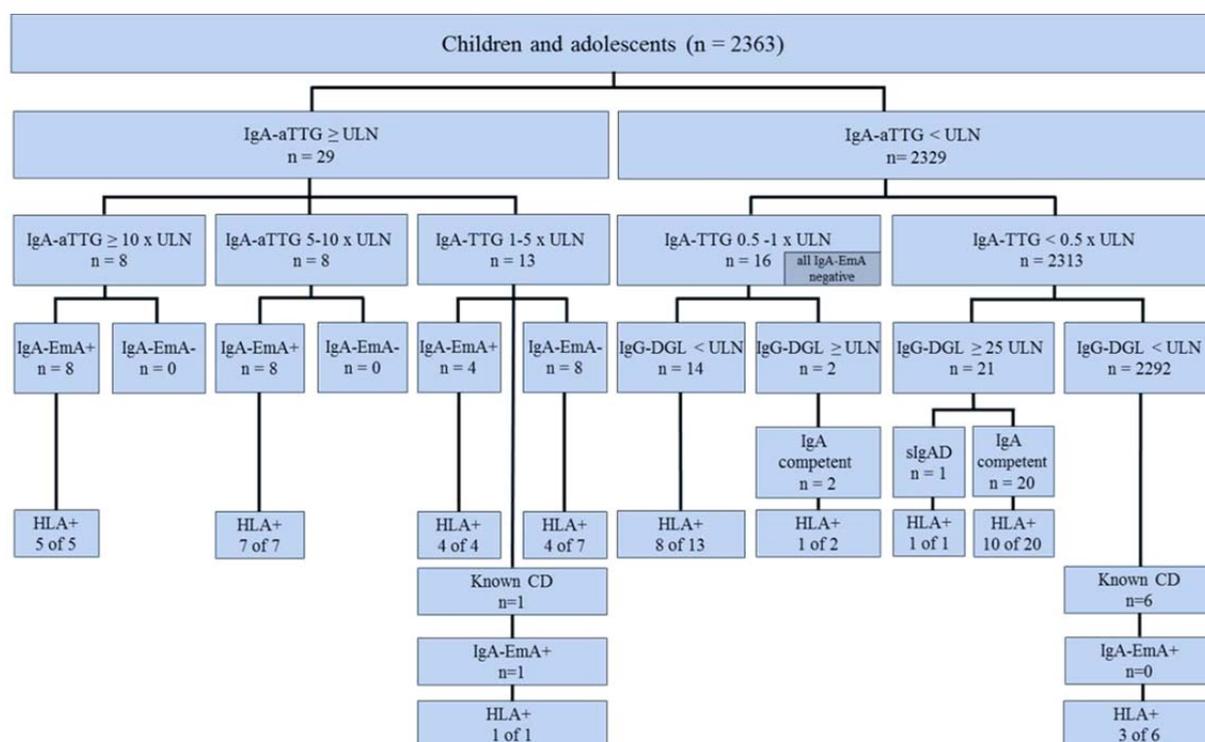


Figure 2. Screening results regarding IgA-aTTG, IgG-aDGL, IgA-EmA and HLA-type. HLA+ HLA-DQ2 and/or DQ8 positive, IgA-EmA+ IgA-EmA positive, slgAD selective IgA deficiency, ULN upper limit of normal

None of the participants with solely IgG-aDGL positivity was positive for IgA-EmA. Otherwise, all participants with IgA-aTTG ≥ 5 x ULN were also positive for the immunofluorescence test. In the group of probands with IgA-aTTG between 1 and 5 x ULN, there were only eight of 13 sera in which IgA-EmA were detected. Further, none of the sixteen patients (0.67%) with IgA-aTTG between 0.5 and 1 x ULN show IgA-EmA positivity. These findings contradict the observations of a large Swedish screening study in which 25% of the IgA-TTG negative probands (between 0.5 and 1 x

ULN) were IgA-EmA positive [8]. This is possibly due to the use of a lower cut-off for IgA-EmA (1:5) or, more likely, to a low sensitivity of the ELISA test.

Finally, positive antibody tests were verified by HLA-genotyping if DNA was available. All participants with IgA-EmA positivity were either HLA-DQ2 and/or DQ8 suggesting that HLA-typing is not necessary if patients show IgA-EmA positivity. Otherwise, probands with IgA-aTTG between 1 and 5 x ULN without IgA-EmA positivity were only positive for HLA-DQ2/8 in four of seven cases. Furthermore, patients positive for only IgG-aDGL have a false-positive rate of 50% indicating the necessity for HLA-typing in these cases. This supports the recommendation to genotype IgA-aTTG seronegative patients with CD-related signs [9] to evaluate the need of subsequent endoscopic evaluation. In case of HLA-DQ2/8 negativity CD seems to be very unlikely and other differential diagnoses have to be considered. We found one participant with sIgAD who was positive for IgG-aDGP carrying HLA-DQ2.

Summarising, we obtained the following frequencies:

Group 1 (known CD)	= 0.16% (CI _{95%} 0.04-0.41)
Group 2 (seropositivity)	= 1.39% (CI _{95%} 0.97-1.98)
Group 3 (potential CD)	= 1.06% (CI _{95%} 0.70-1.58)
Group 4 (probable CD)	= 0.51% (CI _{95%} 0.28-0.91)

In comparison to the most recent German prevalence study [4], the proportion of children being seropositive or having known CD (sum of groups 1 and 2) is higher in our screening study (1.39% vs. 0.9%). As expected, the number of probands in group 3 is lower than in group 2. This is mainly due to sera with IgA-aTTG <5 x ULN but IgA-EmA negative. Potential CD is actually defined as IgA-aTTG, IgA-EmA and HLA-DQ2/8 positivity without severe intestinal impairment (\leq Marsh 1). Due to the fact that all EmA-positives were also HLA-DQ2/DQ8 positive we put back HLA-typing for discussion. Omitting HLA-typing in case of IgA-EmA positivity in addition to IgA-aTTG >1 x ULN seems meaningful for the clinical application to reduce costs. This contradicts current diagnostic recommendations [10] for symptomatic patients with IgA-aTTG \geq 10 x ULN suggesting confirmatory assays of IgA-EmA and HLA. Children in group 4 seem have CD with high probability [6]. It was shown that IgA-aTTG levels \geq 10 x ULN predict mucosal damage \geq Marsh 3A in a population based-screening independently from the patient's symptomatic state. Contrary to our findings, the proportion of probands with IgA-aTTG \geq 10 x ULN was higher (0.48% vs. 0.33%). In addition, Laass *et al.* [4] found a frequency of 0.4% for this group of seropositive probands but concluded that all seropositives and those with known CD (without HLA confirmation) reflect the actual prevalence in Germany. Interestingly, the frequency of group 4 exceeds the estimated German prevalences for biopsy-proven CD [1,2].

