

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

**STRUCTURAL ANALYSIS AND CLINICAL SIGNIFICANCE OF CELIAC  
DISEASE ANTIBODY EPITOPES ON TRANSGLUTAMINASE 2**

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### **Supplement 1.**

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## ABBREVIATIONS

aa= amino acid

APC= antigen presenting cell

CD = celiac disease

DC= dendritic cell

DGP= deamidated gliadin peptide

DH= dermatitis herpetiformis

DTT= dithiothreitol

ECM= extracellular matrix

EDTA=ethylenediaminetetraacetic acid

ELISA= enzyme-linked immunosorbent assay

EMA= endomysial antibodies

ESPGAN= European Society for Paediatric Gastroenterology and Nutrition

FBN= fibronectin

fXIII= Factor XIII

GA= gluten ataxia

GDP= guanosine-diphosphate

GFD = gluten-free diet

GTP= guanosine-triphosphate

HLA= human leukocyte antigen

HRP= horseradish peroxidase

HUVEC= human umbilical cord vein cells

IEL= intraepithelial lymphocyte

IFN= interferon

IL= interleukin

MAb= monoclonal antibody

MHC I= major-histocompatibility-complex class I

MMP= matrix metalloproteinase

PMSF= phenylmethanesulfonyl fluoride

PVDF= Polyvinylidene Fluoride

ScFv= single chain variable fragments

SDS/PAGE= Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TBS= tris-buffered saline

TG2 = tissue transglutaminase, type-2 transglutaminase

TG3= transglutaminase 3

TNF= tumor necrosis factor

Wt= wild type

## 1. INTRODUCTION

Celiac disease is a pathologic immune reaction to ingested gluten proteins of wheat, rye and barley. In subjects with genetic predisposition, gluten-derived gliadin peptides induce chronic intestinal inflammation, jejunal villous atrophy and production of disease-specific autoantibodies against the enzyme type-2 transglutaminase (TG2). The presence of celiac antibodies correlates with gluten consumption and disease activity, and they also were shown to bind to the patients' own TG2 antigen within diseased tissues. These antibodies, particularly those of IgA class, are currently central in the medical diagnostic workup and monitoring of the disease. The pathomechanism of the disease is not entirely known. TG2 bears multiple cellular and biochemical functions and in addition to being an autoantigen it modifies gliadin peptides and thus strengthens the immune response. TG2-specific celiac autoantibodies influence TG2 activity, which can affect the whole pathogenic process. Extensive work has been done to localize the disease-specific binding epitopes of anti-TG2 celiac antibodies, but so far the results were controversial and the number and exact localization of the binding sites are still unknown. Identification of specific celiac epitope(s) on transglutaminase 2 would be important to understand better the disease pathogenesis and might offer a tool to evaluate the interference with antibody binding as a possible future therapeutic approach.

## 2. THEORETICAL BACKGROUND

### 2.1. CELIAC DISEASE

The first description of celiac disease (CD) can be found as a malabsorptive syndrome with chronic diarrhea by Aretaeus of Cappadocia, a Greek physician between the first and second centuries AD (Adams F 1956). The word “celiac” is the translation of the Greek *koiliakos* (abdominal) and was adopted by the pediatrician Samuel Gee (Gee S 1888). He gave the first modern-day study of the disease in 1888 and linked it to food ingestion: ‘The allowance of farinaceous foods must be small, but if the patient can be cured at all, it must be by means of diet.’ Various diets were tried in the early 20<sup>th</sup> century, but the main toxic component remained unclear. The relation between celiac disease and wheat was not made until the 1950s, when Willem-Karel Dicke and colleagues observed that removing wheat and rye from the patients’ diet resulted in improvement of their condition (Dicke WK et al., 1953). In the same decade a team from Birmingham proved that the gluten component was the toxic part of wheat (Anderson CM 1952), and since then gluten free diet (GFD) was introduced for the treatment of CD.

Small intestinal villous atrophy was reported by John W. Paulley in 1954, by the examination of biopsy samples derived from patients with abdominal operations (Paulley JW 1954). The histological classification of CD was established many years later by Marsh (Marsh MN 1992), and it is range from hyperproliferative crypts with intraepithal lymphocytosis to total villous atrophy.

Important progress was made in the 1980s, when association of CD with particular DQ molecules was found (Louka AS and Sollid LM 2003), and later intestinal CD4<sup>+</sup> T-cell clones were identified, which recognized gliadin peptides presented by DQ2 or DQ8 molecules (Lundin KE et al., 1993, Lundin KE et al., 1994). This time it was known that celiac patients produce antibodies to gliadin and to an unknown component of endomysium. The identification of this component as tissue transglutaminase (TG2) and its role as the main celiac autoantigen was published by Dieterich et al. in 1997 (Dieterich W et al., 1997).



### ***2.1.1. Clinical characteristics***

#### **2.1.1.1. Epidemiology**

Once CD was considered as a rare disease with 0,1% prevalence and limited to young children. Nowadays, it is revealed that CD can affect the adult population also, moreover, the prevalence increases with age (Vilppula A et al., 2009). The prevalence of the disease is higher in women with a female:male mean ratio 2:1 (Ciclitira PJ et al., 2001). Based on population screening studies in Europe and in the United States the prevalence of CD can be estimated as high as 1% (Fasano A et al., 2003, Korponay-Szabo IR et al., 2007, Mustalahti K et al., 2010, West J et al., 2003), therefore it is considered as one of the most common chronic diseases. CD can be found in North Africa, India and in the Middle East, while only rare occurrence was described in China and Japan (Wu J et al., 2010). In Finland the prevalence of CD has doubled in two decades from 1% to 2%, in line with increasing occurrence of other autoimmune diseases like diabetes mellitus type I (Lohi S et al., 2007). Interestingly, on the other side of the border, the Russian Karelian population with Finnish ancestry has almost the lowest prevalence (0,2%) of CD, despite the fact that both countries have similar wheat consumption levels and human leukocyte antigen (HLA) haplotype frequencies. This suggests that other environmental factors (e.g. reduced microbial contact at an early age resulting in upregulating immunity) can contribute to the presentation of the disease (Kondrashova A et al., 2008).

#### **2.1.1.2. Genetic background**

CD is a multigenetic disorder; the concordance rate in monozygotic twins is 80%, while in dizygotic twins it is 11%, similar to other first-degree relatives (Dube C et al., 2005, Nistico L et al., 2006).

The HLA locus, which is a part of a conserved extended haplotype region, has the strongest genetic influence on the disease. The primary genetic association in CD is with DQ2 (DQA1\*0501/DQB1\*0201, termed HLA-DQ2.5 hereafter) (Sollid LM et al., 1989) and to a lesser extent to DQ8 (DQA1\*0301/DQB1\*0302) (Spurkland A et al., 1997). The disease-associated HLA-DQ2.5 molecule presents boarder range of gluten peptides than the non-disease-associated HLA-DQ2.2 (DQA1\*0201/DQB1\*0202); this could explain why HLA-DQ2.5, but not HLA-DQ2.2 haplotype, confers a high risk to develop CD. The number and

quality of HLA-DQ2 molecules can influence the probability of developing CD: HLA-DQ2.5 homozygous and HLA-DQ2.5/2.2 heterozygous genotypes are high-risk, HLA-DQ2.5/X heterozygous genotypes are medium-risk, while HLA-DQ2.2 genotypes are low-risk (Vader W et al., 2003). People with neither DQ2 nor DQ8 have extremely low prevalence and thus are not at risk (Karell K et al., 2003).

