SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Prospective investigation of humoral anti-gliadin immune response in children at risk of celiac disease

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1. Introduction

Celiac disease (CeD) is a T-cell mediated, conditional autoimmune disorder triggered by gluten ingestion in genetically predisposed individuals. Gluten comprises prolamin peptides found in wheat (gliadins and glutenins), barley (hordeins) and rye (secalins), and among them wheat gliadin peptides are the most immunogenic for CeD patients. The hallmark of CeD is the T-cell mediated adaptive immune response to gliadin peptides coupled with antibody production against the self-protein tissue transglutaminase (TG2), followed by villous atrophy in the small intestine.

Antibodies to gliadin peptides are not only found in CeD patients, they can be present in various other disorders (e.g. irritable bowel syndrome, IgA nephropathy, rheumatoid arthritis) and in some apparently healthy individuals as well. In contrast, antibodies to TG2 are highly disease specific and their detection is the first-line diagnostic test for CeD.

Autoimmune response in CeD can be stopped by the exclusion of gluten from the diet, which leads to the restoration of all reversible damages. However, reintroduction of gluten into the diet will restart inflammation and the autoimmune process, hence a lifelong gluten-free diet is the only currently known treatment for CeD patients.

There is a strong relation between the immune response to gliadins and TG2, and TG2 contributes to CeD pathogenesis by deamidating gliadin peptides, thereby increasing their immunogenicity. Gliadin peptides, besides T- and B-cell activation, also can trigger the innate immune system and induce cellular stress responses.

Antibodies to gliadin peptides can often be detected well before the manifestation of CeD, however, we have only limited knowledge about this early type anti-gliadin immune response. In the present study we followed up the humoral anti-gliadin immune response in CeD risk patients and compared the pre-manifest response with the one exerted at the time of CeD diagnosis.

2. Review of the literature

Celiac disease as an extraordinary autoimmune condition

CeD is a chronic T-cell dependent autoimmune disorder triggered by gluten ingestion in genetically predisposed individuals. The global prevalence of CeD is around 1% in the Caucasian population. CeD was first described by Aretaeus, an ancient Greek physician as an intestinal disorder associated with malabsorption. Gluten prolamins as the disease triggering antigens were discovered at the end of the 1940's by Willem-Karel Dicke, a Dutch

paediatrician. Afterwards, HLA-DQ alleles, the main genetic risk components of CeD and TG2-specific autoantibodies were identified.

Gastrointestinal symptoms of CeD are due to the intestinal inflammation that leads to small bowel villus atrophy and malabsorption. In CeD, the risk for other autoimmune disorders is increased, such as for type I diabetes mellitus, autoimmune thyreoiditis, autoimmune liver disorders, Sjögren's syndrome and rheumatoid arthritis; the reason behind these associations might be the presence of a shared genetic background.

The traditional diagnostic tool for the diagnosis of CeD is the histopathological evaluation of the small bowel biopsy specimens graded according to the Marsh classification based on small bowel villus height/crypt depth ratio and the number of intraepithelial lymphocytes. Marsh III grade lesions are diagnostic for CeD, but also Marsh II can be accepted for CeD diagnosis in the presence of TG2-directed antibodies. In recent years, serologic diagnosis of CeD also became possible following strict rules formulated by current international guidelines.

Antibodies to gliadin peptides are not perfect diagnostic tools for CeD because they can be also detected in other disorders and may be absent in some CeD patients. After the spreading of recombinant peptide technology and the recognition of the role of deamidation, deamidated gliadin peptide (DGP)-based ELISA assays have been developed, which provided enhanced specificity and sensitivity for CeD diagnosis and are still currently used in many diagnostic laboratories. Discovery of TG2 autoantibodies has opened a new era with outstanding specificity and sensitivity values for CeD diagnosis. ELISA tests utilizing recombinant human TG2 enzyme and the EMA (anti-endomysium antibody) immunohistochemical test both have high diagnostic performances. Although the EMA test cannot be performed as an automated measurement and requires high skills with expertise, this test is still relevant because of its high reliability.

Beyond their diagnostic values, TG2-specific antibodies might play some roles also in the pathomechanism of CeD. TG2-specific antibodies deposit in many tissues along the capillaries containing TG2 in their walls where they can damage the blood supply of various organs and thereby also their function. These autoantibody deposits might be in relation with the extraintestinal manifestations of CeD, such as liver, kidney, muscle and cardiovascular abnormalities, gluten ataxia, osteoporosis.

In contrast to most autoimmune disorders, CeD antibodies target a few favoured epitopes of gliadins and TG2. In case of TG2, there are two main epitopes which are clustered at the N-

terminal part of the enzyme (epitope 1, with key anchor residues K30, R116, H134 and epitope 2, with key anchor residues R19, E153).

This highly targeted immune response is well demonstrated by the biased usage of certain heavy and light chain variable domains of B-cell receptors (BCR) across different CeD patients and the used segments contain only restricted number of somatic hypermutations. These observations are true for both anti-gliadin and anti-TG2 antibodies, moreover, gliadin-specific T-cell receptors (TCR) are also characterized by the conserved utilization of some variable α and β-chain gene segments in pediatric CeD patients as well as in adult patients.

The pathomechanism of CeD is not yet fully elucidated. The most widely accepted explanation is the hapten-carrier model how an exogeneous antigen-driven adaptive immune response could be directed to a self-protein. According to this model, gliadin peptides and TG2 can form a covalent complex as a result of the cross-linking enzymatic activity of TG2. If this complex is taken up by TG2-specific autoreactive B-cells and presented to gliadin-specific T-cells, they can give activation signals to the autoreactive clones. This model, although well describes the gluten dependent autoantibody production even in the absence of TG2-specific T-cells, has not yet been experimentally proven neither in respect of the *in vivo* formation of the hapten-carrier complex nor for the factors contributing to the activation of the otherwise anergic B-cell clones. It is an open question as well whether the immune tolerance for gliadin is inherently missing or an existing tolerance mechanism will be disrupted at the disease onset. Gliadin-reactive T-cells cannot be detected in healthy individuals, probably because an active immune tolerance mechanism is operative, or, in HLA-DQ incompatible subjects, antigen presentation is supposed to be ineffective.

Adaptive immune response to gliadins

Gluten prolamins include the prolamin proteins found in wheat (gliadins and glutenins), barley (hordeins) and rye (secalins). Prolamin peptides of oat (avenins) belong to a distinct group, and usually do not trigger an immune response in the majority of CeD patients. The immunodominant antigens for CeD patients are the gliadin peptides of wheat, and these can be divided into three subgroups: α, γ and ω gliadins.

Gliadins are characterized by proline and glutamine rich repetitive sequences, which make these peptides highly resistant to human intestinal protease processing. Hence potentially immunogenic, 20-30 residues long, undigested oligopeptides will remain that can occupy the major histocompatibility complex II (MHC II) molecules' peptide binding groove. It has been

demonstrated in the case of 33-mer α-gliadin and 26-mer γ-gliadin peptides, that they remain intact even after extensive peptic-tryptic digestion.

The partially digested, potentially immunogenic peptides can traverse the intestinal barrier by transcellular transport or by endocytosis. In case of manifest CeD patients, the paracellular gliadin transport is increased as well, due to the loosened epithelial tight junction links.

The main genetic components of CeD are the disease predisposing HLA-DQ alleles, which encode MHC II molecules responsible for the antigen presentation toward T-cells of peptides of exogeneous (e.g., food) origin. HLA loci are highly polymorphic in the general population and the HLA-DQ2.5 genotype is the most common in CeD patients. HLA-DQ8 is also frequent, while a small proportion of patients bears the HLA-DQ2.2 genotype. The presence of these disease predisposing alleles is indispensable, however, *per se* not sufficient for the development of CeD, since the incidence of CeD is far less (~1%), than the estimated frequency of the risk alleles in the human population (~30%). It seems that the presence of further genetic and environmental factors is required for CeD development.