We obtained 41 of 61 requested follow-up sera including those from children with associated diseases. We observed seroconversion from positive to negative for six participants (Fig. 3A). Interestingly, these sera had an initial IgA-aTTG value between 0.5 and 1 x ULN. One was also positive for IgG-aDGL. All except one were HLA-DQ2/DQ8 positive. Of these, only one was IgA-EmA positive. Concerning these results, there is evidence that there is a fraction of probands with suspected CD that should be followed-up if initial IgA-aTTG values are between 0.5 and 1xULN. In contrast, Fig. 3B represents IgA-aTTG values of participants which decreased from positive values to concentrations below the threshold at follow-up. One was a patient with known CD and the decrease can be attributed to a GFD. For the other two, we have currently no information on possible reasons (GFD or false positive at T0).

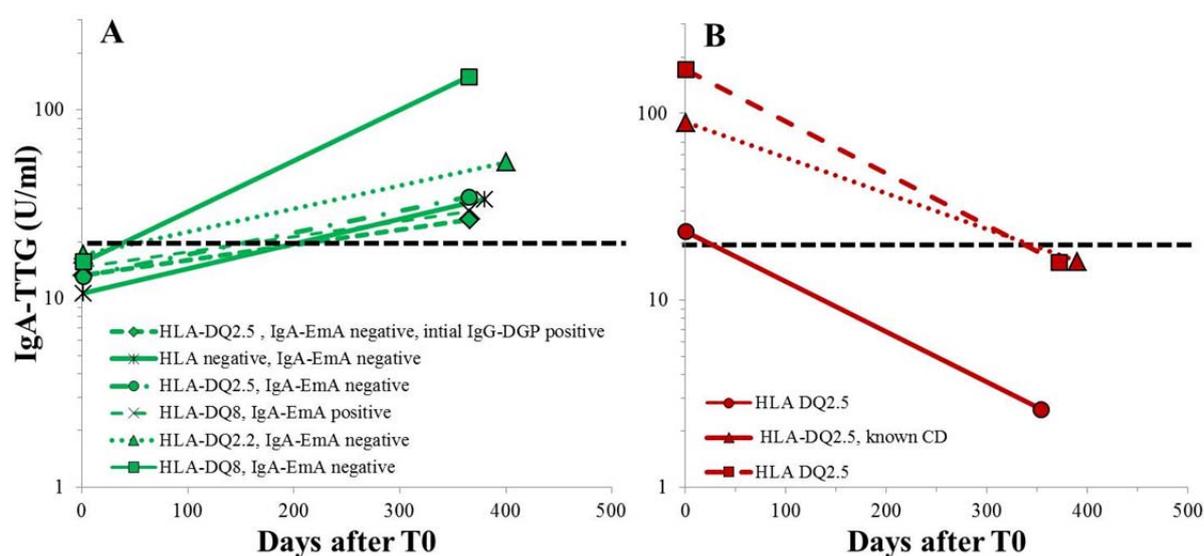


Figure 3. Seroconversions of IgA-aTTG during follow-up (T1). A) Seroconversion from negative to positive B) Seroconversion from positive to negative

Conclusion

The prevalence of CD in the LIFE Child study cohort ranges from 0.16 to 1.39% depending on the kind of calculation/estimations and exceeds previous observations. The percentage of patients with highly probable CD is 0.51%. Calculation of CD prevalence relying only on antibody values above manufacturer's cut-off without further confirmation by EmA or HLA testing results in overestimation. Cases of so-called "already known" CD have to be considered with caution and should be controlled by genotyping. Finally, children with IgA-aTTG values between 0.5 and 1 x ULN should be followed up. We are currently trying to clarify the cases of suspected CD in our screening. For that purpose, the participants are invited for an individual interview and, if necessary, we recommend an appointment with a paediatric gastroenterologist.

Acknowledgements

The study was partially funded by the research fund of the *Deutsche Zöliakiegesellschaft*. We also thank EUROIMMUN AG (Conny Daehnrich, Annika Jahnke, Kathrin Axel und Ulf Steller) for providing the ELISA kits and analysing EmA and HLA-DQ2/Q8.

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5.8 Results of the prospective multicentre trial of antibody diagnostics in coeliac disease (AbCD)

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Introduction

Diagnosis of coeliac disease (CD) relies on a combination of clinical, genetic, serological and duodenal morphological findings. The European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) published new guidelines in the year 2012 that permitted diagnosis of CD without biopsies in symptomatic patients with a concentration of IgA antibodies to tissue transglutaminase (aTTG) ≥ 10 times the upper limit of normal ($10 \times \text{ULN}$) upon confirmation with assay of IgA-endomysium antibodies (EMA) and HLA-typing, but noted that this needs to be evaluated prospectively [1].

A retrospective study proposed two diagnostic procedures based on (a) IgA-aTTG and total IgA (TTG/IgA-procedure) and (b) IgA-aTTG and IgG antibodies to deamidated gliadins (TTG/DGL-procedure). This study uses both one- and tenfold ULN, resulting in the classification "CD", "non-CD" or "biopsy required" [2].

A search in PubMed revealed that until now there was only one paediatric prospective study with 183 children in which the “10×ULN rule” did not lead to any false-positive findings [3]. The usefulness of another antibody species, IgG-antibodies against deamidated gliadin peptides (aDGL) is still under discussion.

The prospective multicentre AbCD study was performed to validate positive / negative predictive values (PPV / NPV) of these procedures.

Materials and methods

Thirteen centres from Germany, the United Kingdom and Austria enrolled paediatric patients scheduled for duodenal biopsy to confirm or rule out CD. Antibodies were measured blinded by a laboratory (EUROIMMUN, Dassow, Germany) and tissue sections underwent reference pathology. After follow-up, the paediatricians made a diagnosis largely following routine clinical procedures.

We defined two antibody procedures as follows:

TTG/IgA-procedure (sIgAD excluded):

CD if IgA-aTTG $\geq 10 \times \text{ULN}$;
no CD if IgA-aTTG $< 1 \times \text{ULN}$;
otherwise: biopsy necessary.

TTG/DGL-procedure:

CD if IgA-aTTG $\geq 10 \times \text{ULN}$ or IgG-aDGL $\geq 10 \times \text{ULN}$;
no CD if IgA-aTTG $< 1 \times \text{ULN}$ and IgG-aDGL $< 1 \times \text{ULN}$;
otherwise: biopsy necessary.

The trial was registered on the Internet Portal of the German Clinical Trials Register (https://drks-neu.uniklinik-freiburg.de/drks_web/setLocale_EN.do; DRKS00003854).

Results and discussion

For 898 of 949 participants, serum, biopsy, and follow-up data were available (592 CD, 345 non-CD, 24 no final diagnosis). A strength of our trial is, that we did not exclude the 24 patients in whom the diagnostic process did not lead to a final diagnosis, who are commonly neglected in diagnostic studies. These patients were included into our calculation of PPV and NPV as false-positive or false-negative cases.

The TTG/DGL-procedure, based on the assay of IgA-aTTG and IgG-aDGL can safely diagnose (IgA-aTTG or IgG-aDGL $\geq 10 \times \text{ULN}$) or exclude CD (IgA-aTTG and IgG-aDGL $< 1 \times \text{ULN}$) irrespective of the presence of symptoms in more than three quarters of children and adolescents (positive as well as negative predictive values $> 95\%$). The TTG/IgA-procedure, based on the assay of IgA-aTTG and of total IgA (IgA-aTTG $\geq 10 \times \text{ULN}$ or $< 1 \times \text{ULN}$ for IgA-competent patients) is almost identical in its ability to diagnose CD, but has a slightly narrower prevalence range over which it can safely

exclude CD. The lower limit of 95% confidence bound (LCB) is above 0.9 for each procedure.