HLA-DQ2 and HLA-DQ8 are also commonly found in healthy individuals, which indicates that these HLA genotypes are necessary, but not sufficient for disease development. The risk effect of these two molecules on CD development is approximately 35% (Hunt KA et al., 2008).

Additionally to the DQ-encoding genes, other immune-related genes can be found at the HLA locus, which may contribute to CD development. Major histocompatibility class I-related genes (MICA, MICB) and tumor necrosis factor (TNF) molecules have been suggested (Louka AS and Sollid LM 2003), however, these findings should be interpreted with caution, since the chance for linkage disequilibrium between these genes and DQ genes is high.

As not all individuals with HLA-DQ2 and HLA-DQ8 will develop CD, additional genes have been suggested to contribute to CD development. In genetic linkage studies several chromosomal regions have been identified that likely contain CD-related genes in families with a high prevalence of CD. Region 2q33 with genes such as CD28, CTLA4 and ICOS (all take part in T-cell response) was described (van Heel DA et al., 2005). Linkage was also identified for region 5q31-33 (Greco L et al., 2001) and 19p13.1 (Van Belzen MJ et al., 2003). Recently, additional genetic loci were identified by large-scale case control-based genome-wide association studies using single nucleotide polymorphisms (SNPs) (Dube C et al., 2005, Hunt KA et al., 2008) and most of the proteins encoded in these regions take part in immunity.

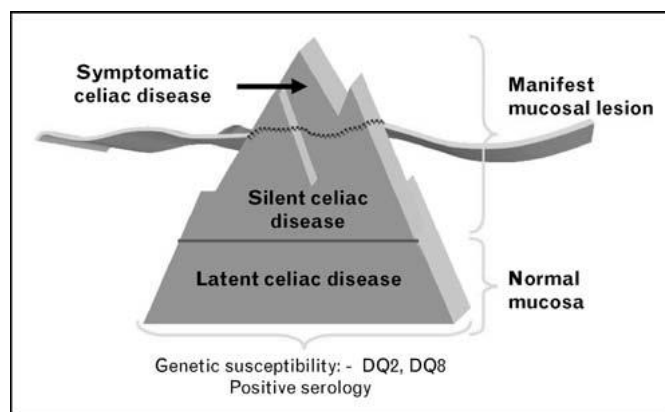
The first important pathway worth noting is T-cell development in thymus. Thymus-expressed molecule involved in selection (THEMIS) is one of the CD-candidate genes, and it is a key regulator in positive and negative T-cell selection in late thymocyte development. Additional molecules in thymocyte maturation such as RUNX3, TNFRSF14 and ETS1 transcription factor were also identified. The second pathway is the innate immune detection of viral DNA. In this case TLR7-TLR8 can be mentioned, which molecules recognize viral RNA, and since viral infection is a putative trigger to CD-development, these genes can well fit into the pathogenesis of the disease. A third pathway involves T- and B-cell-stimulation with molecules that control the response to antigens by T- and B-cells (CTLA4-ICOS-CD28,

CD80, TNFRSF9, TNFSF4). The final pathway includes cytokines, chemokines and their receptors –e.g. interleukin receptor cluster (IL18RAP), chemokine receptor cluster (CCR5). The up to now identified 49 loci altogether explain about 20% of total CD variance, and together with the major HLA-DQ2.5 and less common HLA-DQ8 variants, represent some 40% of the genetic variance (Dubois PC et al., 2010). Many of these loci also predispose to other autoimmune or chronic inflammatory disorders like ulcerative colitis, diabetes mellitus and asthma (Abadie V et al., 2011).

### 2.1.1.3. Presentation forms and symptoms

Clinical manifestations of CD are highly variable. In the case of children diarrhea, abdominal distension and failure to thrive are general presentations; however, vomiting, irritability, anorexia, anaemia, delayed puberty and short stature may also be present. In adults, the classic presentation of the disease is chronic diarrhea, may be with abdominal pain, weakness and malabsorption (Green PH and Cellier C 2007). A great variety of nutrient malabsorptions can be found in CD patients such as anaemia due to deficiency of iron and folic acid, while serum calcium and fat-soluble vitamins (vitamin D and K) level can be low (Collin P et al., 2002).

Nonetheless, CD may be present in patients without the above described symptoms. Many newly diagnosed patients have only mild abdominal discomfort, bloating, and present extraintestinal features, such as dermatitis herpetiformis (DH), anaemia, osteoporosis, infertility and neurologic problems. Some patients are asymptomatic (silent CD), but also these do have manifest mucosal lesion and after introduction of GFD patients report favorable changes in their conditions. Due to these diverse manifestations, many of the patients have a previous diagnosis of irritable bowel syndrome (IBS) (Sanders DS et al., 2001) and are overweight (Dickey W and Kearney N 2006).



**Figure 1. The celiac iceberg model.** Figure taken from (Fasano A 2006).

Latent CD patients show normal small-bowel villous architecture, but they may develop small-bowel villous atrophy and crypt hyperplasia in the disease process (Fig. 1.). In individuals with positivity for endomysium- and TG2-antibodies and high density of  $\gamma/\delta$  -positive intraepithelial T-lymphocytes present in the gut, latent CD may be suspected (Maki M and Collin P 1997).

Since CD shows wide range of symptoms, in many cases it remains undiagnosed, the diet treatment is delayed and, consequently, the risk of long-term complications increases.

Since CD has numerous extraintestinal features, it is hard to distinguish, if these associations are causal or coincidental. Similarly, differentiation between extraintestinal symptoms and complications of CD is often ambiguous.

Dermatitis herpetiformis (DH) is the best known among the extraintestinal manifestations of CD affecting approximately 10-20% of CD patients (Reunala TL 2001). It consists of papulovesicular skin lesions and anti-TG2 in serum and anti-transglutaminase 3 (TG3) IgA deposits in the dermal papillae (Sardy M et al., 2002), and it is often linked to villous atrophy and crypt hyperplasia. The prevalence of HLA-DQ2 and -DQ8 in DH is the same as in CD and it is possible that there are other genes which determine whether patients have only CD or DH. The DH rash can be treated by dapsone, but for a long-term improvement a gluten-free diet is needed (Maki M and Collin P 1997).

CD can be associated with numerous autoimmune diseases like type I diabetes mellitus, autoimmune thyroid diseases and Sjögren's syndrome (Table 1.)(Collin P et al., 2002). The primary link is the common genetic background among these diseases, most importantly in the HLA region of chromosome 6 (carrying HLA DQ2 or DQ8). Beside these CD has been reported to coexist with several hepatic disorders such as autoimmune hepatitis, primary biliary cirrhosis and primary sclerosing cholangitis (Ludvigsson JF et al., 2007).