Prolamin peptides favour the polyproline II. conformation in soluble form, which is advantageous for their binding to the MHC II molecules. HLA-DQ2 or DQ8 molecules, unlike other HLA molecules, do accept proline-rich sequences in their peptide binding groove, despite that proline is unable to form a hydrogen-bridge. In HLA-DQ2 or DQ8 negative subjects lacking these peptide binding features, it is unlikely that a productive antigen presentation and adaptive anti-gliadin immune response will occur. Accordingly, gliadin-specific T-cells have not been detected in serum or biopsy samples of such individuals.

HLA-DQ2 and DQ8 molecules prefer to bind negatively charged residues in certain positions, however prolamin peptides contain very few negatively charged side chains in their native forms.

Through posttranslational modification, TG2 creates negatively charged residues in prolamin peptides by deamidating certain glutamine residues to glutamate. TG2 is ubiquitously present intra and extracellularly in many tissues and possesses multiple enzymatic activities (transamidase, deamidase, GTPase) that require mM order of magnitude Ca^{2+} ions and contribute to many physiological processes (e.g., cell adhesion, extracellular matrix organization, G-protein coupled signalling, apoptosis).

Sequence motifs (QXP, QXPF/Y or QXXF/Y) preferred by TG2 for deamidation can be found in almost all discovered celiac T-cell epitopes. The TG2-mediated deamidation exerts a selection bias for gluten epitopes, since deamidation increases the antigenicity of gluten

prolamins by ensuring more stable peptide binding by the HLA molecules and more frequent presentation toward the T-cells - hence CeD is an example of human disorders in which posttranslational modification plays a crucial role in the pathogenesis.

Up till now 38 T-cell gluten epitopes have been described in CeD patients. Most of them, i.e., 25 originate from gliadin peptides and 27 are HLA-DQ2.5 restricted. These facts reflect very well why gliadins are the main immunogens for CeD patients and why the HLA-DQ2.5 genotype has the highest risk for CeD development. Among gliadins, α and γ-gliadin peptides are similarly potent immunogens for T-cell activation, but the immunodominant T-cell stimulating sequence is the so called 33-mer peptide, which contains repetitions of the p57-68 fragment of α-gliadin (equivalent to the DQ2.5glia-α1 T-cell epitope).

Presentation of gliadin peptides results in an adaptive Th1 mediated immune response, and when the antigen presentation is performed by gliadin-specific B-cells, the activated B-cells differentiate into IgA and IgG antibody secreting plasma cells. Gliadin peptides do not have a tertiary structure, thus besides their HLA restricted T-cell epitopes, gliadin-derived B-cell epitopes are also linear. Moreover, these epitopes often overlap with each other and show high sequence similarity.

Multiple independent peptide library screening studies and pull-down experiments confirmed that the γ-gliadin derived QPQQPFP motif and its deamidated form QPEQPFP (collectively indicated here as QP(Q/E)QPFP) are the immunodominant antigenic sequences for gliadin-specific antibodies of CeD patients. Regarding α-gliadins, the QXQPFP motif present in the p31-43 and p57-68 peptides is also a proven antigen. Deamidation of gliadin peptides is essential for their T-cell mediated recognition, similarly, gliadin-specific antibodies display improved binding toward the deamidated epitopes. However, B-cell clones of CeD patients do not distinguish deamidated and non-deamidated peptides and only a fraction of the B-cells binds exclusively deamidated sequences.

The p31-43 α-gliadin fragment (LGQQQPFPPQQPY) is also known to evoke innate immune activation and stress signals called as a ‘toxic’ effect. In Caco2 cell culture and CeD patient derived biopsy samples the p31-43 peptide triggers increased cell proliferation, actin rearrangement, production of reactive oxygen species (ROS), activation of TG2 and NFκB. Reason for these stress responses is that the p31-43 peptide can interfere with the maturation of the early endocytic vesicles, thereby increasing endosome recirculation and cell surface exposure of the epidermal growth factor / epidermal growth factor receptor (EGF/EGFR) complex as well as the interleukin-15/interleukin-15 receptor α (IL-15/IL-15Rα) complex. IL-15 mRNA expression is also increased by p31-43, which contributes to IL-15/IL-15Rα complex

overrepresentation, a phenomenon that is considered to lead to the activation of intraepithelial lymphocytes and to the break of oral tolerance against gluten. By altering vesicle circulation, the p31-43 peptide may escape the lysosomal pathway and the antigen presentation via MHC II. Instead, it can activate the innate immune system in the toll like receptor 7 (TLR7) dependent pathway and induces interferon-α (INFα) production.

As in the case of many other disorders, we are in lack of information about the immune response preceding the disease manifestation of CeD and there is no proper animal model to study CeD development. The gluten-dependent TG2-specific autoimmune response and subsequent intestinal villous atrophy could not be so far properly established even in humanized mouse models. The most appropriate method to study CeD pathomechanism is still the investigation of clinical samples collected in prospective studies.

3. Aims

In the present study our aim was to monitor the humoral anti-gliadin immune response from early age, and to compare the characteristics of serum antibodies detected in the pre-disease phase and at the diagnosis of CeD. For this purpose, we took advantage of serum samples collected in prospective, international clinical studies (ProCeDE, PreventCD) and investigated the CeD relevant antigens utilizing recombinant proteins and synthetic peptides containing the immunodominant epitopes.

We analysed the TG2 mediated deamidation of p31-43 and p57-68 α-gliadin peptides by the wild type Val^{224} TG2 enzyme and compared the antigenicity of the native and the deamidated α and γ-gliadin peptides utilizing CeD patients' serum samples. We examined the possible cross-reactive recognition of the similar gliadin peptide sequences by individually affinity purified antibody populations utilizing single peptides as antigen.
We monitored the appearance of the anti-gliadin antibodies of CeD risk subjects from the first gluten intake and investigated their recognition and binding affinity to the native versus deamidated immunodominant epitopes. We also explored whether anti-gliadin antibodies predicted later disease manifestation. Further, we investigated the epitope specificity of the emerging TG2-specific antibodies.

4. Patients and methods

Patients

Serum samples utilized in the present study originated at one hand from the ProCeDE international study, comprising 69 untreated Hungarian CeD patients (age 1.8 – 17.3 years, median 6.2). The ProCeDE study investigated the accuracy of diagnostic tests among TG2 antibody positive subjects. Furthermore, we utilized 720 serum samples of 122 infants who participated in Prevent CD study (http://www.preventceliacdisease.com), a prospective clinical study enrolling newborns of CeD affected families with high risk to develop CeD. Blood samples were collected at the ages of 4, 6, 9, 12, 24, and 36 months and at the time of CeD diagnosis (if the diagnosis not coincided with the aforementioned timepoints). Enrolment into the PreventCD study took place in 8 countries between 2007 and 2010. First, the HLA-DQ genotype of the infants was determined and HLA-DQ2.5, DQ2.2 or HLA-DQ8 positive subjects were randomized into an intervention group (Group A) and a placebo group (Group B). Infants in Group A received 200mg of vital gluten (Danone Research BV, Wageningen, The Netherlands) between the age of 4 and 6 months in hope to induce oral tolerance, while infants in Group B received lactose containing placebo in the same period in a double-blind fashion. Infants in both groups consumed gradually increasing amounts of gluten between the age of 7 and 10 months, with no restriction thereafter. Prospective follow up of the PreventCD children is still going on, they are now 11-14 years old.

The CeD diagnosis was confirmed in both patient cohorts with EMA positivity and the presence of Marsh III lesion in the duodenum by histopathology examination. Enrolment and sample collection were performed upon informed consent from the parents that their child's serum will be used for different antibody testings related to CeD in different laboratories. For the current study 122 children from Germany (n = 60) and Hungary (n = 62) were included. The ProCeDE and PreventCD studies were conducted in accordance with the Declaration of Helsinki and approved by the medical ethical committees of the participating centers.