Calculation of predictive values for the subset of our patients with a final diagnosis results in even higher predictive values.

Model based extrapolation shows that PPV and NPV remain above 0.95 with LCB > 0.9 even at a prevalence as low as 4%. Endomysium antibodies and HLA-typing did not improve PPV in samples with IgA-aTTG $\geq 10 \times$ ULN. Notably, the discrepancy rate between pathologists is 4.2% and comparable to the error rate in the serological procedures.

Conclusion

AbCD is the first prospective multi-centre trial providing evidence on the reliability of antibody tests including IgG-aDGL in diagnostics of CD based on a large number of children and adolescents. Our results have major personal and health care implications in clinical practice. They demonstrate that duodenal biopsy can be avoided in three quarters of paediatric patients with suspected CD, thereby reducing costs, endoscopy waiting times, patient risks, and delay of treatment. Furthermore, our study shows that other diagnostic tests such as HLA-typing or assay of EMA are not required in unequivocal cases.

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6 Enzymatic gluten degradation

6.1 Identification of novel and food-grade gluten-degrading enzymes

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Introduction

Gluten proteins have received much attention ever since it was discovered that they are the causative agents in coeliac disease (CD), a T-cell mediated inflammatory disorder of the small intestine [1-4]. Despite the discovery of the gluten-CD causal relationship in the 1950s [5], there is still no pharmacological therapy for CD, and individuals diagnosed must follow a strict life-long gluten-free diet. Several factors contribute to the toxicity of gluten proteins for CD patients. One critical feature of gluten proteins is that the domains comprising the immunogenic epitopes resist degradation by mammalian digestive enzymes, and thus are not neutralised during gastro-duodenal transport [6]. Luminal enzyme therapeutic approaches aim to abolish gluten with exogenous enzymes exhibiting cleavage specificities in immunogenic epitopes [7-11]. The immunogenic gluten epitopes all contain glutamine residues that can be selectively deamidated by tissue transglutaminase, and have consecutive proline (P) and glutamine (Q) residues in their sequences [12]. Our research on the peptidome of human saliva “accidentally” identified an enzyme that hydrolyses the XPQ sequence after Q in salivary proline-rich proteins [13]. This paper summarises the isolation of the gluten-degrading oral species from mixed culture biofilm, and the isolation of the enzyme. The discovery of a food-grade natural variant of this enzyme [14] offers novel and readily available therapeutic perspectives for patients suffering from gluten intolerance disorders, including CD.

Materials and methods

Dental plaque was collected according to a protocol approved by the Institutional Review Board for human subjects research at Boston University. The plaque was suspended in phosphate-buffered saline and plated on blood agar containing haemin and vitamin K. Individual colonies were then subcultured on agar plates containing wheat gluten as the sole protein source. Strains growing well on this agar were further subcultured, alternatingly on blood agar and gluten agar, to purity. Microbe-associated enzyme activities were evaluated in four enzyme assays. In the first, a series of

paranitroanilide-derivatised gluten-derived tripeptide enzyme substrates were employed. In the second assay, gliadin-degrading activity was investigated in-gel in a gliadin zymogram assay. Third, anti-gliadin activity in solution was studied by SDS-PAGE. Lastly, the enzymatic activity towards the immunogenic, protease-resistant 33-mer peptide was investigated by RP-HPLC. Strains of interest were identified by 16S rDNA analysis. The gluten-degrading enzyme from *R. mucilaginosa* was isolated from cells treated with lysozyme and a mild detergent, followed by cell sonication, ultracentrifugation, DEAE-chromatography, and gliadin zymography. Enzyme-active bands were excised, trypsinised, and subjected to LC-ESI-MS/MS for protein identification. Epitope abolishment by the purified *Rothia* enzyme as well as commercially available *Bacillus* enzymes was investigated using ELISA assays employing the R5 and G12 antibodies. The experimental details of the above experiments have been published [14-19].

Results and discussion

Mixed gliadins, incubated in a suspension of dental plaque bacteria, were degraded, and the half-life of 250 µg/ml gliadins in a suspension with an OD₆₂₀ of 1.2 was determined to be 6 h [17]. The isolation of the gluten-bacterial species among the over 1,000 different microbial species contained in dental plaque [20] was accomplished by a selective gluten agar approach [16,19]. Several strains grew well and were then evaluated in the enzyme assays employing gluten-derived substrates. The strains active in at least two of the four assays were speciated as *Rothia aeria*, *Rothia mucilaginosa*, *Actinomyces odontolyticus*, *Neisseria mucosa*, *Capnocytophaga sputigena*, and two *Streptococcus* species [15,19]. Among these, the *Rothia* bacteria were active in all four enzyme assays. Suspensions of *R. aeria*, or *R. mucilaginosa*, degraded mixed gliadins very rapidly, at a 25-fold increased rate compared to mixed dental plaque suspension. The tripeptide substrates hydrolysed most rapidly were Z-YPQ-pNA and Z-LPY-pNA.

The apparent molecular weights of the enzymes from *R. mucilaginosa* and *R. aeria* were between 70,000 - 80,000. The optimum pH for activity was in the neutral pH range, with *R. aeria* showing residual activity at pH values as low as 3.0 [19]. We confirmed earlier reports [6] that the immunogenic 33-mer gliadin peptide was entirely resistant to pepsin, trypsin and chymotrypsin (Fig. 1). The 33-mer, however, was completely degraded by *Rothia*-associated enzymes, within 2 h of incubation (Fig. 1). The sequenced degradation fragments showed evidence for preferential cleavage activity after XPQ↓ and XPY↓, consistent with the results obtained with the synthetic tripeptide substrates [18,19]. The enzyme was subsequently isolated from *R. mucilaginosa*, for which the full genomic sequence was available at the time. The enzyme was found to be cell-bound, and therefore was extracted from a cell sonicate [14]. The proteins in the extract were separated by DEAE anion-exchange chromatography. Proteins with enzymatic activity eluted early in the chromatogram and were thus mildly anionic in nature. The proteins contained in these fractions exhibited molecular weights of ≈125,000, ≈75,000 and ≈80,000. Enzyme activity was

associated with the 75,000 and 80,000 bands, but not with the 125,000 band, as revealed by zymography. The excised and in-gel trypsinised bands (75,000, 80,000 and 125,000) were analysed by mass spectrometry. This led to the identification of a mixture of the hypothetical proteins C6R5V9 and C6R5W1 in the inactive 125,000 band, C6R5V9 in the active 80,000 band, and C6R5W1 in the active 75,000 band.

