Proceedings of the 33rd Meeting

WORKING GROUP on PROLAMIN ANALYSIS and TOXICITY

Edited by
Peter Koehler

10 - 12 October 2019
Urbino, Italy

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Impressum

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Cover picture* and picture of participants
Peter Koehler

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* Cover picture: View of the city of Urbino, location of the 33rd PWG-meeting, 2019

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Preface

At the 2018 meeting of the Working Group on Prolamin Analysis and Toxicity (PWG) in Ayr, Scotland, the location of the preceding meeting was still unknown. When I mentioned this in the executive meeting, Carlo Catassi spontaneously volunteered to be the host for the next meeting in 2019. Carlo suggested Urbino as the location, and this was a very good choice. I think that all participants agree that this city was a unique site to extend the knowledge on celiac disease on one side and to learn about the Renaissance in Italy on the other. The meeting was held at Palazzo Battiferri, a historic building belonging to the University of Urbino. Carlo and the girls from the event management company Congredior were present during the entire meeting. Carlo was also available after the official programme and organised a joint dinner with the participants that stayed for another day after the meeting. Apart from the group members the audience comprised an invited speaker, guests from academia, industry, and international coeliac societies. Representatives from cereal starch producers, producers of gluten-free foods, as well as manufacturers of kits for gluten analysis participated from industry. The participants had very intense one-and-a-half days of presentations, discussions and networking.

Analytical and clinical work in the field of CD, non-coeliac gluten/wheat sensitivity (NCGS/NCWS), wheat allergy, gluten and amylase-trypsin inhibitors done by PWG members as well as by guests and the invited speaker were presented in 14 talks and lively discussed at the meeting. In addition, one presentation was focussed on regulatory aspects of gluten analysis and labelling. In particular, this feature of gluten and coeliac disease provoked an intense discussion. This time, the analytical part was not as pronounced as in previous meetings and should be extended again in the future. The symposium “Six years of research on ATI – Results and consequences” with three presentations of internationally recognised experts highlighted the latest advances in the field of constituents that got into the focus of research during the last years.

I would like to express my thanks to all participants of the meeting for their active contributions and the open discussions that resulted thereof. I am in particular grateful to Carlo Catassi and the girls from Congredior for their enthusiasm and hospitality, which made this meeting a great success. Finally, I express my gratitude to all friends, colleagues, sponsors and participants for their inspiration and ongoing support of the PWG and the meeting.

Esslingen, December 2019 Peter Koehler

Table of Contents

1 Executive summary

Fifteen presentations covered all aspects related to gluten, amylase-trypsin inhibitors (ATI), coeliac disease (CD) and other relevant hypersensitivities, as well as legal issues. All authors have sent abstracts that are compiled in this proceedings book. The PWG-members decided during the executive meeting that the format of the proceedings will be changed. They will no longer be published as a printed book but will be available in electronic form available for download from the PWG website. The contributions are included mostly as one-page abstracts of the presentations.

Analytical session

With only three presentations, the analytical part was not as important as in previous meetings. Therefore, this part of the meeting should be strengthened again in future meetings. One presentation reported an international collaborative study on gluten detection using a new ELISA test kit that uses a combination of monoclonal antibodies that show reactivity with both the prolamin and the glutelin fractions of gluten. Also, monoclonal antibodies can be used to monitor excreted gluten peptides to study the compliance to the gluten-free diet. Finally, the CRISPR/Cas technology and other approaches to eliminate coeliac disease epitopes from gluten were described.

Clinical session

This session included eight presentations, with widespread topics that included a review-type presentation on coeliac disease pathogenesis, a report on persisting gastrointestinal symptoms in treated coeliac patients, talks on problems and consequences of the gluten-free diet, presentations on the pathomechanism from a genetic and protein (peptide) point of view, the use of recombinant ATI to produce antibodies towards ATI and aspects of wheat allergy.

Symposium: Six years of research on ATI - Results and consequences

The symposium included three presentations of recognised experts in this field of research. The first talk described the “discovery” of ATI and their relevance in innate immunity as well as an adjuvant for chronic inflammatory diseases. Furthermore, research was reported that showed the ability of the wheat ATI CM3 to directly target human toll-like receptor 4 (TLR4). Modelling of the interface opened the possibility to synthesise an oligopeptide that specifically binds to the receptor and could inhibit its interaction with ATI. Finally, one talk was focussed on the quantitation of the 13 most relevant ATI types in a selection of wheat cultivars that were approved between 1890 and 2010. There was no clear change in the ATI contents over time. From these results it can be suggested that breeding did not contribute to an increase of putatively immunoreactive ATI during the last decades.