Neurologic disorders are also common extraintestinal features of CD; 10-30% of CD patients suffer from these diseases. Cerebellar ataxia, peripheral neuropathy, chronic headache, depression, epilepsy can be mentioned among others (Bushara KO 2005). Elevated antigliadin-antibody levels in the serum (Bushara KO et al., 2001, Hadjivassiliou M et al., 2006b) and anti-TG2 IgA antibodies in the gut and brain of patients with sporadic cerebellar ataxia (also known as gluten ataxia (GA)) were shown (Hadjivassiliou M et al., 2006b), even in the absence of an enteropathy. Autoantibodies against neuronal transglutaminase (TG6) were also identified in serum samples and in cerebellar deposits of patients with GA (Hadjivassiliou M et al., 2008). Additionally, lymphocytic infiltration in the central and

peripheral nervous system (Hadjivassiliou M et al., 2006a) and slight improvement to GFD (Hadjivassiliou M et al., 2003) was reported which indicates an immune-mediated mechanism of pathogenesis in GA. Advanced neural disease might be irreversible and represents one of the most severe complications of CD.

Osteoporosis and infertility also can be regarded as complications of CD, since they are only partially reversible on GFD.

Endocrine disorders	Neurologic disorders	Liver diseases	Other
Type 1 diabetes	Cerebellar ataxia	Primary biliary cirrhosis	Anemia
Autoimmune thyroid disorders	Peripheral neuropathy	Autoimmune hepatitis	Osteoporosis
Addison disease	Cognitive impairment	Autoimmune cholangitis	Dermatitis herpetiformis
Reproductive disorders	Psychosis		Selective IgA deficiency
	Epilepsy		Turner syndrome
	Migraine		Idiopathic dilated cardiomyopathy
			Down syndrome
			Malignancies

**Table 1. Some of the disorders believed to be associated with celiac disease.** (Briani C et al., 2008)

Due to the absence or the atypical nature of symptoms, big proportion of CD remain unrecognized, which can cause certain cancers like non-Hodgkin lymphoma, enteropathy-associated T-cell lymphoma, small intestinal adenocarcinoma and esophageal carcinoma (Green PH et al., 2003, Lohi S et al., 2009). However, early treatment with strict GFD can prevent complications of CD and reduces the risk of some malignancies (Green PH et al., 2003).

Increased prevalence and mortality were reported by (Rubio-Tapia A et al., 2009) based on a cohort with almost ten thousand samples collected at Warren Air Force Base between 1948 and 1954. Sera were tested for anti-TG2 antibodies, if abnormal, for endomysial antibodies; survival was measured during a follow-up period of 45 years. These data were compared with two recent cohorts with either similar age of birth or similar age of sampling. The study yielded 2 major findings: 1) undiagnosed CD was associated with a nearly 4-fold increased risk of death compared with subjects without serologic evidence of CD and 2) the prevalence of CD has increased dramatically in the United States during the past 50 years. Increased risk for malignant lymphoma was also presented with odds ratio of 4.5 (Delco F et al., 1999).

#### 2.1.1.4. Diagnostic criteria and work-up

Because of the heterogeneity and the lack of specificity of many presenting symptoms, the clinical diagnosis of CD is a challenge even for experts.

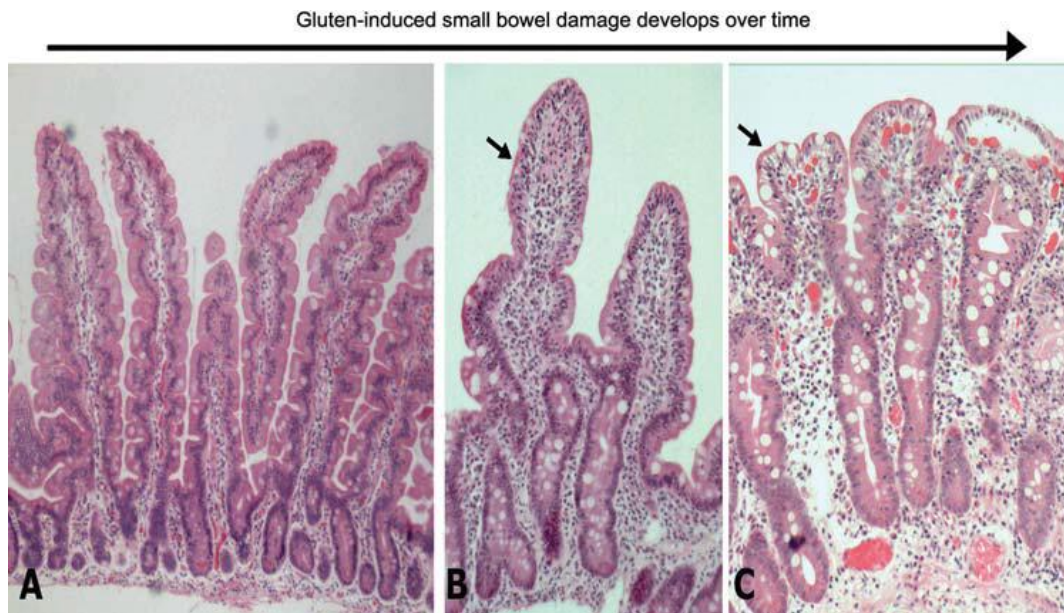
The diagnostic criteria were first formulated by the European Society for Paediatric Gastroenterology and Nutrition (ESPGAN, later renamed as ESPGHAN) in 1970 (Meeuwisse GW 1970), and included three intestinal biopsies: the first when CD was recognized and this should show small-bowel mucosal atrophy, the second during the improvement and showing mucosal recovery on GFD, and the third after the reintroduction of a gluten-containing diet with the proof of the relapse of villous morphology. These criteria were modified in 1990 (Walker-Smith JA et al. 1990), which state hereafter that the histological demonstration of characteristic small-bowel atrophy before treatment and clinical recovery on a GFD is essential, while gluten challenge is only used if needed (e.g. in patients whose first biopsy sample result was ambiguous). Circulating anti-TG2 antibodies and their disappearance on GFD supports the diagnosis (Maki M and Collin P 1997). In 2011, ESPGHAN announced new criteria (Husby S 2011), and according to these small bowel biopsy will no longer be compulsory in children showing symptoms compatible with CD and presenting with very high positivity for both anti-TG2 and endomysial serum antibodies and HLA DQ2 or DQ8. This new position paper also provides a new definition for CD, describing it as a systemic disorder consisting of the variable combination of gluten-dependent symptoms, TG2 antibodies, HLA-DQ2 or DQ8 and enteropathy.

##### *Small intestine biopsy*

Several years ago, when CD was a new disease, diagnosis was solely based on the small intestine biopsy (Shiner M 1956); since villous atrophy is the most relevant feature of the disease, histology became the gold standard of detecting CD. Recently, the accessibility of new diagnostic tools (e.g. serological tests, HLA typing) result that there is a decline in the use of this diagnostic method.