HLA-DQ2 or DQ8 negative infants were excluded from the original PreventCD study, however, after the end of the prospective enrolment period, some of the younger sibs of the participating children with unknown HLA-DQ background also underwent gluten intervention for parental request. The intervention protocol was identical with that of the original study, however the vital gluten was obtained from other source (Sedamyl, Saluzzo, Italy). One single blood withdrawal was performed in these children at the age of 6 months after the intervention period to determine HLA-DQ genotype and serum antibodies and 11 DQ2/DQ8 negative infants were found. These children were included in the present study as controls.

Peptides and reagents

Synthetic gliadin peptides with N-terminal biotinylation and >95% purity were purchased from GenScript (Leiden, The Netherlands) with the following sequences: γGlia_Q (SGGPLQPQQPFP), γGlia_E (SGGPLQPEQPFP), γGlia_sh (SGGPEQPFP), p31-43 (LGQQQPFPPQQPY), p31-43_E34 (LGQEQPFPPQQPY), p31-43_E40 (LGQQQPFPPEQPY), p57-68 (QLQPFPQPQLPY), p57-68_E65 (QLQPFPQPELPY) and an irrelevant sequence (VVKVGGSSSLGW) as negative control. A complex gliadin peptide utilized for antibody purification from PreventCD serum samples was the gift from Inova Diagnostics (San Diego, CA, USA). The deamidated gliadin peptide antigen used in Gliadin DP ELISA clinical kit and its non-deamidated version mounted on the MeDALL chip was from Thermo Fisher Scientific (Freiburg, Germany).

Investigation of deamidation of gliadin peptides by tandem mass spectrometer coupled with liquid chromatography (HPLC-MS/MS)

Deamidation of p31-43 and p57-68 synthetic gliadin peptides was tested in MOPS buffer (50mM, *p*H 6.8) containing 5mM $CaCl_2$ and 1mM dithiothreitol, utilizing 50 pmol of recombinant human TG2 (Val^{224} variant) enzyme. The reaction mix was incubated at 37ºC for 2 hours with a TG2 : gliadin peptide molar ratio of 1:150. The reaction was stopped by heat inactivation of TG2, then peptide products were separated from the enzyme by a centrifugation step using Amicon ultra 10K membrane (Merck, Darmstadt, Germany). Prior to the LC-MS/MS analysis, peptides were further purified by C18 PierceTip (Thermo Scientific, Rockford, IL, USA) following the manufacturer's instructions. The LC-MS/MS analysis was performed at the Proteomics Core Facility of the University of Debrecen by Prof. Dr. Éva Csösz and Dr. Gergő Kalló with Orbitrap Fusion tribrid mass spectrometer (Thermo Scientific, Rockford, IL, USA). Results were visualized using the Scaffold software (Proteome Software Inc. Portland, OR, USA).

Affinity purification of gliadin-specific antibodies

For antibody quantification calibration and antibody cross-reaction testing, individual affinity purification was performed with single α and γ-gliadin peptides from the serum samples of two CeD patients. Biotinylated gliadin peptides were immobilized to Pierce™ High Capacity Neutravidin Agarose resin (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Serum samples were diluted in phosphate buffered saline containing 0.1% Tween 20 (TPBS *p*H 7.4) and incubated with the peptide bound resins.

Antibodies were eluted with 100mM glycine buffer *p*H 2.5, followed by buffer exchange to PBS using 50K Amicon® (Merck, Darmstadt, Germany) ultra-centrifuge filters. Protein concentration was determined by Bio-Rad Protein Assay (Biorad Laboratories, Hercules, CA, USA) using human IgG (Thermo Scientific, Rockford, IL, USA) as a standard.

To investigate kinetic parameters of gliadin-specific serum antibodies of PreventCD infants (n=10), cyanogen bromide-activated sepharose 4B resin (Thermo Scientific, Rockford, IL, USA) linked with synthetic gliadin peptide reagent (Inova Diagnostics, San Diego, CA, USA) was applied for antibody purification. Affinity purification was performed as described above.

Quantification of gliadin-specific antibodies from serum samples by bio-layer interferometry

To determine serum concentration of anti-gliadin antibodies, label free bio-layer interferometry (BLI) device (Personal Assay BLItz, PALL FortéBio, Fremont, CA, USA) and streptavidin (SA) biosensors (FortéBio, Fremont, CA, USA) were applied. The BLI instrument registers antibody binding in real time based on the optical density change on its biosensor tip upon molecular interactions. Biotinylated peptides were immobilized onto SA biosensors and the measurements were performed in TPBS buffer. Separate calibration curves were created with each α and γ-gliadin peptide utilizing antibodies affinity purified with the given peptide. Based on the calibration curves, peptide-specific antibody concentrations were calculated.

Measuring relative binding rates of the affinity purified antibodies by bio-layer interferometry

To determine the relative binding rates of the affinity purified anti-gliadin antibodies to the different α and γ-gliadin peptides, antibodies diluted in TPBS buffer were added in 150nM concentrations to each peptide and binding rates were generated from the binding curve by logarithmic regression where the slope is proportional to the quantity of the bound analyte. After the subtraction of the values obtained with the negative control peptide, duplicates were averaged and normalized in the way that the binding rate to the peptide whereby the antibodies had been purified was set to 100%.

Measuring the binding strength of gliadin-specific antibodies by bio-layer interferometry

Equilibrium dissociation constants (K_D) of affinity-purified anti-gliadin antibodies were measured by BLI using SA biosensors (FortéBio, Fremont, CA, USA) loaded with biotinylated gliadin peptides. The purified antibodies were added in 240, 120, 60, and 30nM concentrations

diluted in TPBS buffer. Kinetic analysis included 5 min association and 5 min dissociation steps measured in duplicates. K_D values were calculated by the BLItz Pro 1.2.1.5 software, using 1:1 kinetic model.

Testing for the biological effect of gliadin peptides in Caco2 cell culture

To examine the 'toxic' effect of gliadin peptides a previously published method was used. Caco-2 cells (American Type Culture Collection, Rockville, MD, USA) were grown to confluency in Minimal essential medium. Before the experiment, FBS content of the culture media was reduced to 1% for overnight and cells were incubated with 100 μg/mL biotinylated synthetic peptides for 24 hours at 5% CO_2 and 37ºC. Cells were fixed with 4% paraformaldehyde and permeabilized by 0.05% TritonX followed by incubation with monoclonal mouse anti-ZO1 antibodies (1:200; Zymed Inc. San Francisco, CA, USA) at 4ºC overnight. Alexa Fluor 488 labelled goat anti-mouse IgG antibodies (1:1000; Invitrogen, Carlsbad, CA, USA) were applied as secondary antibodies. Cell membranes and tight junction structures were visualized and evaluated by using Olympus IX81 microscope (Olympus, Hamburg, Germany) in the laboratory of Prof. Dr. János Szöllősi at the Department of Biophysics and Cell Biology of the University of Debrecen.

Detecting serum antibodies by MeDALL protein microarray

MeDALL protein microarray was loaded with the clinically validated deamidated gliadin peptide antigen of the Gliadin DP ELIA diagnostic kit (Thermo Fisher Scientific, Freiburg, Germany) and its non-deamidated counterpart, which were gifts from Thermo Fisher Scientific for the study. Wild type tissue transglutaminase (TG2) with valine at position 224 (Val^{224} natural variant) and its double mutant form (TG2 RE) where the Arg^{19} and Glu^{153} key anchor residues for celiac epitope 2 antibody binding had been mutated to serine, both produced earlier by our research group, were tested in the microarray as well. Serum samples were measured by the MeDALL microarray method at the Department of Pathophysiology and Allergy Research, Medical University of Vienna by Dr. Bharani Srinivasan in research collaboration. Antigens were spotted in 0.3 mg/mL concentrations onto the surface of pre-activated glass slides (VBC-GENOMICS) by automatic pipetting (Affymetrix 417 Arrayer, Affymetrix, Santa Clara, CA, USA) and linked to the surface with amine coupling covalent binding. Serum samples were diluted 1:5 in TTBS buffer and incubated with the antigens for 60-180 minutes at 37°C by 150rpm rocking. Bound IgA and IgG antibodies were detected by Alexa Fluor 546 labelled mouse monoclonal anti-human IgA or IgG (Pharmingen, San Diego, CA, USA) diluted 1:1000

in the assay buffer. Fluorescent signals were read by Affymetrix 418 scanner (Affymetrix), and raw data were analysed by GenePix Software Version 3.0 (Axon Instruments, Union City, CA, USA). Signals were regarded as positive if the average of the triplicate values exceeded five times the signal obtained with human serum albumin.