Participants of the 33rd Meeting of the Working Group on Prolamin Analysis and Toxicity (PWG), Urbino, Italy, 10 – 12 October 2019

2 List of Participants

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3 Programme

THURSDAY, 10 October 2019

20:30 Arrival of Prolamin Working Group and all participants
Informal get-together with dinner
Location: Urbino

FRIDAY, 11 October 2019

Meeting venue: University of Urbino, Palazzo Battiferri, Via Aurelia Saffi, 42

09:00 Opening of the meeting (*Carlo Catassi, Peter Koehler*)

09:10 Analytical research reports

- *Chirdo, Ciclitira, Gianfrani, Koehler, Lundin, Scherf, Schuppan, Smulders, Tranquet; guests*

10:25 Clinical research reports

- *Catassi, Chirdo, Ciclitira, Gianfrani, Lundin, Schuppan, Tranquet; Troncone; guests*

11:05 Coffee break

11:10 Clinical research reports (continued)

12:50 Lunch

14:00 Clinical research reports (continued)

15:40 Coffee break

16:00 The Prolamin Working Group Executive Meeting (members only)

17:00 Return to the hotel

17:30 Guided tour of “Palazzo Ducale”, Urbino

19:00 Bus departure from “Palazzo Ducale” for all participants to the joint dinner
Location: Urbino dei Laghi
Tenuta Santi Giacomo e Filippo
via San Giacomo in Foglia 7
61029 Urbino PU

22:30 Bus transfer to the hotel

SATURDAY, 12 October 2019

SYMPOSIUM

09:00 Six years of research on ATI – Results and consequences
Chair: Carmen Gianfrani, Naples, Italy

09:05 History and physiological effects of ATI
Detlef Schuppan, Mainz, Germany

09:50 Functional characterization of amylase trypsin inhibitors from several *Triticum* species: The Good, the Bad and the Ugly
Massimiliano Cuccioloni, Camerino, Italy

10:30 ATI in wheat cultivars from 1890 to 2010
Katharina Scherf, Karlsruhe, Germany

11:10 Coffee break

11:40 General discussion of current developments concerning gluten analysis, clinical and legal aspects

Statements by participating organisations, representatives from industry, and guests

Outline: action plan 2020 of the Prolamin Working Group

13:00 Lunch and farewell

Afternoon/evening

Extra time for informal meeting and additional Prolamin Working Group meeting concerning action plan (hotel lobby)

Joint dinner

SUNDAY, 13 October 2019

Departure of the Prolamin Working Group

4 Analytical research reports

4.1 International collaborative study on gluten detection using the Total Gluten ELISA test kit

Markus Lacorn[1], Thomas Weiss[1], Paul Wehling[2], Mark Arlinghaus[2], Katharina A. Scherf[3,4]

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Abstract

According to Codex Alimentarius Standard 118-19179, gluten-free products intended for consumption by celiac disease patients must not exceed gluten contents of 20 mg/kg. The most commonly used tests to analyse gluten contents in foods are enzyme-linked immunosorbent assays (ELISAs). ELISAs based on the R5 monoclonal antibody (mAb) are used most frequently, because this method is endorsed by Codex Alimentarius. However, the R5 method has some limitations. First, gluten from rye and barley is overestimated when using a wheat-based calibration, because the main R5 epitope QQPFP is more abundant in gluten proteins from rye and barley compared to gluten proteins from wheat. Second, the R5 mAb shows little affinity to glutelins, so that the prolamin content detected is converted to gluten using a factor of two (Codex Alimentarius), which leads to overestimation of gluten in many cases, but can also lead to underestimation.

To address these limitations, a new sandwich ELISA (RIDASCREEN® Total Gluten) was developed that includes the R5 mAb, but also three additional antibodies to detect prolamins from wheat, rye and barley, high-molecular-weight (HMW) glutenin subunits (GS) from wheat, HMW-secalins from rye and low-molecular-weight (LMW)-GS from wheat. The samples are extracted with Cocktail solution/ethanol 20:80 (v/v) and the samples are analysed within 50 minutes. A gluten extract from four common wheat cultivars serves as material for calibration.

To assess the performance of the new test, an international collaborative study with 19 laboratories and 42 blind duplicate samples was carried out in addition to the in-house single laboratory validation. The results of the study confirmed that the test kit fulfilled all standard method performance requirements (AOAC SMPR® 2017.021). The limits of detection and quantitation were equal/below 5 mg/kg of gluten and the recoveries ranged from 99 to 137 % for wheat, rye and barley. The relative standard deviation of

reproducibility (RSD(R)) was 20% or lower for incurred homogeneous samples. In case of oat and oats products, the RSR(R) values were mostly 30% or lower, except for oat flours (up to 54%) that seemed to be more inhomogeneous than the other samples. The inhomogeneous distribution of wheat, rye and barley gluten in oat and oats products is well-known and inherent to these. After statistical evaluation and assessment of all collaborative study and in-house validation data by the AOAC International Expert Review Panel on Gluten Assays, the new Total Gluten ELISA was granted AOAC OMA First Action status in December 2018.