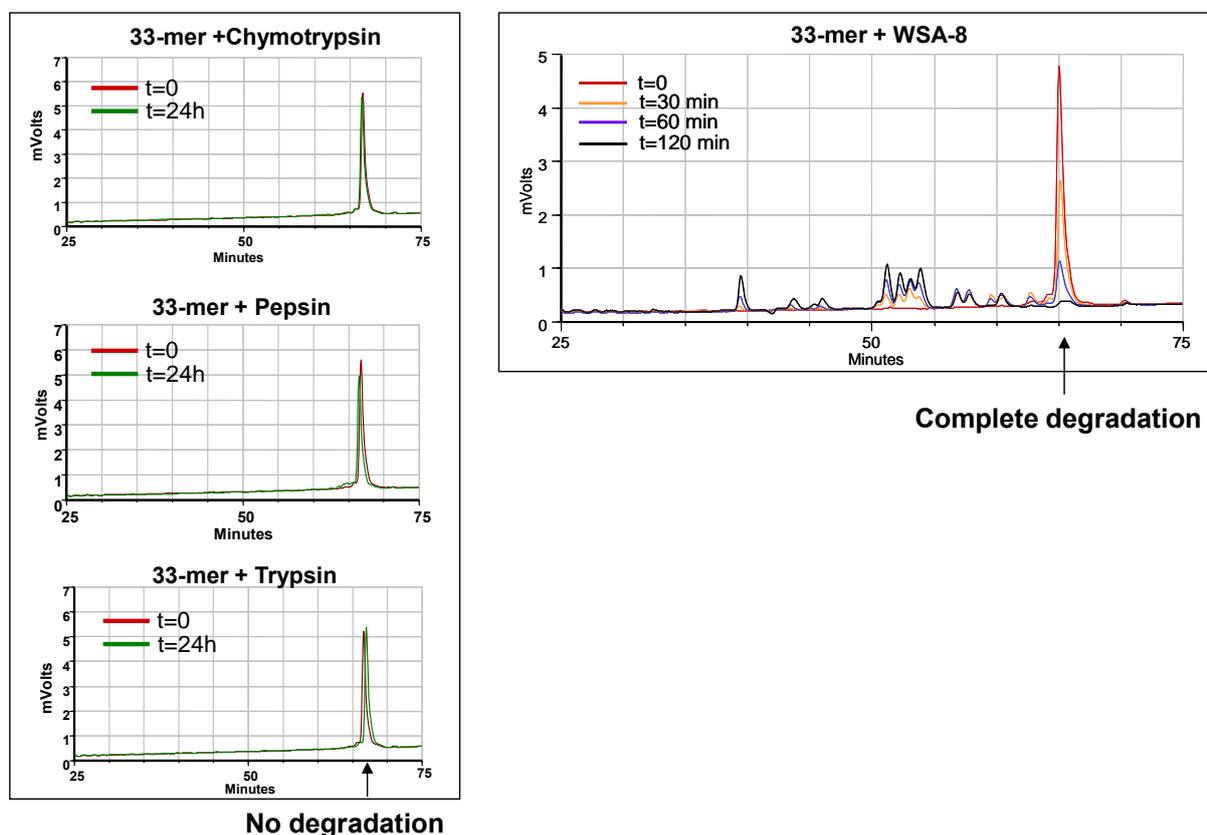


Figure 1. Degradation of the 33-mer gliadin peptide by mammalian enzymes (left panels) or oral microbial enzymes associated with oral strain WSA-8 (right panel). The 33-mer peptide was dissolved to 250 $\mu\text{g}/\text{mL}$ and incubated with 1 $\mu\text{g}/\text{mL}$ chymotrypsin, pepsin or trypsin (left panels) or in a suspension of oral microbial strain WSA-8 (*R. aeria*; $\text{OD}_{620}=1.2$). The incubation buffer composition mimicked the ion composition of human saliva (pH 7.0). Sample aliquots were removed at $t=0$ and $t=24$ h (left panels) or $t=0$, 3 min, 60 min and 120 min (right panel). Degradation of the 33-mer in incubation aliquots was monitored by RP-HPLC

From this result it was concluded that the full length C6R5V9 and C6R5W1 proteins in the 125,000 band are proteolytically processed to yield the active 80,000 and 75,000 enzymes, respectively. Structural analysis of the primary sequences revealed that both proteins are members of the subtilisin enzyme family [14]. The *Rothia* subtilisins are about 3-fold larger in size than the well-known and structurally resolved *Bacillus subtilis*-derived subtilisins, and show very little similarity in primary amino acid sequences. Nevertheless, like *Rothia*, the *Bacillus* subtilisins, specifically subtilisin A, and nattokinase, cleaved gliadins rapidly [14]. *Rothia* subtilisins eliminated both the

R5 and G12 epitopes, while the *Bacillus* subtilisins primarily abolished the R5 epitope (Fig. 2). This indicates some differences in substrate preference among the *Bacillus* and *Rothia* subtilisins that could be clinically significant. The theoretical cleavage specificity of subtilisins, XPX↓, matches the experimentally observed cleavage of the 33-mer at the XPQ↓ and XPY↓ positions. Importantly, these domains are contained in all known gliadin-derived immunogenic epitopes.