Statistical analysis

Statistical analysis was performed by GraphPad Prism7 (San Diego, CA, USA) software. Comparison of two subject groups was made by unpaired two-tailed T-test, while in the case of more than two groups one-way ANOVA with Tukey's test was used. To compare different conditions in the same subject group the paired two-tailed T-test was applied. To analyse correlation between data showing normal distribution the Pearson test was performed, while in other cases the nonparametric Spearman's Rank test was used to examine data sets' correlation. Receiver operated characteristics (ROC) analysis was performed to reveal the predictive potential of antibody results.

6. Results

TG2-mediated deamidation of α-gliadin peptides

We examined deamidation of p31-43 and p57-68 synthetic α-gliadin peptides by recombinant human TG2 Val^{224} enzyme and detected the deamidation sites by tandem mass spectrometer coupled with liquid chromatography (LC-MS/MS). Based on the latest genomic results in the literature the natural human variant of TG2 contains valine at position 224, contrary to widely used earlier generated recombinant TG2 enzymes with glycine in this position. We investigated whether the Val^{224} TG2 enzyme can reproduce the known deamidation pattern of the p57-68 peptide and whether it can deamidate the p31-43 peptide, for which no direct experimental result was available. Our results confirmed the deamidation of the p57-68 peptide at positions Q65 and Q59, the former leads to the generation of the known DQ2.5-glia-α1a T-cell epitope. In case of the p31-43 peptide we detected deamidation of the Q34, Q35 and Q40 glutamine residues either as single changes or in combination. Deamidation of the Q59 and Q35 residues resulted in the QLEPF and QQEPF motifs, respectively, which are already proven to be poor celiac antigens based on experiments in the literature, while deamidation of Q65, Q34, and Q40 may create CeD relevant B-cell epitope motifs.

To investigate anti-gliadin serum antibodies, we chose eight synthetic gliadin peptides based on our deamidation experiment and on the celiac B-cell epitopes described in the literature

(please find sequences in the patients and methods chapter). These peptides represent the α- and γ-gliadin epitopes of interest in native, deamidated and shortened forms.

Comparison of the antigenicity of α and γ-gliadin sequences for CeD patients

We investigated serum antibody bindings towards the native and deamidated α and γ-gliadin peptides, respectively, by utilizing serum samples of 69 anti-TG2 antibody positive, untreated CeD patients who participated in the prospective ProCeDE clinical study. These patients already had been tested by multiple clinical serologic assays (6 anti-DGP, 8 anti-TG2 antibody tests and the EMA test). However, those tests utilize complex antigens and their results are expressed in relative units based on different calibrators, hence comparison of these results is not easy. In our experiments we used a bio-layer interferometry device (BLItz) which detects molecular interactions in real time and in a label-free manner based on the optical density change and which provides absolute concentration (μg/mL) values. For the quantitation of serum antibodies we constructed calibration curves with each synthetic gliadin peptide separately by utilizing affinity purified antibodies isolated from CeD patients' serum samples with the respective peptide. Our results show that anti-gliadin antibodies of CeD patients predominantly recognized γ-gliadin sequences. In case of the p31-43 α-gliadin peptides moderate antibody binding was registered, while p57-68 α-gliadin peptides were very poor antigens for the serum antibodies.

We observed improved binding tendency of serum antibodies to the deamidated α and γ-gliadins compared to the native ones, but this difference did not reach statistical significance.
Serum antibody concentrations obtained by the γGlia_E single peptide antigen containing the PLQPEQPFP sequence were in good correlation with the results of all six clinical anti-DGP IgG antibody tests. At the testing of these correlations we took into account whether the given clinical test was based on logarithmic (optical density based) or concentration proportional (calibration curve based) calibration. The importance of this result is that the manufacturers use complex antigens with proprietary sequences, while in our BLI assay we utilized a short, single peptide. We were able to detect the vast majority of the total anti-gliadin antibody amounts just by utilizing the γGlia_E peptide, confirming that γ-gliadin-specific antibodies represent the predominant anti-gliadin antibody population in CeD patients' serum samples.
When we analysed the serum antibody bindings of patients individually, we observed that none of them displayed higher antibody binding to the α-gliadin peptides than to γ-gliadins.

Remarkable binding to the α-gliadins was only detected in patients with even much higher γ-gliadin-specific antibody levels.

The γGlia_E peptide contained the full deamidated QPEQPFP heptapeptide epitope, the other synthetic peptides with shorter homologous sequences as antigens resulted in significantly reduced antibody binding. Deamidated antigens were better recognized in the shortened sequences than in their native counterparts. In the absence of the PQQ motif the peptides' antigenicity obviously and drastically declined.

First appearing anti-gliadin antibodies target γ-gliadin peptides

We investigated the early humoral anti-gliadin immune response by utilizing serum samples collected in the course of the PreventCD study. This study enrolled infants with high risk for CeD, who were prospectively followed from the first gluten intake. Here we tested by bio-layer interferometry the serum samples of 21 patients who developed CeD (samples were collected at the age of 4 months, 9 months, and beyond the age of 2 years at CeD diagnosis) to compare the antibody binding to α and γ-gliadin peptides.

We detected low levels of γ-gliadin binding antibodies in three cases at the age of 4 months, however, those antibodies were of maternal origin since in each case the mother was having an active, seropositive CeD at giving birth and infants did not consume yet gluten at the time when the samples were collected. Further investigation showed that these antibodies were of only IgG subclass, supporting their transplacental origin.

At the age of 9 months infants were adherent to gluten containing diet and anti-gliadin antibodies were detected, which exclusively recognized γ-gliadin peptides, suggesting that first the γ-gliadin-specific B-cell clones had been activated. Beyond the age of 2 years, at the time of CeD diagnosis with anti-TG2 antibody positivity and villous atrophy, antibodies recognizing the p31-43 and p57-68 α-gliadin peptides also appeared, but the humoral immune response was still dominated by antibodies binding to γ-gliadin peptides.

Cross-reactivity of gliadin-specific antibodies between α and γ-gliadins

To examine the possible cross-reactive feature of the gliadin-specific antibodies we affinity purified antibodies from CeD patients' serum samples utilizing the different synthetic gliadin peptide sequences separately as antigen, then we tested the binding of the obtained antibody populations with all used native and deamidated gliadin peptides separately.

Antibodies isolated by using the γGlia_Q and γGlia_E peptides displayed similar binding features, both recognized preferentially the γ-gliadin peptides and they also bound to the p31-43 peptides (with highest rate to the p31-43_E40 peptide), but their binding to the p57-68 peptides was almost absent. Among the γ-gliadin peptides, γGlia_sh was only moderately antigenic, reason of that might be the reduced length of this peptide. Interestingly, antibodies isolated by the γGlia_E peptide bound similarly to the deamidated (γGlia_E) and native (γGlia_Q) γ-gliadin peptides, which is in line with our serum antibody quantitation results.
Interestingly, when the p31-43 or p57-68 α-gliadin peptides were utilized as the purification antigen, the obtained antibodies preferentially recognized the γGlia_Q and γGlia_E sequences over the α-gliadin peptides, clearly indicating the cross-reactive properties of the antibodies. Antibodies isolated by the p57-68_E65 and p31-43_E34 deamidated peptides preferentially bound to the deamidated epitopes over the non-deamidated ones, not only in the case of α-gliadins, but also in the case of γ-gliadin peptides.