4.2 Monitoring excreted gluten peptides for the management of coeliac disease

Angel Cebolla

Biomedal S.L., Seville, Spain

Abstract

Gluten immunogenic peptides (GIP) have shown to be resistant to gastrointestinal digestion, translocated through the intestinal epithelia, deamidated by brush border transglutaminase and excreted in faeces and urine. The extent of each process is unknown. Immunotechniques based on antibodies reactive to the most immunogenic peptides have allowed determining the excreted GIP after a few hours of gluten intake in urine and after at least one day in stools. The amount of ingested GIP that can be detected was estimated to be less than 0.5% in stool and less than 0.1% in urine, which may suggest that only a minimal part of gluten is not fully metabolized by the human organism. Despite these limitations, determination of excreted GIP has allowed new applications in the management of CD: a) confirming gluten ingestion during diagnosis, b) monitoring gluten-free diet, c) controlling patient's diet in studies for alternative therapies, (d) verifying the absence of gluten intake in non-responsive CD and in difficult to control environments. A prospective observational study was conducted including 23 de novo CD patients and 80 CD patients on GFD. Four patients out of the 23 (about 20%) had low or no GIP in urine at diagnosis. In those patients in follow up, urine samples on three days of the week were collected and GIP were analysed. Anti-tissue transglutaminase antibodies, dietary questionnaire, clinical manifestations and histology were analysed simultaneously. About 25% of CD patients on GFD showed Marsh II-III mucosal atrophy. Among this population with histological damage, 95% had detectable urine GIP in at least one sample, however, between 60-80% of them were asymptomatic, showed negative serology and a good GFD adherence according to dietary questionnaire. In contrast, 97% of CD patients with no detectable urine GIP in any sample showed no villous duodenal atrophy. These results demonstrated a high sensitivity (95%) and high negative predictive value (97%) of GIP measurement with respect to the recovery of the intestinal mucosa. The detection of GIP in urine in patients on GFD allowed detecting transgressions that correlated with the presence of histological lesions. The recurrent absence of GIP in three urine samples appeared to be highly reliable to predict correct GFD compliance and the absence of villous atrophy, decreasing the need for invasive techniques.

4.3 Eliminating coeliac disease epitopes from gluten

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Abstract

Coeliac-safe bread wheat with baking quality cannot be simply produced by combining natural or randomly induced mutations, due to the large number of genes in the gliadin and glutenin gene families to be modified or removed, and because of their occurrence in large blocks or tandem repeats on the three wheat genomes of bread wheat. However, recently developed biotechnological approaches may be combined with classical breeding approaches to change this situation.

Inhibiting the production of gluten proteins in the developing wheat grain by RNA interference (RNAi) with gene-specific mRNAs has been shown to be effective for reducing one or several gliadin families at once, although the level of other gliadins or glutenins may be upregulated, as compensation. As the silencing DNA construct must remain present in the progeny plant lines, these plants are considered GM.

Another approach is gene editing using CRISPR/Cas9, enabling specific targeted mutagenesis at the level of an entire gene family or individual genes. For example, gene editing of alpha-gliadins led to up to 85% total gliadin reduction as measured by the R5 method for total gluten detection (Sánchez-León et al., 2018; https://doi.org/10.1111/pbi.12837). Simultaneous editing of alpha- and gamma-gliadin genes (Jouanin et al., 2019a; https://doi.org/10.1186/s12870-019-1889-5) affected up to 30% of the targeted genes. Editing of epitopes in gliadins to make them coeliac-safe, necessitates the development of novel screening methods for the remaining gluten genes or epitopes, e.g. through droplet digital PCR or targeted DNA capture and sequencing (the GlutEnSeq system) (Jouanin et al., 2019b; https://doi.org/10.1016/j.jcs.2019.04.008).

Because the CRISPR/Cas9 construct, after its targeted mutagenic activity in the primary transformed plant, is removed through segregation in the next generations, the products of this technology are considered non-GMO in many countries world-wide, but in the EU (where, regarding genetic modification, not the product but the process counts) the products of CRISPR/Cas9 gene editing are thus far considered GM. If this is not changed, the foreseen consequence for the EU will be that hypoimmunogenic (coeliac-safe[r]) wheat will not be produced soon here using targeted gene editing (Jouanin et al. 2018; https://doi.org/10.3389/fpls.2018.01523).

5 Clinical research reports

5.1 Coeliac disease pathogenesis: The uncertainties of a well-known immune mediated disorder

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Abstract

Features of CD suggest that it can be considered an autoimmune disease with gluten as an environmental trigger causing activation of a highly specific adaptive immune response. Activated *lamina propria* T cells influence the behaviour of other cell populations through the release of cytokines, including IFNγ, IL-2 and IL-21. An increase in intra-epithelial lymphocytes (IELs) is a classic finding in CD and some with a NK-like phenotype are thought to contribute to enterocyte destruction. The function of other IEL populations is less certain and they may play a local immune regulatory role. There is also evidence that cells of the innate immune system, including eosinophils, mast cells and neutrophils, contribute to disease pathogenesis. A further cell population, myofibroblasts, may play a role and these cells are an important source of TG2 and metalloproteinases. Controversy surrounds the issue of whether non-immune gliadin peptides contribute to the disease process. Some studies report that one such peptide, p31-43, can cause direct damage to enterocytes and also stimulate enterocyte proliferation. The failure to identify a receptor for this peptide has been used to reject its involvement in the disease process. If alternate gluten peptides cause innate cell activation, this will be important in designing future gluten avoidance strategies.