According to the classification of Marsh MN gluten-induced small bowel mucosal lesions develop gradually from mucosal inflammation with increased number of intraepithelial lymphocytes (IELs; Marsh type I lesion) to crypt hyperplasia (Marsh II) and finally to villous atrophy (Marsh III, Fig. 2.) (Marsh MN 1992). It must be emphasized that lesion with increased number of IELs indicate CD only in 10% of cases, since other conditions (including food allergy, gastrointestinal infections, Chrons' disease, ulcerative colitis) can also be the

cause of such phenomenon (Kakar S et al., 2003). And similarly, autoimmune enteropathy, Whipple disease and eosinophilic gastroenteritis also can cause non gluten-dependent villous atrophy (Freeman HJ 2004, Oberhuber G *et al.*, 1999).



**Figure 2. Development of gluten-induced small bowel mucosal lesion over time in celiac disease** from normal villous structure (A) to mucosal inflammation with crypt hyperplasia (B), finally to diagnostic villous atrophy with crypt hyperplasia (C). Arrows show the site of intraepithelial lymphocytosis. (Kaukinen K et al., 2010)

Moreover, the interpretations of the histological samples can often be controversial, since the results depend on many technical factors and incorrect orientation and insufficient size of samples may interfere with the correct evaluation. This can result in both false-positive and negative interpretations (Collin P et al., 2005). Therefore, at least four biopsy samples should be taken; two from the duodenal bulb and two from the distal duodenum. Moreover one should bear in mind when diagnosing CD, that patchy and irregular mucosal regions are as relevant as continuous lesions. Well-oriented biopsies provide good possibility for evaluation of villi/crypt ratio ( $\geq 3 : 1$  in normal mucosa) and an accurate count of IELs (Volta U and Villanacci V 2011).

Given the low specificity and technical difficulties, histology alone cannot provide the diagnosis, it should be evaluated together with the clinical signs, serological markers and HLA haplotypes.

### *Serological tests*

Serology has become a highly relevant part of the diagnosis of CD; it seems that with serological tests CD can be identified in early stages, before the appearance of severe intestinal symptoms.

The available tests measure antigliadin, connective-tissue (antireticulin and antiendomysial) and anti-TG2 antibodies. The sensitivity and specificity of antigliadin antibodies do not show the required level to be suitable for detecting CD – except in children younger than 18 months of age (Rostom A et al., 2005). Introduction of a new antibody test utilizing the deamidated forms of gliadin peptides (DGPs) as antigens significantly improved clinical performance (Kaukinen K et al., 2007, Prince HE 2006). The principle of the test is the finding that celiac antibodies can recognize deamidated hexapeptide motifs QPEQPF and QEQPFP with higher efficacy than the corresponding native peptide motifs (Osman AA et al., 2000). In another study the combination of PLQPEQPFP and PEQLPQFEE peptides are used for detect IgA antibodies of celiac patients. This binding assay had 94% diagnostic efficiency, while assays using conventional gliadin had only 81% (Schwertz E et al., 2004).

For serological screening of CD detection with anti-TG2 IgA antibodies is recommended in cases when the clinical symptoms are subtle or atypical, and also for individuals with increased risk of developing CD by reason of heredity or associated disorders (Volta U et al., 2002). These ELISA tests are highly sensitive for CD (97%) and specificity is greater than 90% in the case of human TG2 antigen (Rostom A et al., 2005). However several studies described false negative IgA TG2 results with IgA endomysium positivity and false positive IgA TG2 results in the absence of IgA endomysium positivity (Wong RC et al., 2002). This is why detection of serum autoantibodies on tissue sections containing TG2 in endomysial or reticulin structures (e.g. human umbilical cord; EMA test) can be employed as a confirmatory test in anti-TG2 positive cases, since its specificity is almost 100%.

Since the EMA tests are based on immunofluorescent staining, they are observer dependent, while anti-TG2 ELISA tests can be performed on automated instruments. However, the accuracies of these tests are antigen-dependent, since the first generation ELISAs (using guinea pig TG2 as antigen) are less precise than the second generation ELISAs applying human recombinant or purified TG2 as antigen (Rostom A et al., 2005). Recently a new diagnostic kit was developed (AESKULISA, CeliCheck New Generation), utilizing TG2 covalently crossed-linked to deamidated gliadin peptides and which can detect three types of antibodies: anti-TG2, anti-DGP and the newly formed neo-epitopes of the complex (Matthias T et al., 2010).



Celiac autoantibodies can be deposited in the small intestine as well as in other organs like in the liver, lymph nodes, kidney, skeletal muscles, etc., where they target the autoantigen TG2 (Korponay-Szabo IR et al., 2004). Staining of these deposits became used in the diagnosis of CD, since these deposited antibodies appear in the mucosa before they can be detected from serum or before any changes in the morphology of the mucosa would occur (Koskinen O et al., 2010, Salmi TT et al., 2006). The majority of these serological tests are based on the detection of IgA class antibodies, only in the case of selective IgA deficiency are IgG class antibody tests used (Korponay-Szabo IR et al., 2003).

Based on the detection of IgA celiac antibodies a rapid test was also developed, which uses a fingertip blood of the patient as sample and self-red blood cells-derived TG2 as antigen. This test offers good sensitivity (96.7%) and specificity (93.5%) (Korponay-Szabo IR et al., 2007, Raivio T et al., 2006) and can be used for detecting CD and monitoring treatment.

#### *HLA typing*

HLA typing can be used for controversial cases when there is a discrepancy between histology and serology results and in the case of first-degree relatives of CD-patients, since CD is highly unlikely when the patient is both HLA-DQ2 and -DQ8 negative (Karell K et al., 2003, Kaukinen K et al., 2002). However, the predictive value of a positive DQ2 or DQ8 result is low, since 30% of the normal population also have these alleles (Husby S, et al 2011).

#### 2.1.1.5. Treatment

Due to our present-day knowledge of celiac disease, the only accepted treatment is lifelong gluten-free diet. There is regular relapse with the reactivation of the immune process whenever the diet is stopped. This strict diet requires the elimination of wheat, rye and barley, which may severely affect the lifestyle and social activities of the patient. So there is an apparent need for alternative therapies (Sollid LM and Khosla C 2011).

### ***2.1.2 Molecular mechanism***

The pathogenesis of CD includes multiple interactions between environmental, genetic and immunologic factors. The external trigger of the disease is gluten, which activates the innate and adaptive immune system and leads to mucosal damage and production of anti-gliadin and anti-TG2 antibodies (beside others). HLA-DQ2 and -DQ8 are strongly linked to the disease and can be found almost in all of the patients. Additionally, non-HLA genes also contribute to the disease development.

#### **2.1.2.1. Structural basis of gluten intolerance**

The term “gluten” refers to the main storage proteins of wheat of which visco-elastic properties are essential for dough formation and provide unique texture and taste for bread. Gluten is widely used in the food industry: besides the products that are originally made of wheat like bread, pasta and cookies, many other foods can contain hidden gluten like sauces, instant soups and sausages. Rye and barley contain proteins similar to gluten (secalin and hordein, respectively) and can trigger CD as well.