Biological effect of the highly similar gliadin sequences on Caco2 cell line

Since the antibodies displayed high degree of cross-reactions between the p31-43 and γ-gliadin peptides, we examined whether the γ-gliadin peptides are also able to exert the well-known cellular (so called 'toxic') effect of the p31-43 peptide in Caco2 cell line due to their sequence homologies. The p31-43 peptide triggers the loss of tight junction connections in Caco2 cell culture and this can be visualized by the immune fluorescent labelling of ZO-1 protein in the cell membrane giving a straightened appearance with the loss of the wavy interdental structure. We were able to produce this known biological effect with the p31-43 peptide, however, neither the p57-68, nor γ-gliadin peptides triggered the loss of tight junction connections, despite their highly similar sequences. Based on these results the 'toxic' effect of the p31-43 peptide is unique and not related to the sequence parts of homology with the γ-gliadin peptide sequences.

Investigating the samples of the PreventCD study – clinical outcome

Next, we investigated in prospectively collected serum samples the anti-gliadin antibody response of patients who participated in the international PreventCD study. The PreventCD study gave a unique opportunity to follow up the immune response of infants at risk of CeD development from the first gluten intake, furthermore, to compare the immune response of children who later developed CeD and of not affected children.

The primary aim of the original PreventCD study was to attempt the prevention of CeD development by the early introduction of small doses of gluten to infants at genetic risk to

develop CeD. Infants in the intervention group received vital gluten powder from 4 to 6 months of age (Group A), while in the control group infants took placebo from 4 to 6 months of age (Group B). After the age of 6 months, infants in both groups received gluten in gradually increasing amounts between 7 to 10 months of age, with no restriction thereafter. The scheduled appointments for blood tests were 4, 6, 9, 12, 24, 36 months of age, additional samples were collected at the time of CeD diagnosis that can differ from the previous timepoints. Based on the outcome of the PreventCD study, the primary endpoint was not met, since there was no significant difference in CeD prevalence between the intervention and placebo groups. However, availability of these prospectively collected serum samples gives a unique opportunity to investigate the effect of uniformly dosed gluten in early infancy and compare it with age-matched controls.

We investigated the changes of the humoral immune response targeting the main antigens of CeD (non-deamidated gliadin peptides – NGP, deamidated gliadin peptides – DGP and tissue transglutaminase – TG2) by the MeDALL protein microarray technique for the simultaneous monitoring of multiple antibody concentrations and to compare the antibody response of children who developed CeD and who remained disease-free.

Analysis of the early antibody response after the primary gluten exposure

Infants in Group A displayed remarkable antibody production in response to the 8 weeks gluten intervention. Both IgG and IgA antibodies were detectable at the age of 6 months in the serum samples which recognized NGP and DGP as well, but TG2-specific antibodies (indicative for CeD) were absent. During this early antibody response, antibody binding to the NGP antigen was significantly higher compared to the DGP antigen. Presence of gliadin-specific IgA antibodies proved that infants responded by their own adaptive immune system to the ingested food antigen, since this immunoglobulin isotype cannot be transferred transplacentally or by breast feeding. Infants in Group B took placebo until the age of 6 months. Accordingly, these serum samples did not show any reactive antibody positivity at 6 months, however, at the age of 9 months when children in Group B also consumed a gluten-containing diet, infants displayed similar antibody production as in Group A with binding to NGP and DGP.

Evaluation of the early antibody response in relation to future CeD manifestation and genotypes

In Group A, infants carrying the HLA-DQ2 and DQ8 risk alleles responded with similar IgA and IgG antibody production to the gluten intervention irrespective of the future development

of CeD, moreover newborns of CeD affected families but negative for the HLA-DQ risk alleles also showed a similar antibody response. In each subgroup, including the children developing CeD, antibodies preferentially bound to NGP over DGP during this early anti-gliadin response.

Interestingly, we observed that infants carrying HLA-DQ2.5 and thus have higher risk to develop CeD, produced significantly lower levels of IgA and IgG anti-gliadin antibodies in response to the same gluten dose than infants with HLA-DQ2.2 or HLA-DQ2.3 genotypes predisposing to CeD with a much lower risk. HLA-DQ2.5 homozygous infants, who have the highest risk to develop CeD, produced the lowest levels of antibodies, moreover, antibody response measured in serum after the primary gluten intake was totally absent in some cases.

Changes in NGP/DGP binding during the early antibody response and at the manifestation of CeD

When we compared the NGP and DGP antibody response of risk infants in Group A who developed CeD or remained healthy, CeD developing infants displayed two peaks in antibody production: the first at the age of 6 months, i.e. at the early antibody response to the primary gluten intake, and the second at the time of the diagnosis of CeD. In-between the antibody production decreased. Infants who did not develop CeD showed one single peak, the early antibody response after the primary gluten intake. Irrespective of future CeD development, the NGP recognition was preferential over DGP binding. The children who did not develop CeD continued to have the preferential recognition of NGP during the whole follow up time, while those children who developed CeD started to have significantly increased antibody production towards DGP by the time of CeD diagnosis both for IgG and IgA antibodies (IgA response was less uniform, absent in some cases).

Similarly, also in Group B both CeD developing and non-developing children produced antibodies at the age of 9 months, after their primary gluten intake, which preferentially recognized NGP (until the age of 6 months they took placebo). This tendency only changed in children developing CeD. At the time of CeD diagnosis, the antibody binding preference shifted in them towards DGP.

Our results with multiple antibody monitoring suggest that this shift in anti-gliadin antibody targeting towards DGP occurs at the same time when the TG2-specific antibodies appear.

Affinity maturation towards the PEQPFP epitope at the time of CeD manifestation

We examined by synthetic single peptides and bio-layer interferometry whether the anti-gliadin antibodies produced by CeD risk children during the early immune response targeted the CeD-

relevant epitopes or not, and whether antibody binding strength changed during disease manifestation.

To this end we affinity purified gliadin-specific antibodies from serum samples collected at the age of 6 months from children developing CeD (n=3), not developing CeD (n=3) or from serum samples collected at the time when CeD was diagnosed (n=4). We determined the equilibrium dissociation constant (K_D) of the antibodies utilizing the following peptide sequences: non-deamidated γGlia_Q (PLQPQQPFP), deamidated γGlia_E (PLQPEQPFP) and short deamidated γGlia_sh (PEQPFP) peptide, each consisting of a single epitope, thus offering monovalent binding.

Gliadin-specific antibodies produced at the age of 6 months by future CeD children or not affected children both bound γGlia_Q (mean K_D 2.5×10^{-8} M and 1.2×10^{-7} M respectively) and γGlia_E (mean K_D 3.5×10^{-8} M and 1.7×10^{-7} M respectively) peptides with considerably high affinity, suggesting that - irrespective of later CeD development - the early antibody response to the ingested gluten targeted the immunodominant gliadin epitope. The antibodies produced at the age of 6 months did not bind in relevant manner to the short γGlia_sh peptide (mean K_D 1.3×10^{-4} M and 1.8×10^{-4} M respectively). However, the antibodies produced at the time when CeD was diagnosed showed high affinity interaction not only with the γGlia_Q and γGlia_E peptides (mean K_D 3.6×10^{-8} M and 1.6×10^{-8} M respectively), but also with the γGlia_sh peptide, confirming a significant affinity maturation to the deamidated PEQPFP motif (mean K_D 5.8×10^{-7} M).

Mean K_D values of antibodies for the γGlia_Q and γGlia_E peptide did not show significant differences between the patient groups. However, when we calculated the K_D γGlia_E / K_D γGlia_Q ratio in the same individual and established means of these values, it was obvious that antibodies produced at the age of 6 months displayed significantly higher affinity towards the non-deamidated γGlia_Q peptide, while antibodies produced at the time of CeD diagnosis had significantly higher affinity towards the deamidated γGlia_E peptide. These changes in antibody affinity were in line with the observed preferential binding of the serum antibodies to NGP at the time of the early immune response and with the significant increase in previously underrepresented DGP recognition at the manifestation of CeD.