5.2 General health and persisting gastrointestinal symptoms in treated coeliac patients

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Abstract

Objectives: Strict adherence to a gluten free diet usually leads to clinical and histological remission in coeliac disease (CD). However, few studies have investigated the prevalence of persistent symptoms in a general CD population. Our main aim was to describe general health and specific symptoms in treated CD patients. Secondary, we described the prevalence of persisting GI symptoms, and factors associated with GI symptoms.

Methods: In a web-based national survey in adults with CD, respondents filled in the questionnaires Coeliac Symptom Index (CSI), Gastrointestinal Symptom Rating Scale-IBS version (GSRS-IBS), Coeliac Disease Adherence Test (CDAT) and additional background questions. Chi-square test, t-test, analysis of variance, linear regression and Pearson correlation coefficient (r) were used for statistical analysis.

Results: Of 4028 participants (mean age 47 years; 82% women), 19% had CSI score ≥45, indicating reduced quality of life. Furthermore, 54% reported GI symptoms the previous week, of which 9.5% had mean score ≥4, indicating moderate to severe discomfort. Participants scored highest on the GSRS-IBS domains "bloating syndrome" (23.5% scored ≥4) and "pain syndrome" (20.0% scored ≥4). Adequate adherence shown by CDAT score ≤12 was reported by 43%. i.e. adequate adherence. Self-reported adherence was very good in 88%. Moderate correlation was found between CSI and GSRS-IBS ($r=0.66$, $p<0.001$), CSI and CDAT ($r=0.61$, $p<0.001$) and CDAT and GSRS-IBS ($r=0.35$, $p<0.001$).

Conclusion: In this national cross-sectional study, we found a high symptom burden, indicative of reduced quality of life. Persistent GI symptoms was frequent; pain and bloating were the most prominent GI complains. Although GI symptoms was correlated with poorer dietary adherence, more research is needed to find the cause of persisting GI symptoms, and potential treatment methods.

Conflict of interests: None.

5.3 Nutritional status and dietary intake of children with celiac disease on a gluten-free diet: a case-control, prospective study

Elena Lionetti, Niki Antonucci, Michele Marinelli, Beatrice Bartolomei, Elisa Franceschini, Simona Gatti, Giulia Naspi Catassi, Anil K. Verma, Chiara Monachesi, Carlo Catassi

Department of Pediatrics, Università Politecnica delle Marche, Ancona, Italy

Abstract

Introduction: Nutritional adequacy of the gluten-free diet (GFD) is a controversial issue.

Aims: To evaluate the nutritional status, the dietary intakes and the adherence to the national recommendations of intakes and to the Mediterranean diet of Italian CD children on a GFD.

Design: This is a case-control prospective study. All children diagnosed with CD on a GFD for ≥2 years were recruited. Controls were age and gender-matched healthy children not affected with CD. In both groups anthropometric measurements and energy expenditure information were collected. Dietary assessment was performed by a 3-days food diary. The adherence to the Mediterranean diet was estimated by the KIDMED index.

Results: The daily intake of lipids was significantly higher in the celiac group, while the consumption of carbohydrates and fiber was lower in the CD group. The median KIDMED index was similar in both groups, with a suboptimal adherence to the Mediterranean diet in both groups.

Conclusions: The diet of celiac children is nutritionally unbalanced with higher intake of fat, and lower intake of carbohydrates and fiber, highlighting the need of a dietary counselling.

5.4 Expression analysis of HLA class II risk genes in relation to the anti-gluten T cell immunity in celiac disease patients

Giovanna Del Pozzo[1], Laura Pisapia[1], Stefania Picascia[2], Mariavittoria Laezza[1], Federica Farina[2], Serena Vitale[2], Pasquale Barba[1], Carmen Gianfrani[2]

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Abstract

In celiac disease (CD), the great majority of patients carry the DQA1*05 and DQB1*02 alleles of the Major Histocompatibility Complex (MHC/HLA) class II genes. The HLA DQA1*05/DQB1*02 alleles encode for the DQ2.5 heterodimer, a surface molecule with a key role in the immune response to gluten. In fact, the formation of complexes between DQ2.5 and gluten peptides on antigen-presenting cells (APCs) is necessary to activate pathogenic CD4+ T lymphocytes.

Previous studies have indicated that subjects homozygous for DQA1*05 and DQB1*02 alleles have the highest risk of developing CD, and the DQ2.5 gene dose correlates with the intensity of CD4+ T cell response to gluten [1,2]. We demonstrated that the expression of HLA DQA1*05 and DQB1*02 alleles is much higher than non-CD-associated alleles when in heterozygosity, either in HLA DR1/DR3 and DR5/DR7 haplotypes [3-5]. This influences the levels of DQα105 and DQβ102 proteins and determines a comparable expression of DQ2.5 heterodimers between DQ2.5 homozygous and heterozygous APCs. According to these findings, the magnitude of the anti-gluten CD4+ T cell response is more prominently dependent on the gluten dose and less on the DQ2.5 gene configuration of APCs. Furthermore, our findings support the concept that the expression level of DQ2.5 alleles is an important risk factor in CD. The preferential expression of certain HLA alleles, and the prominent antigen binding properties, provides a new functional explanation of why HLA genes are so frequently associated with autoimmune disorders, such as CD. Further studies are required to dissect the molecular mechanisms responsible of this differential expression of CD-risk genes.