Gliadin is the alcohol-soluble fraction of gluten and can be subdivided to  $\alpha$ -,  $\gamma$ - and  $\omega$ -gliadins. They are 250-300 residues long with 25-75 kDa molecular weights. Gliadins are monomers, while the other protein fraction of gluten named glutenins form large polymeric structures and can be subdivided to high-molecular-weight (650-800 residues long) and low molecular-weight (270-330 residues long) glutenins (Sollid LM 2002).

Gluten proteins are rich in amino acids proline (15%) and glutamine (30%), whereas the glutamic acid and aspartic acid content is unusually low. The high proline content causes resistance to degradation by gastrointestinal enzymes and even 33 amino acids long highly immunogenic peptides of these peptides remain in the intestinal lumen after ingestion of gluten (Shan L et al., 2002, Shan L et al., 2005). Under certain conditions, e.g. intestinal infections like rotavirus (Stene LC et al., 2006) when the intestinal permeability is increased the degradation-resistant gluten peptides can pass through the epithelium and interact with antigen-presenting cells in the lamina propria.

The proteins of rice and corn contain glutamine and proline in lesser proportion and can be consumed by celiac patients without harm. The proteins of oat (avenins), however, have an intermediate amino acid composition, and moreover, products with oats are often contaminated with wheat or rye, that is why the introduction of oat into GFD is controversial (Lundin KE et al., 2003).

According to the classification by Ciccocioppo et al. gluten peptides can be further subgrouped according to the type of the reaction they induce (Ciccocioppo R et al., 2005). Some fragments are 'toxic', capable to induce mucosal damage *in vivo* or *in vitro*, while some fragments are 'immunogenic', as they are able to stimulate HLA-DQ2 or HLA-DQ8 T-cell mediated specific immune response. Several gliadin peptides were synthesized from A-gliadin (Kasarda DD et al., 1984) (a 266 amino acids long fraction of  $\alpha$ -gliadin) and identified as 'toxic' peptides (de Ritis G et al., 1988, Maiuri L et al., 1996), while even more have been tested recently for their immunogenicity. Some peptides are found to be immunodominant (Anderson RP et al., 2000, Shan L et al., 2002, van de Wal Y et al., 1998), which means that these peptides induce strong T-cell response in intestinal biopsies of almost all patients. Moreover, some inactive peptides become very potent T-cell stimulators if their defined glutamine residues are converted to glutamic acid by enzymatic deamidation by TG2 (Arentz-Hansen H et al., 2000, van de Wal Y et al., 1998).

#### 2.1.2.2. Antigen presentation and adaptive immune response

Gliadin peptides that could enter the lamina propria will be processed and presented by antigen presenting cells (APC, like dendritic cells (DCs), macrophages or B cells) via their HLA-DQ2 or DQ8 molecules. Both molecules prefer binding of negatively charged amino acids in their peptide-binding groove, namely P4, P6, P7 anchor points in the case of HLA-DQ2 and P1, P4, P9 for HLA-DQ8 (Sollid LM 2002). Gluten peptides usually do not contain negatively charged amino acids in their natural form, while with deamidation by TG2 the non-charged glutamine can be converted to negatively charged glutamic acid. The deamidation process preferably occurs when pH is below 7.3 (Fleckenstein B et al., 2002), and since the pH is ~6.6 in the proximal small intestine and increased TG2 expression can be observed in celiac epithelial brush border (Molberg O et al., 1998), deamidation can occur there. During antigen presentation TG2 may also be internalized by e.g. DCs (which presents TG2 on their cell surface), and the enzyme could also work when the pH is low (Sollid LM 2002). The deamidated form of gluten will bind to HLA molecules with almost 25 fold higher affinity compared to the non-deamidated one (Kim CY et al., 2004).

Since TG2 can be found mainly intracellularly, some events should trigger the externalization of the enzyme, like gastrointestinal infections (which can also increase intestinal permeability) or tissue damage, e.g. by the innate immune response to gluten discussed below. If these occur, a self-amplifying loop will be created: more and more TG2 will be liberated,

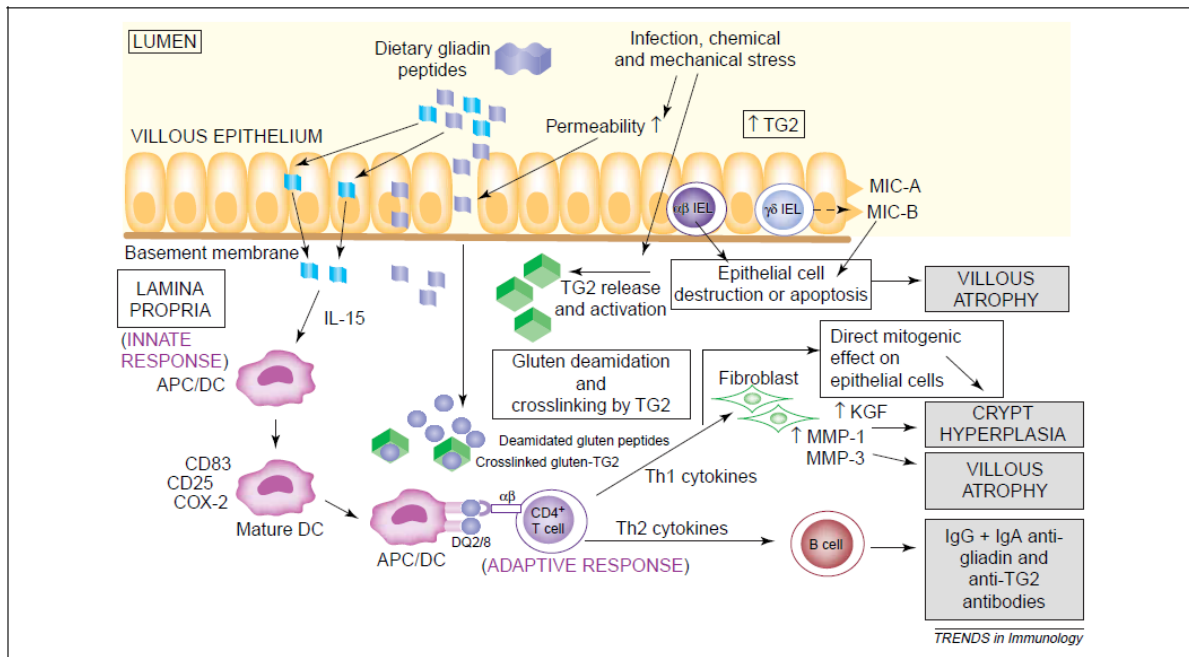
which will deamidate more and more gliadin peptides and will trigger more potent immune reaction by the expansion of gluten-specific CD4<sup>+</sup> T cell repertoire (Tjon JM et al., 2010). These gluten-specific CD4<sup>+</sup> T cells will secrete pro-inflammatory cytokines such as tumor necrosis factor (TNF)  $\alpha$  and interferon (IFN)  $\gamma$  and thus stimulate fibroblasts and cytotoxic T cells to produce matrix metalloproteinases (MMPs). Moreover, activation of T cells will result in production of Fas ligand and granzyme, which will lead to apoptosis of enterocytes. These events together may lead to mucosal damage, matrix remodeling, and finally to villous atrophy which is characteristic for CD. Activation of gluten-specific CD4<sup>+</sup> T cells will also result in clonal expansion of gluten-specific B-cells and release of gluten-specific antibodies, while TG2-specific B-cells may become activated through intermolecular help by the same CD4<sup>+</sup> cells (Fig. 3.) (Briani C et al., 2008, Ciccocioppo R et al., 2005).