Anti-gliadin antibodies produced in the absence of anti-TG2 antibodies are poor indicators of CeD

Antibodies to NGP and DGP were commonly found in our experiments in children at risk of CeD but who remained healthy. We examined by ROC analysis whether we could distinguish

the CeD developing and non-developing children based on the antibody concentrations produced against NGP or DGP and how the appearance of anti-TG2 antibodies influenced this prediction.

The ROC curve for antibody positivity to NGP or DGP at any age produced in the absence of anti-TG2 antibodies resulted in low area under the curve (AUC) values (AUC=0.51 for NGP IgG and AUC=0.53 for DGP IgG), thus it cannot distinguish at any cut-off concentration the children who develop CeD or who do not. However, when we took only those positive NGP or DGP antibody values into account which were coupled with anti-TG2 antibody production, the ROC analysis resulted in significantly improved AUC values (AUC=0.94 for NGP IgG and AUC=0.99 for DGP IgG). ROC analysis of the IgA antibodies to NGP or DGP had similar outcome with slightly inferior AUC values.

Our results argue that neither the early anti-gliadin antibody production following the primary gluten introduction, nor anti-gliadin antibodies at any later age would *per se* discriminate CeD and non-CeD cases. Only TG2-specific antibody production can successfully distinguish these two groups, underpinning that TG2-positivity is the gold standard for CeD diagnosis.

The first appearing TG2-specific antibodies target the epitope 2 surface

During the clinical follow up, 33 out of the 122 children (27%) investigated from the PreventCD study were diagnosed with CeD by duodenal biopsy and Marsh III lesions, at the median age of 3 years, range 2-5.5 years. Anti-TG2 antibody positivity was present in all these children - as measured by clinical ELISA and EMA tests - at the time of CeD diagnosis. For the MeDALL microarray measurements only 24 serum samples were available from the time of the diagnosis, since CeD manifested in 9 children after the inclusion of the samples for the MeDALL analysis.

Monitoring the appearance of TG2 autoantibodies, elevated serum IgA to TG2 was detected exclusively in the 24 CeD cases, and only in the samples collected at the time of CeD diagnosis. Anti-TG2 IgG antibodies were detected in 20/24 of them and in low concentrations (there was no IgA deficient CeD patient in the present study). Elevated levels of antibodies to NGP and DGP occurred in all CeD cases at diagnosis, but their serum concentrations did not correlate with those of anti-TG2 antibodies based on Pearson's test (TG2 IgA vs NGP IgA r=0.21 and TG2 IgA vs DGP IgA r=0.16).

We also examined the epitope specificity of anti-TG2 autoantibodies by utilizing the double mutant TG2 RE enzyme characterized by our research group in earlier studies. In TG2 RE, two key anchor residues of the celiac epitope 2 surface (Arg^{19} and Glu^{153}) are mutated to serine, thus epitope 2-specific antibody binding is abolished. Serum antibody binding to this double

mutant enzyme was only 17% compared to the wild type TG2 enzyme, suggesting that vast majority of the produced anti-TG2 antibodies target the epitope 2 surface. Antibodies to epitope 1 and to other TG2 surfaces were thus responsible for approximately only one-sixth of the total anti-TG2 antibody population.

6. Discussion

In the present study we have demonstrated by using label-free quantitative analysis and antibodies affinity-purified by single gliadin peptides, that although γ-gliadins are the main target of the humoral immune response, α-gliadin sequences might be immunogenic for the B-cells as well and their antigenicity could be increased by TG2-mediated deamidation.

Two α-gliadin peptides have major importance in the pathomechanism of CeD: the p31-43 and the p57-68 peptide fragments. The p57-68 peptide is the immunodominant DQ2.5 restricted T-cell activator gliadin peptide, of which the immunogenicity is significantly increased by TG2-mediated deamidation, while the p31-43 peptide is able to activate the innate immune system by evoking a cellular stress response. The TG2-mediated deamidation of gliadin peptides was previously tested mostly in complex gluten mixtures, initially by using guinea pig TG2 enzyme and afterwards by recombinant human TG2 Gly^{224} enzyme. However, the most common natural variant of the human TG2 enzyme is containing valine at the position 224. We thus examined the TG2-mediated deamidation of p31-43 and p57-68 peptides by the Val^{224} natural TG2 variant since the glycine/valine change might influence the enzyme's activity. Based on the mass spectrometry results deamidation occurred in positions Q34 and Q40 in case of the p31-43 peptide, which resulted the QEQPF and PEQPY motifs, respectively. Our results are in line with data in the literature that TG2-mediated deamidation preferentially occurs at QXP motifs. In the case of the p57-68 peptide, deamidation at Q65 was abundant, confirming the generation of the well-known PELPY motif.

Our bio-layer interferometry results did indicate serum antibody binding of CeD patients to the native and these deamidated forms of the p31-43, and p57-68 α-gliadin peptides, but with much lower antibody concentrations than to γ-gliadin peptides which predominated in the humoral immune response from the beginning of the seroconversion. Moreover, the first appearing gliadin-specific antibodies recognized exclusively the γ-gliadin peptides. Although the serum antibodies bound slightly better to the deamidated α-gliadin peptide forms compared to the non-deamidated ones, the difference was not statistically significant and none of the patients had higher reactivity to p31-43 or p57-68 α-gliadins than to γ-gliadins, further underpinning the dominant role of γ-gliadins in CeD patients' B-cell immunity.

In fact, antibodies measured by using the single γGlia_E peptide antigen (core target peptide: QPEQPFP) in bio-layer interferometry assay showed good correlation with the results of the anti-DGP IgG clinical diagnostic tests, indicating that this reactivity represents the majority of anti-gliadin antibodies. At the same time, this result also validated our measurements with the bio-layer interferometry method.

To our knowledge this is the first time to take advantage of using different single peptide antigens for antibody purification from CeD patient's serum samples and investigate the antigen-binding preferences and cross-reactivity of the obtained antibodies. We found that antibodies purified with any of the gliadin peptides recognize the QP(Q/E)QPFP γ-gliadin motif the most efficiently. Antibodies recognizing α-gliadins appeared only at later age and were always in minority. These observations indicate that γ-gliadins are the main antigens, but antibodies cross-reactive with α-gliadins also emerge during the humoral immune response.

Gliadin-specific serum antibodies of CeD patients also displayed a remarkable cross-reactivity between the non-deamidated and deamidated γ-gliadin sequences corresponding to the results of the single peptide-based affinity-purified antibody binding assay. This observation is in line with the previous results that gliadin-specific B-cell clones of CeD patients are able to recognize γ-gliadin peptides also in deamidation independent manner.

Cross reaction of serum antibodies among the gliadin peptide sequences also has important clinical implications in diagnostic testing. This feature may substantially enhance the sensitivity of the clinical detection of celiac antibodies, well demonstrated by our serum antibody quantitation measurements obtained with the γGlia_E single synthetic peptide containing the deamidated γ-gliadin QPEQPFP motif, which correlated very well with the results of the clinically used commercial DGP IgG assays. The clinical assays utilize multiple, publicly not known peptide sequences, and the measured values are expressed in relative units. A novel, bio-layer interferometry-based assay would be a suitable tool to standardize the calibration and express specific antibody concentrations in μg/mL. This could overcome the discrepancies between the different commercial clinical tests and clinical centers and could set a more appropriate common diagnostic cut-off value. It is important to note, that 0-39% of antibody values measured in the ProCeDE clinical study by the different clinical tests were outside of the detection range. These high values may be useless for the follow up of the therapeutic effect of a gluten-free diet in the initial phases of the treatment.

Another advantage of the bio-layer interferometry is that it can quantitate IgA and IgG antibodies in one step. Based on the clinical studies, assays measuring IgG antibodies have

higher sensitivity, while assays detecting IgA antibodies have higher specificity; in BLI detection these advantages could be combined.