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5.5 Self-assembly properties may be linked to the in vivo effects of p31-43 gliadin peptide

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Abstract

Celiac disease (CD) is a chronic enteropathy elicited by a Th1 response to gluten peptides in the small intestine of genetically susceptible individuals. However, it remains unclear what drives the induction of inflammatory responses against harmless antigens in food. By studying the biological properties of the p31-43 peptide (p31-43) from α-gliadin, we observed the induction of mucosal damage and innate immune response in the proximal small intestine upon intragastric p31-43 administration in wild type mice. By *in vivo* studies, we demonstrated that mucosal damage triggered by p31-43 requires the NLRP3 inflammasome (NLRP3, ASC and caspase 1). As consequence of inflammasome activation we observed production of IL-1β. Administration of p31-43, but not scrambled or inverted peptides, to normal mice induced histological changes in the proximal small intestine (reduction of Villus height/Crypt depth ratio, and increase in IEL number). Since a cellular receptor for p31-43 has not been identified, this raises the question of how this peptide could mediate different biological effects. With the aim to characterise the conformation of p31-43 different biophysical and *in silico* tools were used. Dynamic Light Scattering (DLS) and Atomic Force Microscopy (AFM) analysis showed p31-43 oligomers with different height distribution. By Circular Dichroism, we observed that p31-43 self-organized in a poly-proline II conformation in equilibrium with β-sheets-like structures, which remained stable in the pH range of 3 to 8. In addition, these findings were supported by Molecular Dynamics Simulation. The formation of p31-43 oligomers may help to explain the molecular etiopathogenesis in the induction of pro-inflammatory effects and subsequent damage at the intestinal mucosa in CD.

[*] these authors contributed equally to this work

5.6 Effects of the spatial organisation (number and distance) of epitopes involved in allergy to hydrolysed wheat proteins on (antibody aggregation and) basophil activation

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UR 1268 Biopolymers Interactions Assemblies, INRA, Nantes, France

Abstract

Basophils and mastocytes are major effectors cells triggering the symptoms of gE-mediated allergy. Degranulation of basophils requires that allergens induce aggregation of IgE/FcεRI complex on cell surface. Among other parameters, such as the affinity of IgE, the spatial organisation (accessibility, number and proximity) of IgE-epitopes on an allergen modulate degranulation. Gluten allergens are characterised by repetition of closely related short sequences that have been shown to be involved in different wheat allergies and particularly in anaphylactic reactions. We explored the effects of the number and the distance between epitopes involved in allergy to hydrolysed wheat proteins (HWP) on antibody aggregation and reactivity and sensitivity of basophils.

Based on the sequence of the main allergens involved in HWP-allergy, peptides containing 1 to 4 epitopes whom distance varied from 3 to 29 amino-acids (AA) were synthetized. The size of the immune complexes formed by the interaction of these peptides with a specific antibody (mAb-DG1) was determined by size exclusion chromatography coupled to light scattering detector. The degranulation induced by the peptides was then monitored with basophils sensitized with human sera or a chimeric mouse/human IgE directed to HWP epitopes (chIgE-DG1).

We observed that the size of the immune complexes varied according to the distance between two epitopes. A correlation between spacer length and both the reactivity and the sensitivity of sensitized basophils was also highlighted. The number of epitopes also affected the size of immune complexes and basophils degranulation.

In this work, we determined the minimal requirements in terms of number and spacing of gliadins epitopes that affect basophils degranulation. Although the epitopes organisation was diversified in our set of peptides, many spatial organisation of epitopes were able to strongly activate basophils. Aggregation of immunoglobulin receptors is a common mechanism for activating or inhibiting immune cells. We observed here, that the very particular structures of repeated domains of gliadins offers numerous possibility for the aggregation of specific immunoglobulins bound to their receptors and thus for acting on the effector cells.

5.7 Production of antibodies against functional recombinant wheat ATIs

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Abstract

Amylase/Trypsin inhibitors (ATIs) account for 0.3% of the whole grain where they are located in the endosperm. They belong to a multigenic family, they are constituted of small polypeptides stabilized by disulfide bonds and arranged in monomers, dimers or tretramers. Several wheat amylase-trypsin inhibitors (ATIs) are major allergens in respiratory allergy (baker's asthma), they can also trigger food allergy in children. They are activators of the innate immunity. They have also been suggested to have a pro-inflammatory role, and might therefore be involved in the onset of food allergy, celiac disease or in the poorly characterized pathology Non Celiac Wheat Sensitivity. Wheat ATIs are usually obtained in an enriched complex fraction called CM-like, they have been characterized by LC MS/MS, and quantified by targeted mass spectrometry. In view of the potential effects of ATIs on the immune system, reliable tools to detect these proteins in raw materials as well as in food are needed. The purpose of this work was to develop antibodies (IgG), polyclonal and monoclonal, directed against ATIs and use them for detection.