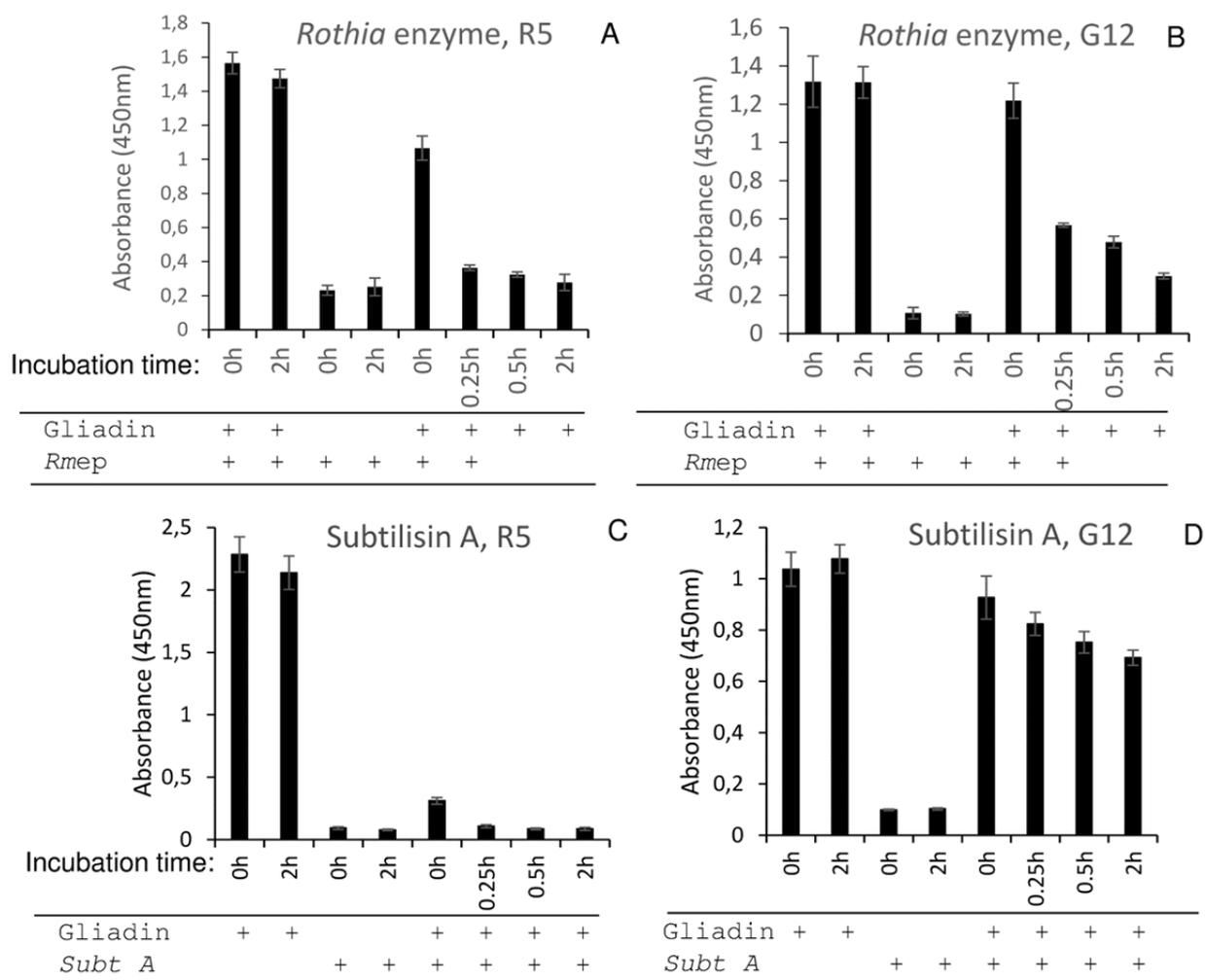


Figure 2. Gliadin epitope abolishment by subtilisins. Mixed gliadins were incubated with *R. mucilaginosa* enzyme preparation (*Rmep*) enriched in subtilisins. Aliquots were analysed for the survival of epitopes QQPFP in the R5 ELISA assay (A) and QPQLPY in the G12 ELISA assay (B). Results obtained with *B. subtilis* subtilisin A as the enzyme source are shown in C and D. Data adapted from Wei et al., 2016 [14]

Conclusion

The subtilisins isolated from non-pathogenic oral *Rothia* bacteria showed a remarkable activity towards mixed gliadins and capacity to neutralise the most immunogenic epitopes implicated in CD. What makes these enzymes of particular interest is the fact that certain members of the subtilisin family are already food-grade, e.g. nattokinase,

which demonstrated significant gluten-degrading activities as well. The latter enzyme is present in the *Bacillus*-containing food product called natto which is widely consumed in Japan. Coincidentally, the prevalence of CD in these regions is low. Our discoveries have opened new therapeutic avenues for the further development of the subtilisin enzymes as dietary supplements to help in the digestion of gluten *in vivo*.

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6.2 Studying kinetics of intestinal gluten degradation using peptide libraries

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Introduction

In Western countries about 1% of the population suffer from coeliac disease (CD) [1-3], a multifactorial chronic inflammatory intestinal disorder that is triggered by cereal storage proteins, the so-called gluten. Gluten consists of gluey, poorly water-soluble, proline-rich polypeptides that constitute about 10% of the mass of cereal flour and are important for the baking process and the taste, texture and appearance of the resulting product [4,5]. Although consumption of gluten is a necessary requirement for the development, manifestation and progression of CD, it alone is not sufficient to cause illness. Additional factors must coincide to boost disease [6]. On the genetic side, this applies mainly to the presence of major histocompatibility genotypes HLA DQ2 or DQ8, as almost all affected individuals express one of these haplotypes [7]. It was shown that peptidic fragments of the gluten fraction avidly bind in particular to these MHC-class II variants giving rise to proinflammatory T cells which in turn foster disease and its persistence [7]. Yet, with a prevalence of about 1% for CD and about 30-40% of HLA DQ2/8 present in the population [1-3] additional environmental or physiological elicitors must exist. Such a factor may be the gastrointestinal degradome and its capacity to cleave gluten peptides, in particular the ones that bind to HLA DQ2/8. If those peptides can be broken down completely before they reach their target antigen-presenting cells in the alimentary tract, gluten-specific T cells cannot be generated and disease cannot develop, even if an individual is HLA DQ2/8 positive.

As a comprehensive degradation profile of CD-active gluten peptides in oro-gastro-intestinal fluids does not exist we wanted to address this question using a recently developed peptide stability assay [8]. A major advantage of the assay in this context is its robustness regarding the proteases and peptides to be used and the respective readout it creates. The assay accepts crude protease mixtures such as gastrointestinal fluids, accommodates every conceivable amino acid sequence motif and yields true half-lives - as long as the stability of the substrate peptide is not orders of magnitude higher than that of the protease mixture itself. In our first round of experiments reported herein we used rat small intestinal juice as model protease mixture [9] and a battery of T-cell epitope-sized gluten peptides whose amino acid sequence was shown to “elicit coeliac disease or to activate MHC class II restricted T cells of subjects with coeliac disease” [10,11] as substrates. The data shall provide information on the feasibility of such a study on human beings and provide a first clue about the gluten

peptide and T-cell epitope half-lives to be expected after exposure to crude intestinal juices.

Materials and methods

A compilation of CD-active gluten sequence motifs was procured from the AllergenOnline database [11]. The selected sequences were synthesised by solid phase peptide synthesis, extracted from the solid support and subjected to proteolysis testing as described in detail by Gorris et al. [8]. Briefly, proline-functionalised cellulose sheets were equipped in a spatially resolved manner with Boc-Lys (Fmoc) to provide an acid-cleavable anchor onto which a Lys- ϵ -biotin - polyethylene glycol - D-diglutamate “detection-insulator-unit” was positioned. Thereafter, the respective peptides were synthesised onto these areas using traditional Fmoc solid phase peptide synthesis before, in a final step, another “insulator-capture-unit” composed of D-diglutamate - polyethylene glycol - aminoundecanoic acid - 2,4-dichlorophenoxyacetic acid was attached on each spot. The synthesis areas were punched out and the residing double-tagged peptides were deprotected and cleaved-off by acid treatment. The crude peptides can be used without further purification in the subsequent digestion assay.

Intestinal juice was harvested from female Wistar rats of 6 weeks of age in undiluted form using absorbent filter wicks [9], eluted by centrifugation and pooled. Animal experiments were approved by the Ministry of Energy, Agriculture, the Environment and Rural Areas of Schleswig-Holstein, Kiel, FRG [No. V312-7224.123-3]. The total proteolytic activity of the intestinal fluid was determined using a commercially available azocasein proteolysis assay (Sigma #A2765, Sigma-Aldrich, Steinheim, FRG; ref. [12]). For proteolysis testing of gluten peptides, the intestinal fluid was serially diluted with buffer mimicking the conditions of the small intestine in terms of pH and ionic strength in microplates. Peptide solutions were added and the mixtures were allowed to react for defined periods of time at 37 °C. The reactions were terminated by addition of protease inhibitors and heating and the respective amounts of remaining intact peptide were determined in a microplate assay using anti-2,4-dichlorophenoxyacetic acid antibodies as capture and streptavidin-peroxidase as detection reagents. Due to the minute amounts of peptide-substrate used (≤ 0.75 pMol per digestion reaction) the kinetics follow pseudo-first-order and the results can be reported as peptide half-lives in undiluted intestinal juices. Data analysis was performed with the Prism v5.0f software package, GraphPad, Ja Jolla, CA, USA.