Anti-gliadin antibodies could be present in the circulation prior to CeD manifestation however, we have limited knowledge about the characteristics of this adaptive immune response preceding the diagnosis of CeD, its predictive value and how it differs from the anti-gliadin response observed at the time of CeD diagnosis.

We took advantage of the international PreventCD study to answer some of these questions. PreventCD was a prospective, dietary intervention study enrolling infants with a high genetic risk for CeD, thus its results are not in all respects relevant for healthy infant populations. Strength of the study was that despite its prospective nature, remarkable number of CeD cases manifested during the follow up time and the gluten intervention was performed with equalized dosage. Anti-gliadin and anti-TG2 antibodies were monitored extensively, ensuring reliable and comparable results and the early detection of disease manifestation.

We applied the MeDALL microarray system containing the CeD relevant antigens (DGP, NGP, wild-type human TG2 and epitope 2-depleted mutant TG2) to quantitatively evaluate antibody production at multiple timepoints. Clinical ELISA/ELIA assays are calibrated for the low range of antibody concentrations to achieve the best possible sensitivity in clinical care and thus signal saturation is common, while the fluorescent detection in the microarray system allowed us antibody quantitation in a broad linear range and made quantitative changes more readable.

To our knowledge, this is the first time when simultaneous follow-up of both anti-NGP and DGP antibody response in infants at risk of CeD and determination of the binding strength of the produced antibodies have been performed.

Results of the German and Hungarian participants in the PreventCD study revealed that the gliadin-specific antibodies produced in response to early-age gluten exposure preferentially recognized non-deamidated gliadins over the deamidated ones. The presence of both IgG and IgA isotype antibodies suggests isotype switching, confirming that the infants responded to the ingested gluten with an active immune process. Furthermore, antibodies produced during this early anti-gliadin immune response targeted the major disease-relevant gliadin epitope QP(Q/E)QPFP irrespective of future CeD development. Our results suggest a T-cell mediated immune response and highlight that anti-gliadin antibodies might also be present in non-celiac subjects at early age and without pathological signs.

Previous studies reported that gliadin-specific T-cells were present in the duodenum of TG2-antibody seronegative CeD risk children with normal mucosal morphology but with high anti-

gliadin antibody serum titres. This observation indicates that the presence of anti-gliadin serum antibodies in non-CeD subjects might be the result of a T-cell mediated immune response.

HLA-DQ2/DQ8 negative subjects in our study were also able to produce gliadin-specific IgA and IgG antibodies in response to the early age gluten intervention. This observation has major importance, suggesting that gliadins are immunogenic also for individuals who lack the CeD associated HLA-DQ risk alleles. Antibodies produced by HLA-DQ2/DQ8 negative children also have undergone an isotype switch and preferentially recognized non-deamidated gliadin peptides similarly to the antibodies produced by HLA-DQ2/DQ8 positive CeD risk children.

Our results raise the possibility that gliadin peptides might be presented to the immune system by other HLA molecules as well and suggest that tolerance to gliadin peptides is not simply the lack of antigen recognition, but an active tolerogenic mechanism should exist in healthy individuals.

Based on this concept, it is interesting that children at higher genetic risk for CeD (especially those homozygous for the HLA-DQ2.5 alleles) displayed weak humoral immune response to the ingested gluten. This led us to speculate that HLA-DQ molecules associated with a higher genetic risk might present gliadin peptides less adequately during the primary gluten intake at early age, and this may cause a deficient tolerogenic response. In fact, HLA-DQ2.5 molecules have earlier been found to have very restricted antigen binding capacity and bound only a small subset of peptides from a mixture of diverse gluten prolamins. The interaction of HLA-DQ2.5 molecules is impaired with the HLA-DM chaperone molecule due to a single nucleotide polymorphism characteristic for this allele. HLA-DM would assist the release of the CLIP peptide from the antigen binding pocket, therefore, HLA-DQ2.5 molecules often expose the CLIP peptide of the invariant chain at the cell surface instead of gliadin peptides. This experimentally proven deficient antigen presentation is also confirmed by the observation that HLA-DQ2.5 subjects mount a poor immune response also to hepatitis B virus vaccination.

The existence of a tolerogenic immune response is supported by the presence of type 1 regulatory T-cells found in CeD patients. Furthermore, differentiation of proliferating, gliadin-specific T-cells into IL-10 secreting, immunosuppressive, type 1 regulatory T-cells was also detected in an HLA-DQ2.5 humanized mouse model. In contrast to that, gliadin-specific effector or regulatory T-cells have not been found in healthy HLA-DQ2.5 subjects, suggesting that such cells became anergic or had undergone negative selection during the anti-gliadin tolerance development.

Statistical evaluation of the differences in the humoral immune response associated with distinct genotypes was limited in our study by the somewhat unequal number of subjects in the groups.

Moreover, without investigating T-cell activation it cannot be disclosed that HLA-DQ2.5 subjects responded poorly to the ingested gluten in all respects. It is also clear that the higher risk for CeD cannot be explained by the observed lower early-age anti-gliadin humoral immune response alone, since not all of the low responders developed CeD and conversely, the high anti-gliadin response did not protect all subjects from CeD development. Still, based on our results and data in the literature, it is worth to reinterpret the HLA-DQ presentation of gliadin peptides, i.e., consider the existence of an HLA-DQ2/HLA-DQ8 independent presentation and note the attenuated humoral response in HLA-DQ2.5 subjects which might be in connection with their increased genetic risk for CeD.

In contrast to the early anti-gliadin immune response, antibodies showed significantly improved binding towards the deamidated gliadin peptides at the time of CeD diagnosis in our study. In case of those risk children who did not develop CeD, non-deamidated gliadin peptide binding remained dominant, suggesting an active role of TG2-mediated deamidation in the pathomechanism of CeD. However, gliadin-specific antibodies produced by CeD risk infants already at the age of 6 months displayed high affinity to the immunodominant QP(Q/E)QPFP gliadin epitope. This observation is in line with the described stereotypic anti-gliadin antibody response of CeD patients, revealing that gliadin-specific B-cells are characterized by limited number of somatic mutations and germ line encoded residues also ensure inherent stable binding of the gliadin peptides. Tough, anti-gliadin antibodies produced at the time of CeD diagnosis displayed significantly improved binding towards the PEQPFP motif, suggesting an affinity maturation during CeD pathogenesis. In addition, antibodies produced at the age of 6 months had higher affinity towards the non-deamidated γGlia_Q peptide, while antibodies produced at the time of CeD diagnosis had higher affinity towards the deamidated γGlia_E peptide. This is well reflected by the significant decrease of the K_D γGlia_E / K_D γGlia_Q ratio, denoting affinity maturation at CeD manifestation towards the deamidated γGlia_E peptide as well. In case of the gliadin-specific T-cells isolated from the duodenal biopsies it was also observed in one other study that T-cells responded more intensely to the deamidated gliadins at the time of CeD diagnosis, while in case of pre-manifest subjects T-cells responded similarly to the native and deamidated gliadins.

Anti-gliadin antibodies *per se* are poor predictors of CeD, irrespective of the age or the deamidation of the peptides. We could not distinguish CeD developing and non-developing children based on the ROC analysis. Our results highlight that antibodies to gliadins are present in the serum well before the appearance of TG2-specific antibodies, but they do not have diagnostic significance at this stage and do not indicate CeD. CeD and non-CeD cases can be

discriminated only by anti-TG2 antibody positivity, thus TG2-specific antibodies are the first line reliable markers for CeD.

The serum concentrations of anti-TG2 antibodies were not in linear correlation with those of anti-NGP or anti-DGP. This might be related to the different mechanisms of their triggering or the observed deposition of anti-TG2 IgA in the different tissues at the surface of the extracellular TG2, lowering the detected serum anti-TG2 concentration.