CM-like fraction and purified recombinant ATIs (CM3 and 0.28) were checked for their bioactivity in an '*in vitro*' basophil model using sera from patients suffering from wheat allergy. These ATIs were then used as antigens for immunization. Polyclonals antibodies (pAbs) were obtained against CM-like fraction and monoclonal antibodies (mAbs) against ATI CM3 and ATI 0.28. These antibodies, pAbs and mAbs, were usable in Western blot (WB) and in ELISA.

Using the mAbs in ELISA, we pointed the presence of ATI CM3 in both *T. aestivum* and *T. durum* species and its lack in *T. monococcum.* The ATI 0.28 was also present in *T. aestivum* and lacking in *T monococcum.* but variable across *T. durum* cultivars. These different contents in ATIs were confirmed with polyclonal antibodies.

The two types of antibodies produced here are usable in WB and ELISA, their specificity has made it possible to highlight differences in content between wheat species but also between cultivars.

5.8 Gluten contamination in the daily diet of treated celiac disease patients

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Abstract

A life-long strict gluten-free diet (GFD) is the only accepted treatment for coeliac disease (CD) to date. Adherence to a strict GFD is difficult to achieve for CD patients. Recent studies have reported that there is contamination with gluten traces during a GFD. However, so far, very little is known about the exact quantity of gluten accidentally consumed by CD patients. In an ongoing study, we are determining analytically the amount of gluten accidentally consumed during 24-h period in children with CD. From October 2018 to October 2019, pediatric CD patients following a GFD for at least two years were recruited for this study after matching inclusion and exclusion criteria. Each patient was requested to maintain a 24-h food diary and document key information of the consumed food and take its weight using kitchen balance. They have also been requested to provide us a portion of food sample in a cold-chain, as soon as we collected the food samples in our laboratory, we stored them at -20 ^{o}C until we performed the analysis. We excluded naturally gluten-free food samples from the analysis e.g. water, milk, fresh fruits, and raw vegetables. All food samples were analyzed for their gluten content by Ridascreen Gliadin sandwich R5 ELISA (R-Biopharm, Darmstadt, Germany), and, until May 2019, also by GlutenTox Sandwich G12/A1 ELISA (Biomedal diagnostics, Spain). Manufacturer's guidelines were strictly followed during the ELISA performance. Food samples with <20 mg/kg (ppm) of gluten were considered gluten-free. This study was divided into two major steps; in the first step (October 2018 to May 2019), we compared the performance of Ridascreen R5 and Biomedal G12 ELISA methods and in the second step (Ongoing) we are quantifying the exact amount of gluten consumed accidentally by CD patients in mg/day. Until May 2019, total 285 food samples were compared by both ELISA methods (R5 and G12). None of the food samples detected with gluten level more than acceptable level of gluten (<20 ppm) except one sample that was quantified with low amount of gluten (i.e. ≤100 ppm of gluten) by both the ELISA methods. Initial outcome shows, there is no major difference in the performance of both ELISA methods. As this study is in ongoing phase, we do not have the final results however, preliminary results of the study suggest that daily unintended exposure to gluten in our Italian cohort of children on a GFD is very low.

6 Symposium: Six years of research on ATI – Results and consequences

6.1 Wheat amylase trypsin inhibitors: Drivers of disease

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Abstract

We identified a common family of wheat proteins, amylase-trypsin inhibitors (ATIs), as activators of innate immunity in wheat and gluten preparations. ATIs stimulate toll like receptor 4 (TLR4) on monocytes, macrophages and dendritic cells. Moreover, ATI cause intestinal barrier dysfunction and directly induce dysbiosis They represent 2-4% of the wheat protein and are largely resistant to baking and intestinal proteolysis. We showed that ATI consumption in a normal wheat based diet induces low level intestinal inflammation in vivo. In animal studies dietary ATIs promote chronic inflammatory diseases, such as the metabolic syndrome, including fatty liver and cardiovascular diseases, autoimmune diseases, such as systemic lupus, rheumatoid arthritis and multiple sclerosis, allergies in general, fibrotic diseases of liver, lungs and skin, and even the growth of cancers.