Results and discussion

A total of 458 CD-active 8-15mer peptides derived from wheat, rye, barley, and oat proteins were tested for their cleavability by rat small intestinal fluid under physiological conditions. As readouts half-lives in rat small intestinal juice at 37 °C were recorded for each peptide. Since reporting all individual readouts tabularly would be of little value to convey our findings we refrain from listing those data in this

report. They will be made available to interested researchers from the authors upon request¹.

Under our experimental conditions, the half-lives of the CD-active cereal protein sequence motifs range from 2 ms to 20 min with a mean value of approximately 8 s (Fig. 1). Although these values seem to indicate rapid digestion, this is actually not the case when juxtaposed with fragments of other typical dietary proteins. Comparison of our data with the half-lives of chicken egg ovalbumin peptides in murine small intestinal juice, which we have determined in a previous work [8], highlights this point. While we could establish that the total proteolytic activities of the mouse and the rat small intestinal juices were comparable, the half-lives of 16mer ovalbumin-derived peptides in murine small intestinal juice were considerably lower than those of the gluten peptides in rat intestinal juice, ranging from 800 μ s to 6.4 s with a mean value of 152 ms. Even shorter, less proteolysis-prone 10mer ovalbumin peptides display a mean half-life of only 1.3 s (range 800 μ s to 40 s) (Fig. 1). In a second round of analysis we focused on CD-relevant gluten epitopes of which the specific T-cell reactivity has been defined in detail, namely those CD-active peptides which contain one of the 9mer core, DQ2- or DQ8-restricted T-cell epitopes compiled by Sollid and colleagues [13]. A total of 112 peptides fulfilled this criterion. Here the mean half-life was slightly lower (5 s) than that of the total pool tested but the overall distribution profile was very similar (Fig. 1).

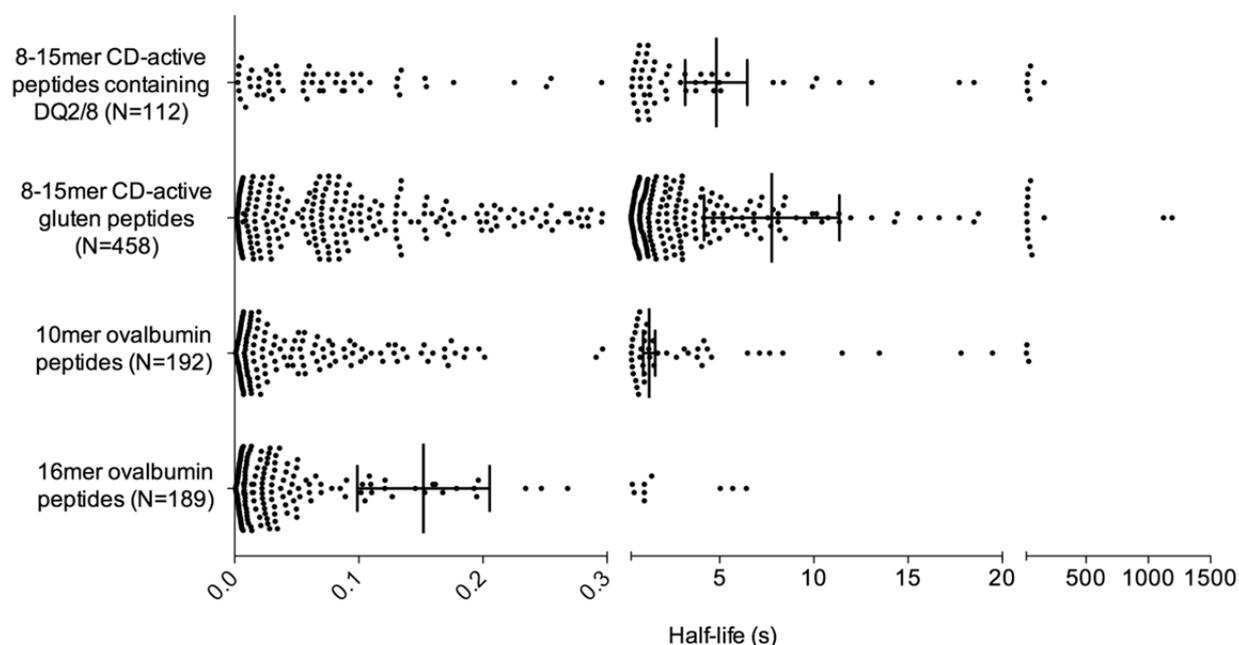


Figure 1. Comparison of the stability of CD-active gluten peptides and peptides derived from chicken egg ovalbumin in rodent small intestinal juice. Individual half-lives of all peptides analysed are depicted. Vertical bars indicate mean \pm SEM

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Thus, the CD-active gluten peptides analysed are significantly ($p < 0.05$, Kruskal-Wallis test with Dunn's Multiple Comparison post hoc test) more stable than those of ovalbumin when tested in the rodent system. Still, when one considers the typical gastrointestinal transit times for rodents of about 90 min to 2 h [14-16] only the most stable gluten peptides should be able to pass the intestines to a considerable extent without being broken down, and therefore may access every cell type of the mucosal lining. The majority of potential MHC class II-restricted gluten T-cell epitopes will not survive transit. This might be an explanation for the fact that rodents do not develop CD-like enteropathy, even if expressing HLA-DQ2 and gluten-specific T-cell receptors [17], although additional factors promoting disease in the human system may also be missing in those models.

Conclusion

The results presented above confirm earlier findings on the high stability of cereal storage proteins and show that even the regular small intestinal degradome of rodents is not ideally suited to cope with cereal storage proteins and to completely clear gluten-borne T-cell epitopes. It will be of great interest to port this assay to the human system and investigate whether in humans, differences in the proteolytic stability of CD-active peptides in the intestine might correlate with a disposition for CD.

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

7.1 News from Codex and regulatory affairs

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Introduction

AOECS, the Association Of European Coeliac Societies, has Observer status in the Codex Alimentarius Commission since 1992. Information about the organisation, the duties and all the extensive work of all Codex Committees is published on their website [1]. In November 2015 and February 2016 two important Codex sessions took place where issues regarding “gluten-free” were on the agenda: CCFNSDU [2] and CCMAS [3].

Codex Committee on Nutrition and Foods for Special Dietary Uses

The thirty-seventh Session of the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) took place from 23 – 27 November 2015 in Bad Soden, Germany. The Committee was attended by delegates from 66 Member Countries, one Member Organisation and 36 International Organisations, one of them was AOECS.

At Agenda item „Matters Referred by the Codex Alimentarius Commission and/or Other Subsidiary Bodies“, sub-item „Matters for Action“, the CCFNSDU has to consider the following text of the Codex Document CX/NFSDU 15/37/2:

“The 36th Session of the Committee on Methods of Analysis and Sampling (CCMAS 36)

Examination of “ELISA G12” as a potential additional method for inclusion in Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten (CODEX STAN 118-1979)

“19. 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.

20. The Committee is invited to consider the above reply.”

When the item was opened for discussion, two Member countries outside Europe were in favour of adopting the G12, one Member Organisation was not in favour.