#### 2.1.2.3. Innate immunity involvement

In active CD the number of intraepithelial lymphocytes (IELs, mainly CD8<sup>+</sup> TCR $\alpha\beta$ <sup>+</sup> and TCR $\gamma\delta$ <sup>+</sup> cells) in the small intestine is markedly increased. They are localized in the basolateral side of the epithelium between intestinal epithelial cells and express natural killer cell receptor NKG2D and CD94/NKG2C. These receptors can be activated by interleukin 15 (IL-15), which plays a role in the migration and expansion of IELs and can be the main effector molecule in the innate response of CD (Londei M et al., 2005). Moreover, gluten induces the production of IL-15 and non-classical major-histocompatibility-complex class I (MHC I) ligands MIC and HLA-E in intestinal epithelial cells. Interaction of NKG2D and CD94/NKG2C on CD8<sup>+</sup> lymphocytes with their ligands MIC and HLE on stressed epithelial cells results enhanced interferon- $\gamma$  (IFN- $\gamma$ ) production and cytolysis leading to tissue damage (Jabri B et al., 2005, Maiuri L et al., 2003). An increased expression of toll-like receptors 2 and 4 was also described (Szebeni B et al., 2007).

It is still a question, how gluten peptides can enter the intestinal mucosa and how they can cross the basal membranes of epithelial cells to get in connection with the immune cells in the lamina propria. Gluten challenge resulted in elevated permeability of tight junctions, mediated by zonulin (Clemente MG et al., 2003), which expression was increased in CD according to previous findings (paracellular route) (Fasano A et al., 2000). Another possibility is the transcellular route, when gliadins get across the epithelial cells using enterocytic vesicles (Van Niel G et al., 2003). In this way gliadin peptides can reach the lamina propria where they can activate dendritic cells, which will produce IL-15. In addition to the effects of IL-15

mentioned above, IL-15 can alter the NK-cell receptor-mediated cytotoxicity (Meresse B et al., 2006), which leads again cell lysis and tissue damage (Fig. 3.).



**Figure 3. Summary of the molecular basis of celiac disease.** APC, antigen presenting cell; COX-2, cyclooxygenase-2; DC, dendritic cell; HLA, human lymphocyte antigen; IEL, intraepithelial lymphocyte; IL-15, interleukin-15; KGF, keratinocyte growth factor; MIC-A, MHC class I chain-related sequence-A; MMPs, matrix metalloproteinases; TG2, tissue transglutaminase; TNF, tumor necrosis factor. Figure taken from (Hadjivassiliou M et al., 2004)

#### 2.1.2.4. Induction of antibodies

Gluten is the trigger of CD and antibody production, but the most characteristic CD antibodies target TG2, a self-antigen. Interestingly, these antibodies are no longer produced after gluten is excluded from the patient's diet, despite the fact that TG2 is still available to the immune system.

The most accepted hypothesis for anti-TG2 antibody production is the hapten-carrier model (Sollid LM et al., 1997). In 1997 TG2 was identified as the main autoantigen of CD (Dieterich W et al., 1997) and after that it was proved that TG2 can crosslink gliadin peptides also to itself (Dieterich W et al., 2006, Skovbjerg H et al., 2004). In this way the initial immune response against gliadin can be diversified to other epitope to another antigen, in this case TG2, via intermolecular help. In this model, gliadin, gliadin-gliadin and gliadin-TG2 complexes can be processed and presented to gliadin-specific CD4+ T cells by APCs. If the gliadin-TG2 complexes are internalized by TG2-specific B cells as non-professional APCs, the gliadin-specific CD4+ T cells will provide intermolecular help to these TG2-

specific B cells to expand and result in production of TG2-specific antibodies. This hypothesis can be supported by the finding, that gliadin-specific T cells but not TG2-specific T cells can be found in the small intestine of CD patients (Lundin KE et al., 2003, Molberg O et al., 1998), and by clinical observations showing a close temporal link in the production of DGP-specific and TG2 specific antibodies in young children (Liu E et al., 2007). However, in a recent study authors were able to detect and expand also some TG2 specific T cells from the peripheral blood of CD patients (Ciccocioppo R et al., 2010).

Another theory for TG2-specific antibody production is based on the possible structural homology between gliadin peptides and TG2. The initial immune reactivity is based on this similarity, while there may be secondary reactions against unique epitopes on the secondary antigen (TG2) resulted by epitope spreading via intramolecular help. This possibility was further investigated in the present work, with DGP-reactive monoclonal anti-TG antibodies.

Additionally, autoantibodies against other members of the TG-family are produced in dermatitis herpetiformis (TG3) (Sardy M et al., 2002) and in gluten ataxia (TG6) (Hadjivassiliou M et al., 2008). Reaction to these analogous molecules can be explained by above mentioned molecular mimicry model: initially patients develop antibodies against gliadin and TG2 and spreading the reaction to other TG molecules results in extraintestinal manifestations. However the cause and effect relation between autoantibodies and CD manifestations is not obvious and require further verification.

Production of TG2-specific autoantibodies can be located to the small intestinal mucosa, while the humoral response against gliadin occurs both peripherally and centrally (Marzari R et al., 2001). The anti-TG2 antibodies will be trapped in the small-bowel mucosa, by binding to extracellular TG2 below the epithelial layer and around blood vessels (Korponay-Szabo IR et al., 2004). Even seronegative patients and patients in the early stage of the disease with normal small-bowel mucosal villous morphology can have these anti-TG2 antibody depositions (Kaukinen K et al., 2005, Salmi TT et al., 2006).

#### 2.1.2.5. Pathologic effects of autoantibodies

CD is mainly considered as a T-cell mediated disease, however, growing number of reports show that circulating and deposited autoantibodies may also play a role in the pathogenesis of the disease and TG2-specific antibodies may not be just innocent bystanders in CD as previously thought.

The epithelium in the small-bowel mucosa is characterized by constant cell renewal and differentiation: epithelial cells proliferate in the crypts, migrate toward the villus and

differentiate to absorptive enterocytes. In untreated CD the number of proliferated cells and cellular turnover is increased, the differentiated cell-number is decreased, and the barrier function of epithelium is altered. Gliadin and proinflammatory cytokines can provoke these events, while several data suggest that CD autoantibodies have similar effects in cell cultures (Caja S et al., 2011). It was reported as early as in 1999 that celiac IgAs inhibit the *in vitro* differentiation of intestinal epithelial cells (Halttunen T and Maki M 1999), and later it was shown that they induce epithelial cell proliferation. In this later study commercial and patient derived monoclonal anti-TG2 antibodies induced a cell cycle S-phase entry of epithelial cells of celiac biopsy samples *ex vivo* (Barone MV et al., 2007). Other recent publication demonstrates that celiac IgA specifically increase the transepithelial passage of gliadin peptides *in vitro* (Rauhavirta T et al., 2011). To insert these information into the puzzle of CD-pathogenesis we can assume that IgA induce translocation of gliadin peptides to intestinal tissue, where they can trigger T-cell immune response, which will lead to tissue damage.