Innate immune cell activation is higher in the mesenteric lymph nodes than in the gut lamina propria, suggesting a rapid propagation of the inflammatory signal to the periphery, likely by emigration of intestinal migratory dendritic cells from the intestine shortly after their contact with ATI. ATIs are present in many plants and play a protective role by inhibition of alpha-amylases from insects and mites. ATIs of gluten containing grains (wheat, barley and rye) have potent inflammatory activity, while structurally less related ATI of oats, maize, rice and legumes have no or low activity. ATIs associate starch and storage proteins in the endosperm and are involved in grain maturation. ATIs are a family of 17 structurally related proteins of 120-150 amino acids. ATI are present as non-covalently linked hetero-tetramers (CM proteins), dimers (0.19, 0.28 and 0.53), and as monomers, such 0.29). Each ATI harbour 10 cysteine residues that form five intramolecular disulfide bonds. This compact, highly disulfide-linked secondary structure of ATI is needed for their biological activity, namely the activation of TLR4. 2 peptide sequences have been identified that inhibit the interaction of CM3 with TLR4 at high concentrations. CM3 and 0.19 are the most prevalent ATI species, show to have comparable bioactivity when expressed recombinantly in eukaryotic cells. The quantity of ATIs in flours, as determined by

mass spectrometry, does not correlate well with their bioactivity that depends on oligomerization and complex matrix effects. Current efforts are targeted at the selection and production of wheats that have a low ATI content for improved health, while their gluten content remains unaffected to secure good baking and textural properties. This is highly relevant, since even in modern wheat ATI bioactivity can vary 6-fold, and since their effect on immune activation is dose dependent, with the clinical recommendation to reduce the consumption of gluten - and thus wheat-containing foods by at least 90%. Several clinical studies in patients with autoimmune and metabolic diseases randomized to an ATI-free vs ATI-containing diet are ongoing.

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6.2 Functional characterization of amylase trypsin inhibitors from several *Triticum* species: The Good, the Bad and the Ugly

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Abstract

ATI are a group of bifunctional hydrolase inhibitors from wheat. HPLC/MS analyses showed non-easily predictable differences (variety- and environmental-dependent) in terms both of total and individual ATI content.

Besides interfering with the normal absorption of nutrients due to their inhibitory properties, ATI can elicit strong innate immune response by activating the TLR4–MD2–CD14 complex according to a lipopolysaccharide-like mechanism, with implications both in gastrointestinal inflammatory disorders (i.e. celiac disease, wheat/gluten sensitivity), and in non-intestinal inflammation. According to a combined use of SPR biosensors and computational methods, we demonstrated the ability of wheat ATI CM3 to directly target human TLR4, the resulting complex showing an equilibrium dissociation constant in the nanomolar range and specific stoichiometry. The complex was mainly stabilized by non-covalent electrostatic interactions, and changes in ionic strength significantly altered its stability (specifically, both kinetic and equilibrium dissociation constants increased with NaCl concentration).

Computational analysis predicted CM3-containing-ATI molecule to favourably accommodate within the β-strand loop of TLR4, in a region other than MD2 binding site and TLR4 self-dimerization interfaces. The mapping of this TLR4-ATI non-contiguous binding interface guided the design of an antagonist hydrolase-resistant 11-mer oligopeptide. When tested for binding to TLR4, the oligopeptide specifically bound to the receptor and prevented the formation of the ATI-TLR4 complex. Based on these promising results, we have reason to believe that this oligopeptide, although demanding adequate customization to enhance its affinity for the target, could have profound physiological and pharmacological implications for both celiac disease and non-celiac wheat sensitivity by blocking the interaction between TLR4 and its activators eventually preventing the inflammatory cascade.

6.3 ATI in wheat cultivars from 1890 to 2010

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Abstract

There are more and more reports on an increasing prevalence of wheat hypersensitivities in the population since the last decades, but the underlying causes remain unclear. In addition to increased awareness and improved diagnostics, there may have been changes on the side of the human immune system, including changes in dietary habits, gut permeability and gut microbiota and low frequencies of infections. On the side of wheat and wheat products, factors such as agricultural practices during wheat cultivation, modern wheat processing techniques and wheat breeding might play a role. Higher yields and increased resistance towards abiotic and biotic stress factors are major breeding goals. These may have contributed to inadvertent changes in wheat protein composition that might cause higher immunoreactivity in sensitive individuals.

To explore this hypothesis, the five most commonly used wheat cultivars per decade from 1890 to 2010 in Germany were selected and grown in Gatersleben in the years 2015, 2016 and 2017. After milling, the protein composition of the flours was analysed by modified Osborne fractionation and reversed-phase high-performance liquid chromatography. The content of amylase/trypsin-inhibitors (ATIs) was determined by liquid chromatography tandem mass spectrometry (LC-MS/MS).

Total protein contents were dependent on the harvest year with an overall median of 98 mg/g in 2015, 80 mg/g in 2016 and 76 mg/g in 2017. When comparing the five cultivars per decade, there was a slight, but non-significant trend towards decreasing protein contents from 1890 to 2010. The source of variation was a lot higher for the harvest year compared to the decade. Considering the protein composition, the contents of albumins/globulins and of gluten did not show significant changes over the time period investigated, whereas the contents of gliadins decreased and those of glutenins increased. The ATIs are primarily found in the albumin/globulin fraction and are subdivided into several types, such as α-amylase inhibitors 0.19, 0.28 and 0.53, α-amylase/trypsin inhibitors CM1, CM2, CM3, CM16 and CM17, trypsin/α-amylase inhibitors CMX1, CMX2 and CMX3, α-amylase/subtilisin inhibitor, chymotrypsin inhibitor and trypsin inhibitor in wheat. Targeted LC-MS/MS analysis of those 13 ATI types based on 21 specific marker peptides revealed that CM3 and 0.19 were the major ATI types making up 20% and 18% of the total ATI content, respectively. The ATIs CM17, CM16, 0.28 and CM2 amounted to 10-12% of total ATIs, whereas all other

types were below 5% each. As observed for the total protein contents, the ATI contents also depended on the harvest year, but there was not clear change in contents from 1890 to 2010. Further work will explore the possible correlation between ATI contents and bioactivity determined on monocytes/macrophages expressing toll-like receptor 4.