In my comment on behalf of AO ECS, I recalled that the threshold for gluten-free was established based on the results given by the R5. It is not known today whether or not the G12 gives the same results in all the different kinds of gluten-free foods, e.g. mixed ingredients from rice and maize or buckwheat or pseudocereals or wheat starch etc. Further on, there are no data published whether the G12 can detect partly hydrolysed wheat protein products or beer or malt extracts etc. Regarding oats products, the G12 shows positive results in some oats samples. Varying results below or above the threshold of gluten-free in the same food sample will cause severe difficulties in terms of food labelling and will lead to confusion for coeliacs, food producers and national food control authorities. Today it is too early to take a decision.

The Chair proposed to establish an ad hoc Working Group with the task to elaborate questions to CCMAS, which was accepted by the Committee.

The Working Group members considered this item very carefully and elaborated questions to CCMAS. After some discussion in the Plenary, the Committee approved the work of the Working Group and the following text for the CCNFSDU report:

“Examination of “ELISA G12” as a potential additional method for inclusion in Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten (CODEX STAN 118-1979)

“10. 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.”*

Other important items on the agenda were the Review of the Standard for Follow-up Formula, the Proposed Draft Definition for Biofortification and a Discussion Paper on a Standard for Ready-to-use Foods.

Codex Committee on Methods of Analysis and Sampling

The thirty-seventh session of the Codex Committee on Methods of Analysis and Sampling (CCMAS) took place from 22 – 26 February 2016 in Budapest, Hungary and was attended by 47 Member Countries, one Member Organisation and Observers from 17 International Organisations, one of them was AOECS.

Before the CCMAS session, the Meeting of International Organisations working in the field of Methods of Analysis and Sampling (Inter-Agency-Meeting – IAM) took place on Saturday, 20 February 2016, to consider the items on the agenda of the CCMAS. Regarding the subject “Comparison of methods for gluten“, the participants published their statement in the Codex Conference Room Document CRD 4:

„Participants noted that both R5 and G12 based procedures were in use to detect gluten in food products. Both methods have been validated and adopted by AACCI with limited scopes based on the range of reference materials and matrices tested. It was not considered feasible for further validation to be performed without this being undertaken by the manufacturers of the test kits. Furthermore, the addition of further matrices and mixed matrices to the scope of application of the tests might be best carried out by the end-users of the tests where these products occur. IAM members indicated that an appropriate place to remind users of these matrix restrictions may be in section 5.2 of the appropriate standard.“

The Codex Working Group on the Endorsement of Methods of Analysis and Sampling was held on Sunday, 21 February 2016, prior to the Plenary session. Among other items, the WG discussed also the subject

“Examination of “ELISA G12” as a potential additional method for inclusion in the Standard for Foods for Special Dietary Use for Persons Intolerant to Gluten (CODEX STAN 118-1979)“

as requested by the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU). After some debate the conclusion was published in the Conference Room Document CRD 2:

“The WG recommended to inform CCNFSDU that (regrettably) the two methods (the R5 and G12 methods) for the determination of gluten are not comparable. Comparison data for the two methods are not available, and mixed matrixes are not included in the scope of either of the methods obtained during their validation. Users confirmed that the two methods provide different results, and that both methods are used in official control. The developers of these proprietary methods might be able to provide further information on the applicability of the methods.”

When the item was discussed in the Plenary session, the recommendation of the Working Group was supported by the Committee and the following text is in the CCMAS report:

“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.”

Since a few years, a further 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 generally considered the subject of equivalency to Type 1 Methods and, finally, CCMAS agreed to the following conclusion as written in the CCMAS report:

“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.”

A further important item on the agenda was

Review and update of Methods in Codex STAN 234-1999

The Committee agreed to continue to work on the review and update of CODEX STAN 234-1999. All Codex Methods, also the R5 Method, are listed in this Codex Standard and maybe there will be some debate in the future whether or not to keep the R5 as Type 1 Method.

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7.2 Considerations concerning methods for gluten quantitation in foods (R5/G12 ELISA)

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Introduction

During the sessions and in the coffee breaks there were lively debates on how novel ELISA methods for the quantitation of gluten could be introduced into practice and which criteria should be set to acknowledge both established methods and newly introduced methods. This was particularly related to the R5 and G12 ELISAs and would also be relevant for test kits introduced into the market in the future. After the meeting a number of experts met in the lobby of the conference hotel to initiate actions to deal with this issue. The following statements summarise the suggestions of the experts and depict a possible way out of this issue.

R5 and G12 ELISAs

The sandwich ELISA based on the R5 monoclonal antibody [1] for the quantitation of intact gluten was endorsed as a Codex Alimentarius type 1 method [2] and has also been adopted as AOAC International [3] and AACC International [4] approved methods. Rice- and maize-based matrices have been used in these studies. A competitive version of the R5 ELISA has also been approved by both standardisation organisations [5,6]. In 2008, sandwich and competitive ELISAs based on the G12 (and A1) monoclonal antibodies were developed [7] and are now commercially available. The G12 sandwich ELISA has also been validated in an international collaborative study using rice-based matrices and is approved as AACC International [8] and AOAC International [9] method.

Codex Alimentarius

Codex analytical methods are being revised every 10 years and revision for the R5 Mendez ELISA is due either in 2017 or 2018. Thus, it is time to discuss how to handle ELISAs for gluten quantitation regarding approval by the Codex Alimentarius. Based

on the matrices used for validation, the R5 method has been recommended for gluten quantitation in maize matrices and the G12 method for the analysis of rice matrices. Both methods fulfil the performance requirements for gluten analysis set by the Codex Standard 118-1979 [10], i.e., a limit of quantitation of 10 mg gluten/kg or less and the detection of coeliac disease-active epitopes.

At the 37th session CCMAS decided to inform the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) that the R5 and G12 methods were not comparable, that comparability data were not available and that the kit producers should be encouraged to provide more information on the applicability of the methods [11]. In the following session of CCNFSDU, the committee decided not to include the ELISA G12 method in the Codex Standard because they noted that the results were not comparable with the R5 ELISA and recommended to wait for comparability data provided by the PWG [12].

Concerns

A major concern, in particular of coeliac societies, coeliac disease patients, food producers and national food control laboratories is the unclear situation if two ELISA methods for gluten quantitation were endorsed. It can be assumed that each analytical laboratory would use one ELISA as the default method for gluten quantitation. Consequently, it would be unclear, if a value obtained by one laboratory with one kit would be comparable to the value provided by a different laboratory with a different kit. This would lead to the question how to handle conflicting results from different laboratories. In general, the possibility of having two type I methods has to be questioned, because the definition of a type I method as “the only method” should exclude approval of a second type I method. On the other hand, if a proprietary method fulfilling the performance criteria of the Codex is on the market and has been approved by suitable collaborative studies, it should not be excluded due to the fact that another method has already been endorsed by the Codex Alimentarius. Both the R5 and G12 sandwich ELISAs have been compared in a number of scientific studies. In summary, the results of these studies strongly suggest that these methods do not yield comparable results. Typical examples are papers published by Bugyi *et al.* [13], Bruins Slot *et al.* [14] and Scherf [15].