Autoantibodies of celiac patients can also activate monocytes by binding to toll-like receptor 4 (Zanoni G et al., 2006), which cells could secrete cytokines like interleukin 8 (IL-8) and TNF  $\alpha$  (Jelinkova L et al., 2004). This leads to attraction of immune cells to the site of inflammation and activation of MMPs through cytokine production mentioned above. In addition, the autoantibodies and gliadin can induce monocyte-mediated cytotoxicity and thus provoke tissue damage. Zanoni and colleagues suggest that autoantibodies can be the link between innate and adaptive immune responses.

Further, a reduced vascular  $\alpha$ -smooth muscle actin expression was found in intestinal biopsy specimens of untreated CD patients, which results in disorganized vasculature (Myrsky E et al., 2009b). TG2-specific celiac autoantibody deposits have been described around the small-bowel mucosal vessels (Korponay-Szabo IR et al., 2004) and TG2 plays role in angiogenesis (Jones RA et al., 2006). CD-specific IgA antibodies disturbed the initial steps of angiogenesis *in vitro* in human umbilical cord vein (HUVEC) cultures (Myrsky E et al., 2008). In another study increased blood vessel permeability was reported for macromolecules and lymphocytes by celiac antibodies (Myrsky E et al., 2009a).

Neurological manifestations occur almost in 7% of celiac patients; they have circulating neuronal antibodies against neurons, like Purkinje cells, which represent a distinct antibody population from anti-TG2 antibodies (Volta U et al., 2002). Sera of patients containing anti-TG2 antibodies with or without neuronal antibodies induce neuronal apoptosis, however, the effect of the former one (with neuronal antibodies) seemed to be more potent (Cervio E et al., 2007). TG2-specific IgA autoantibodies can be found deposited in the small intestinal mucosa

and in the brain vasculature of patients with gluten ataxia (Hadjivassiliou M et al., 2006b). Injection of serum derived from a patient with gluten ataxia or celiac patient-derived monoclonal antibodies to mouse brain resulted in similar symptoms that can be observed in gluten ataxia (Boscolo S et al., 2007).

### ***2.1.3. Animal models and new treatment options***

Since there is no cure for the disorder, several attempts have been made to develop good animal models to further clarify steps of the pathogenesis and to test experimental therapies. At the level of symptoms gluten fed rabbits (March JB 2003), juvenile rhesus macaques (Bethune MT et al., 2008), Irish setter dogs (Hall EJ et al., 1992) and a human DQ8 transgenic mouse model for dermatitis herpetiformis (Marietta E et al., 2004) appeared to be promising. However, none of these models produced circulating IgA class anti-TG2 antibodies, apparently an essential hallmark of the human disease and also the small intestinal damage was much milder than in patients. Newer studies revealed that the presence of HLA-DQ restricted gliadin specific CD<sup>+</sup> T lymphocytes is not enough to induce villous atrophy (de Kauwe AL et al., 2009) and additional inflammatory hits, such as IL-15 overproduction or infections are needed in humanized models (D'Arienzo R et al., 2009, DePaolo RW et al., 2011, Freitag TL et al., 2009). That is why the testing of new potential therapeutics seems not to be easy and should preferably be done on simplified biological systems, like cell cultures.

Since compliance with the GFD is complex and often unsuccessful, other attractive alternatives have been proposed. One of these introduces oral enzyme supplementation designed to accelerate gastrointestinal degradation of proline-rich gluten, especially its proteolytically stable antigenic peptides (Shan L et al., 2002, Stenman SM et al., 2009). Interference with activation of gluten-reactive T cells by inhibition of intestinal TG2 activity to prevent selective deamidation of gluten peptides and blocking the binding of gluten peptides to the HLA-DQ2 or HLA-DQ8 molecules is another alternative, though proper TG2 inhibitors for such a purpose have not been found yet. In a very recent paper interference with T cell activation could be instead demonstrated *in vitro* by using recombinant analogues of HLA molecules linked to gliadin peptides (Huan J et al., 2011).



## 2.2. TRANSGLUTAMINASES

The first transglutaminase discovered was blood coagulation Factor XIII responsible for the stabilization of fibrin monomers during blood clotting (Laki K and Lorand L 1948). Transglutaminase 2 (protein-glutamine  $\gamma$ -glutamyltransferases, E.C. 2.3.2.13) was identified in 1957 by Heinrich Waelsch as a liver enzyme of guinea pig, which incorporates amine into proteins (Sarkar NK et al., 1957). Later seven more TG enzymes were described in mammals. Papain, papain-like cysteine proteases and TGs belong to the same enzyme superfamily based on the similarities in the catalytic triad (Cys-His-Asp or Cys-His-Asn) and in reaction mechanism: formation of acyl-enzyme thioester intermediate between active site Cys and a Gln of the substrate, and the reaction of the intermediate with a suitable nucleophile.

### 2.2.1. Transglutaminase superfamily

TG enzymes can be found in all over the human body with diverse functions: fXIII plays essential role in fibrin clot stabilization and wound healing, TG1 and the epidermal TG3 are involved in the terminal differentiations of keratinocytes (TG3 was also identified as an autoantigen in DH), the prostatic TG4 plays role in semen coagulation, TG6 is in connection with gluten ataxia and the catalytically inactive homologue band 4.2 protein is a membrane skeletal component of erythrocytes (Table 2.)(Lorand L and Graham RM 2003). Features of TG2 are discussed in details below.

Identified forms of Tgase	Synonyms	Residues (molecular mass in kDa)	Gene	Gene map locus	Prevalent function
Factor XIII A	Catalytic A subunit of Factor XIII found associated with B subunit in plasma as A2B2 heterotetramer. Fibrin stabilizing factor	732 (83)	F13A1	6p24-25	Blood clotting and wound healing
Type 1 Tgase	Keratinocyte Tgase	814 (90)	TGM1	14q11.2	Cell envelope formation in the differentiation of keratinocytes
Type 2 Tgase	Tissue Tgase	686 (80)	TGM2	20q11-12	Cell death and cell differentiation, matrix stabilization, adhesion protein
Type 3 Tgase	Epidermal Tgase	692 (77)	TGM3	20q11-12	Cell envelope formation during terminal differentiation of keratinocytes
Type 4 Tgase	Prostate Tgase	683 (77)	TGM4	3q21-22	Reproductive function involving semen coagulation particularly in rodents
Type 5 Tgase	Tgase X	719 (81)	TGM5	15q15.2	Epidermal differentiation
Type 6 Tgase	Tgase Y		TGM6	20q11 15	Not characterized
Type 7 Tgase	Tgase Z	710 (80)	TGM7	15q15.2	Not characterized