We also investigated the epitope specificity of the produced anti-TG2 antibodies by applying the double mutant TG2 RE enzyme, where epitope 2 antibody binding is abolished and proved that the vast majority of the emerging autoantibodies produced by CeD patients target the epitope 2 surface. The histology results of the subsequently performed intestinal biopsies showed that the presence of epitope 2-specific TG2 antibodies was associated with villous atrophy regardless of age.

Despite the regular gluten intake and the continuous presence of TG2 protein in the extracellular matrix, it seems that TG2 as an autoantigen and deamidated gliadin epitopes come into the crosshairs of the immune system only later on. The preferential recognition of NGP after starting gluten intake might mean that deamidation and consequent availability of DGP is less frequent at this early (pre-disease) immune response and becomes more abundant later on due to cellular stress or other mechanisms. It has been reported by multiple studies that the cellular stress induced by gliadin might eventually lead to TG2 activation and further cascade of innate immune and inflammatory responses. Though CeD manifestation may be delayed in predisposed persons even for decades on a regular gluten-containing diet indicating that other trigger factors (e.g., intestinal infections, changes in the microbiome) might also be necessary for the pathologic onset. It is of great interest to see what directs TG2's activity toward the gliadin peptides and whether the deamidase or the cross-linking activity is making a change in CeD pathogenesis. It has been shown in *in vitro* studies that TG2 can covalently cross-link gliadins to itself, leading to a possible hapten carrier formation. However, the *in vivo* formation of such a complex has not yet definitively been proven, and a recent study suggested that the gluten–TG2 complex might be rather a temporary enzyme–substrate intermediate in *in vivo* conditions. This led us to speculate that enhanced complex formation is associated with the enhanced deamidation of gliadin, or enhanced isopeptidase activity of TG2 could exert the same effect. However, the role of TG2 as a pander and a victim remains still elusive.

For a more comprehensive picture on the anti-gliadin response of CeD and non-CeD subjects, the gliadin-specific T cells would be of great interest to examine, but this was beyond the scope of the present study. However, 11 of the PreventCD patients investigated here participated in

another clinical study as well, where gliadin-specific T-cell lines were cloned from their duodenal biopsy samples and tested for their antigen specificity by a large panel of different gliadin peptide sequences. In case of these manifest CeD patients we could compare the gliadin-specific T-cell and B-cell response.

From the successfully processed biopsy samples of 10 patients altogether 81 gliadin-specific T-cell clones have been established, among them 9 were specific for α-gliadins (including the immunogenic 33-mer peptide), 22 were specific for γ-gliadin sequences (5 of them cross-reacted with ω-gliadins) and 2 were specific for ω-gliadins (both cross-reacted with γ-gliadins). In case of the further gliadin-reactive T-cell clones peptide specificity could not be determined.

In our measurements, serum antibodies to the p57-68 α-gliadin peptide were detected only in one of the four patients who had α-gliadin reactive T-cells. In contrast, all patients produced antibodies to the QP(Q/E)QPFP γ-gliadin derived motif, who had γ or ω-gliadin reactive T-cells.

Limitation of the clinical studies is that in the case of HLA-DQ2/DQ8 negative subjects prospective follow up and invasive duodenal biopsy sampling cannot be performed by ethical reasons and thus the normal immune response to the ingested gluten cannot be directly investigated.

7. Summary

In the present study, we confirmed the TG2 mediated deamidation of p31-43 and p57-68 α-gliadin peptides in isolated reactions, that resulted in the production of CeD relevant B-cell epitopes, hence may influence antigenicity of the peptides. By examining serum antibodies of CeD patients we observed that antibodies mainly targeted γ-gliadin peptides, while α-gliadin peptides, despite of their highly similar sequences, were poor antigens. Quantitative results obtained with the PLQPEQPFP peptide were in linear correlation with the results of clinical diagnostic kits, indicating that this single γ-gliadin derived peptide has key role in the humoral immune response of CeD patients. Results of the affinity purifications performed by single peptides confirmed that there was a high degree of cross-reaction, since antibodies isolated by α-gliadin peptides recognized the γ-gliadin peptides more efficiently. The first appearing antibodies in CeD risk patients recognized γ-gliadin peptides exclusively, while we observed α-gliadin reactive antibodies only at the time of CeD diagnosis. Level of α-gliadin antibodies were always much lower compared to γ-gliadin antibodies further indicating that they might develop from γ-gliadin specific antibodies during CeD immune maturation.

CeD risk infants exerted more intense antibody response towards the non-deamidated gliadin peptide after the early age gluten introduction, despite the remarkable overlap seen in non-deamidated versus deamidated peptide binding. Preferential binding of non-deamidated peptides continues in the children who do not develop CeD, while in the cases where CeD developed, antibody binding to deamidated peptides significantly improved at the time of CeD diagnosis. Moreover, besides the qualitative changes, antibodies displayed affinity maturation towards the deamidated epitope. These observations suggest changes in TG2 enzyme deamidase activity that might be in connection with disease manifestation.

Interestingly, also infants who lack disease predisposing HLA-DQ alleles were able to produce IgA and IgG antibodies targeting the immunodominant epitope of gliadin in early age. This observation suggests that in response to the primary gluten intake, a non-celiac type, symptomless, non-pathological anti-gliadin immune response takes place that might be part of the normal processing of gluten in healthy subjects leading to immune tolerance development without anti-TG2 antibody production. In the course of this early antibody response, HLA-DQ2.5 homozygous children, who are at the highest genetic risk for CeD, were the poorest responders, that might have an impact on the later developing CeD, presumably because the proper tolerogenic processing of gluten cannot be performed. To confirm and validate these observations, detailed investigation of the antigen presentation and gliadin specific T-cell response needs to be done.

Anti-gliadin serum antibodies are not appropriate diagnostic markers of CeD, which is also reflected by the fact, that based on their positivity, CeD and non-CeD cases cannot be distinguished. Proper CeD prediction can be assured only if we test for TG2-specific antibody positivity, hence TG2-specific IgA antibodies are the first line reliable clinical markers for CeD diagnosis.

We examined epitope specificity of the autoantibodies, emerging at the time of the CeD diagnosis. Based on our results, the majority of TG2-specific antibodies (with 83% median values) targeted the epitope 2 surface of the enzyme, hence this seems to be the dominant celiac epitope of TG2.

List of Publications

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Candidate: Ádám Diós
Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. **Diós, Á.**, Srinivasan, B., Gyimesi, J., Werkstetter, K., Valenta, R., Koletzko, S., Korponay-Szabó, I.: Changes in Non-deamidated versus Deamidated Epitope Targeting and Disease Prediction during the Antibody Response to Gliadin and Transglutaminase of Infants at Risk for Celiac Disease.
   Int. J. Mol. Sci. 23 (5), 1-16, 2022.
   IF: 5.923 (2020)

2. **Diós, Á.**, Elek, R., Szabó, I., Horváth, S., Gyimesi, J., Király, R., Werkstetter, K., Koletzko, S., Fésüs, L., Korponay-Szabó, I.: Gamma-gliadin specific celiac disease antibodies recognize p31-43 and p57-68 alpha gliadin peptides in deamidation related manner as a result of cross-reaction.
   Amino Acids. 53 (7), 1051-1063, 2021.
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   IF: 3.52 (2020)

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List of other publications

3. Birinyi, Z., Réder, D., **Diós, Á.**, Korponay-Szabó, I., Hunyadi-Gulyás, É., Florides, C. G., Juhász, A., Gell, G.: Immunoanalytic investigation of grain proteins antigenic for celiac disease patients in an einkorn collection.
   Food Chem. 371, 1-11, 2022.
   DOI: http://dx.doi.org/10.1016/j.foodchem.2021.131148
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4. Miczi, M., **Diós, Á.**, Bozóki, B., Tőzsér, J., Mótyán, J. A.: Development of a Bio-Layer Interferometry-Based Protease Assay Using HIV-1 Protease as a Model.
   Viruses-Basel. 13 (6), 1-20, 2021.
   DOI: http://dx.doi.org/10.3390/v13061183
   IF: 5.048 (2020)

Total IF of journals (all publications): 22,005
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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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