Suggestions

Both ELISAs have gone through several rounds of FAPAS (Food Analysis Performance Assessment Scheme) proficiency studies. The number of laboratories that used the R5 ELISA is by far higher compared to the G12 ELISA. However, a cumulative consideration and statistical evaluation of all available FAPAS data, in particular those studies in which the same matrices were analysed by both the R5 and the G12 ELISA are not available to date. Thus, data from FAPAS testing starting at least from 2012 should be acquired and digitalised. This would enable appropriate

statistical analysis and would help to objectively compare the methods. Then knowledge gaps can be identified and a decision can be made whether additional analyses of samples (e.g., defined food samples from different categories) are necessary.

The CCNFSDU 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 [16]. This could be interpreted by kit producers that if the matrix would be restrained very clearly and if the method would be fully validated for this matrix, more than one Codex type I method would be possible. Consequently, each kit producer would validate its own kit for a specific matrix and multiple type 1 methods would evolve. This would lead to even more confusion than with ‘only’ the two current methods based on the R5 and G12 antibodies. This cannot be the goal for the future.

Position of the Prolamin Working Group (PWG)

Therefore, the PWG suggests that ELISA methods should be approved using a combination of

- (1) information on the method that has been used to provide the analytical value,
- (2) strict performance criteria and
- (3) a pre-defined set of maximum five matrices.

This would be similar to the Standard Method Performance Requirements (SMPR) published by AOAC International for allergen-containing commodities such as whole egg, milk, peanut and hazelnut [17].

Performance criteria

Performance criteria include the correct setup and statistical evaluation of validation studies [18-20] as well as the fulfilment of the requirements for standard method performance [21]. The minimal performance requirements set in AOAC SMPR 2016.002 [17] for whole egg, milk, peanut and hazelnut can be adapted to gluten, and the suggested values are given in Table 1. AOAC suggests low recovery rates of 60%, but the PWG feels that, in general, the recovery range should be between 80 and 120 %, which is in line with Abbott *et al.* [20]. A recovery rate of 60% should only be tolerated if repeated analyses with different kits have shown that the low rate is reproducible among different methods. A range of recovery rates between 50 and 150% for difficult matrices as suggested in [19] should not be accepted with modern gluten ELISAs. In this context the term “difficult matrix” is not well defined and should be omitted. Relative standard deviations that have to be tolerated appear to be rather high (up to 30%), but this seems to be an inherent issue of ELISA methods as compared to other analytical methods. Nevertheless, method developers should aim to

gradually improve these parameters. With respect to limit of detection and limit of quantitation both R5 and G12 ELISAs perform well [4,5,8].

Table 1. Method performance requirements for gluten ELISAs.

Parameter	Value/Range
Analytical range (mg/kg)	10 - 100
Limit of detection (LOD) (mg/kg)	3
Limit of quantitation (LOQ) (mg/kg)	10
Recovery (%)	80 - 120
Repeatability relative standard deviation (RSD _r) (%)	20
Reproducibility relative standard deviation (RSD _R) (%)	30

Matrices

Matrixes should not be based on botanical origin (e.g., rice- or maize-based), but on constituents that most likely affect the interaction of the antibodies with the gluten antigens. Possible matrixes should be categorised into protein-based, starch-based, fat-based, polyphenol-rich and fibre-rich foods. Table 2 suggests categories and examples for foods from each category. Examples are limited to three per category to keep the number of required analyses in validation studies in a range that can be handled.

Table 2. Suggested matrix categories and examples for foods from each category.

Category	Examples
Protein-based	Coated meat, sausage, protein isolate/concentrate
Starch-based	Starch, baked goods, sauce
Fat-based	Cookie, cake, ice cream
Polyphenol-rich	Chocolate cake, cocoa powder, beer
Fibre-rich	Cereal bran, breakfast cereals, legume seed flours

Practical considerations

Kit manufacturers are encouraged to agree on a set of matrixes which should be comparatively analysed using their methods. In case of conflicting R5/G12 results, in particular in the concentration range of the 20 mg/kg threshold, the higher concentration value should be considered relevant in the interest of the coeliac consumers. In future analyses it should then be avoided having to do two ELISAs. For any analysis value, the ELISA method that was used should be indicated alongside the results.

Conclusion

The PWG acknowledges that more than one ELISA method for the analysis of gluten in foods are currently used and that the results of these methods are not comparable. The group does not support the policy of the Codex Alimentarius to allow approval of

more than one type 1 method because this is in contrast to the definition of a type 1 method. The Codex Alimentarius should decide soon how to proceed, if methods are available that fulfil all performance criteria such as the R5 and G12 ELISAs. The PWG suggests that performance data of both ELISAs obtained with identical or at least comparable matrixes should be compared. If existing data is not sufficient, comparative studies should be carried out on a set of foods suggested in this statement paper. This could result in a kind of guidebook that suggests specific ELISAs for specific foods.

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8 Perspectives and action plan of the PWG

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The Prolamin Working Group executive meeting and joint discussion held on 23 September 2016, led to the decisions and statements outlined below.

Action plan

I. Analytical

- Peter Koehler is responsible for the PWG gliadin reference material (peter.koehler@tum.de).
- PWG gliadin will continue to be the reference material supported by the group. Material for 5 - 10 years is still on stock.
- Regarding new reference material, suitable wheat cultivars are currently identified by the MoniQA initiative. The PWG thinks that flour is not a suitable reference material and supports a protein sample as reference material. The flours identified by the MoniQA initiative could be used as starting materials for isolating protein reference materials for gluten from wheat, rye, and barley.
- A statement of the PWG on the use of ELISA kits for the analytical determination of gluten will be worked out and is published in this book.
- Katharina Scherf will be invited to the 2017 meeting to speak on the state-of-the-art in gluten quantitation by LC-MS.

II. Clinical

- For the symposium of the 2017 meeting the topic “The role of intestinal microbiota in coeliac disease” has been selected. Three speakers will be invited.

III. Members, Policy

- Olivier Tranquet and Rudolf Valenta are new members of the group. René Smulders will replace Luud Gilissen as a group member.
- Potential new group members have to be identified in the near future.
- Fernando Chirido 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) and by the help of Mrs. Anneliese Stoiber and Dr. Gaby Andersen, Deutsche Forschungsanstalt für Lebensmittelchemie (Freising, Germany). It will be distributed among leaders of opinion in gluten analysis and clinical medicine. An electronic version can be downloaded free of charge from the PWG website.

Next meeting: 2017

We are very pleased to announce the venue for our meeting in 2017:

Minden, Germany**Host:**

Dr. Markus Brandt
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DE-32427 Minden, Germany
Phone: +49 0571 83799-43
Fax: +49 0571 83799-20
E-mail: Markus.Brandt@sauerteig.de

Time: 28 - 30 September 2017**Focus of the meeting:**

- Role of intestinal microbiota in coeliac disease
- Gluten quantitation: LC-MS vs. ELISA

The meeting will be limited to 55 participants and attendance is by invitation only. Invitations have been sent by March 2017. Registration deadline will be June 15, 2017.

For registration please contact:

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Very special thanks to the hosts for this kind invitation!

ISBN 978-3-00-055981-5

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