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

7.1 News from Codex and Regulatory Affairs

Hertha Deutsch

AOECS Codex Delegate, Austrian Coeliac Society, Vienna

Abstract

AOECS, the Association Of European Coeliac Societies, has Observer status in the Codex Alimentarius Commission since 1992. In the past months, some items important for coeliacs regarding the gluten-free diet were discussed in the Codex Committee on Food Hygiene (CCFH) in November 2018, in the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) in November 2018, in the Codex Committee on Methods of Analysis and Sampling (CCMAS) in May 2019 and in the Codex Alimentarius Commission (CAC) in July 2019. The reports of the sessions are published on the Codex website www.fao.org. A short summary:

CCFH: all AOECS written comments for the Proposed Draft Code of Practice on Food Allergen Management for Food Business Operators were accepted by CCFH and the texts in this paper were changed accordingly.

CCNFSDU: Proposed Draft Guidelines for Ready to Use Therapeutic foods: AOECS highlighted the need for further consideration whether gluten-containing cereals should be permitted in such products because gluten intolerance in SAM (severe acute malnutrition) infants and children may result in life threating situations.

CCMAS: AACCI suggested to delete „Gluten-free Foods“ in the Commodity of the Codex Standard 234 and replace it with „Corn- and Rice-Based Gluten-Free Foods“ and „Oat-Based Gluten-Free Foods“. AOECS highlighted the importance not to change the Commodity “gluten-free foods”: Additional to special gluten-free dietary products, the claim “gluten-free” was also permitted for foods for normal consumption in the Codex Standard 118-1979 (section 4.3) and in several national food legislations. The gluten-free market was increasing rapidly in the world trade, therefore it is essential for avoiding health problems of coeliacs and trade barriers to use the same and most reliable method for gluten determination. CCMAS agreed to refer the AACCI proposal to CCNFSDU for consideration and further agreed that the methods for “gluten free” would not be considered in the continued review of the methods of analysis in the cereals, pulses and legumes workable package.

CAC: The Proposed Draft Code of Practice on Food Allergen Management for Food Business Operators was adopted at Step 5. The Revision of the General Standard for the Labelling of Prepackaged Foods regarding allergen labelling and guidance on precautionary allergen or advisory labelling was approved as a new work.

8 Perspectives and action plan of the PWG

Peter Koehler

Biotask AG, Esslingen, Germany

The Prolamin Working Group executive meeting and joint discussion held on 11 October 2019, led to the decisions and statements outlined below.

Action plan

I. Analytical

- The PWG gliadin reference material is available from Arbeitsgemeinschaft Getreideforschung e.V. (Association of Cereal Research), Mr. Tobias Schumacher, Schuetzenberg 10, 32756 Detmold, Germany, E-mail: info@agf-detmold.de. The PWG has set the price for one batch (100 mg) to 150 Euro.
- The MoniQA initiative has extensively characterised wheat cultivars from around the globe. A flour of a blend of five wheat cultivars will shortly be available as a reference material for purchase in 2020.
- The PWG considers flour not as a suitable reference material and supports a protein sample as reference material. Further action is planned in this field.

II. Clinical

- The PWG keeps considering to become a working group under the umbrella of the International Society For The Study Of Celiac Disease

III. Members, Policy

- Stefania Masci, University of Tuscia, Viterbo, will be invited to the next meeting and is a potential new member of the group.
- To improve the visibility of the group, an Open Access publication entitled *Recent progress and recommendations from the Working Group on Prolamin Analysis and Toxicity on celiac disease* will be completed shortly and published in "Frontiers in Nutrition".
- Publications on topics covered by the PWG will replace the classical proceedings in the future. Proceedings will no longer be published as printed books. They will be available free of charge in electronic form from the PWG website (http://www.wgpat.com) and will contain 1-page abstracts of the meeting presentations.
- New group members have to be identified because some members will retire in the next years.

Next meeting: 2020

We are very pleased to announce the venue for our meeting in 2020:

Merano, Italy

Host:

Ms Jacqueline Pante
Dr. Schär AG / SPA
E-mail: Jacqueline.Pante@drschaer.com

Time: 15 - 17 October 2019

Focus of the meeting:

- Triggers and drivers of coeliac disease
- Analytical aspects of gluten and ATI

The meeting will be limited to 55 participants and attendance is by invitation only. Invitations will be sent by April 2020. Registration deadline will be June 15, 2020.

Very special thanks to the hosts for this kind invitation!