**Table 2. Members of the transglutaminase enzyme family, characterized at protein level. (Griffin M et al., 2002)**

### ***2.2.2. Transglutaminase 2***

Ubiquitous expression, widespread localization and hydrolysis of guanine nucleotides –are some of the unique features of TG2 that distinguish TG2 from other TGs. TG2 catalyze the post translational modification of proteins by incorporation of primary amines into the  $\gamma$ -carboxamide group of glutamine residues or by the cross-linking of proteins via  $\epsilon$ -( $\gamma$ -glutamyl) lysine bonds. Beside these it can catalyze deamidation of glutamine to glutamic acid-at low pH, in the absence of a suitable amine when water can act as an alternative nucleophile. TG2 can also function as an isopeptidase (Fesus L and Piacentini M 2002); able to bind guanine nucleotides and hydrolyze guanosine-triphosphate (GTP). The enzymatic activities of the enzyme are regulated by the binding of GTP or  $\text{Ca}^{2+}$ : binding of GTP inhibits crosslinking activities, while binding of  $\text{Ca}^{2+}$  inhibits GTP-binding (Achyuthan KE and Greenberg CS 1987). TG2 was found to be identical with the  $\alpha$ -subunit of an atypical, high-molecular-weight G-protein, which mediates the activation of phospholipase C (PLC) by the  $\alpha_{1B}$ -adrenergic (Nakaoka H et al., 1994), and certain thromboxane and oxytocin receptors (Iismaa SE et al., 2000). Moreover, TG2 shows protein disulfide isomerase (Hasegawa G et al., 2003) and protein kinase (Mishra S and Murphy LJ 2004, Mishra S and Murphy LJ 2006) activities, it also act as a BH3-only protein and bind to pro-apoptotic Bcl-2 family member proteins (Rodolfo C et al., 2004).

In physiological conditions the transamidation function of TG2 is inactive, while in pathological states like tissue injury (Siegel M et al., 2008), or during apoptosis (Fesus L et al., 1987) the crosslinking activity of the enzyme is manifested. In the process of apoptosis the expression of TG2 is elevated, it interacts with mitochondria and increases the mitochondrial membrane potential (Piacentini M et al., 2002). Moreover, in apoptosis the intracellular  $\text{Ca}^{2+}$ -level also increases, which will switch TG2 to crosslink intracellular proteins (like actin and retinoblastoma protein) to stabilize the structure of the dying cells before being phagocytosed. This prevents the release of harmful intracellular components.

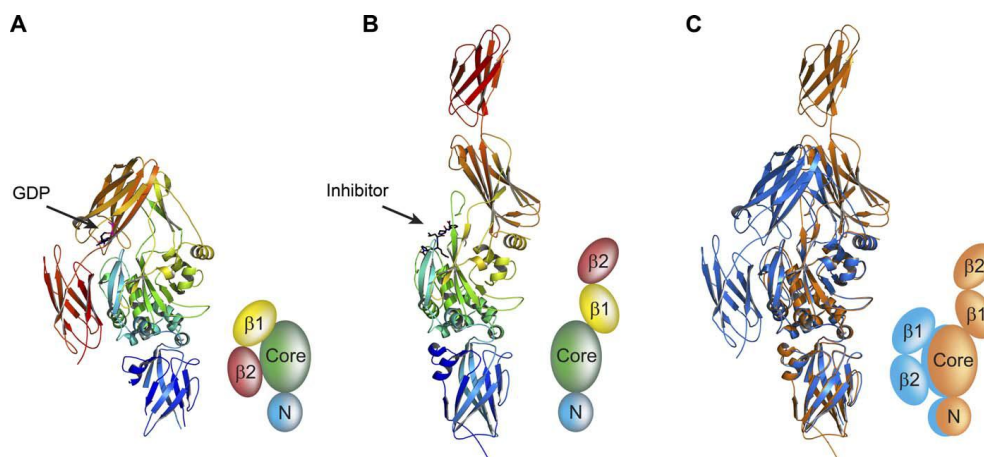
Although TG2 is mainly a cytosolic protein, it can be found in the nucleus (Tatsukawa H et al., 2009), and it is secreted to the cell surface and to the extracellular matrix (ECM) as well (Balklava Z et al., 2002). In the nucleus it can crosslink histones, SP1 and thus may affect gene expression. The enzyme is located on the cell surface in complex with fibronectin (FBN) and integrins, and this interaction facilitates adhesion, spreading and motility of cells (Akimov SS and Belkin AM 2001, Balklava Z et al., 2002). Moreover TG2 plays a role in wound healing and angiogenesis (Haroon ZA et al., 1999) and in the assembly, remodeling

and stabilization of ECM by crosslinking fibrinogen/fibrin, von Willebrand factor, collagen V and several other molecules (Aeschlimann D and Thomazy V 2000).

Despite the various functions of TG2, knockout mice are anatomically, developmentally, and reproductively normal.

### 2.2.2.1. Structure of transglutaminase 2

Similarly to other TG family members TG2 has four domains: I. domain: N-terminal  $\beta$ -sandwich (aa 1-139, with a short helix and several  $\beta$ -strands); II. domain: catalytic core (aa 140-460, with four  $\alpha$ -helices and many  $\beta$ -strands), III. and IV. domains: C-terminal  $\beta$ -barrels 1 and 2 (aa 472-585 and aa 586-687, arranged as antiparallel  $\beta$ -strands). Two conformations of the enzyme have been revealed by crystallizing it either complexed with guanosine-diphosphate (GDP) (Liu S et al., 2002) or with an inhibitor that mimics inflammatory gluten peptide substrates (Pinkas DM et al., 2007) (Fig. 4.).



**Figure 4. Overall structures of GDP-bound 'closed' (A) and inhibitor-bound 'open' (B) TG2.** The N-terminal  $\beta$ -sandwich and the catalytic core domains of the two structures are superimposed (C). The 'closed' conformation is shown in blue, the 'opened' is shown in yellow. Figure taken from (Pinkas DM et al., 2007).

The first domain of the molecule contains the FBN- and the integrin-binding sites; mutations of Asp94 and Asp97 significantly decreased the affinity of TG for FBN, which indicates that these residues play crucial role in FBN-binding (Hang J et al., 2005). TG2 also interacts with PLC through a binding site located in the IV. domain of the enzyme (Hwang KC et al., 1995). In the signaling complex TG2 is complexed with calreticulin (a  $\text{Ca}^{2+}$ -binding protein), which inhibits the GTP-binding capacity of TG2 (Feng JF et al., 1999).

In the GDP-bound form the catalytic triad (Cys277, His335 and Asp358) is located deeply within domain II., and access is blocked by the conserved aa Tyr516. This forms hydrogen bonds with active site Cys277 and inhibits the substrate entry. Trp241 is also crucial for the transamidating activity, it stabilizes the transition-state intermediate after substrate binding (Griffin M et al., 2002). On the contrary, in the abovementioned inhibitor-bound form of TG2 (Pinkas DM et al., 2007) the catalytic site is exposed, disclosing that catalysis occurs in a tunnel, while the relative positions of the two C-terminal  $\beta$ -barrels shift. This big conformational change may be the result of  $\text{Ca}^{2+}$ -binding in addition to the substrate binding, but the order of these binding steps and the exact way of activation require further analysis.

In TG2 a unique guanine-binding site can be found in a cleft between the catalytic core and the first  $\beta$ -barrel (aa 476-482, aa80-583 on  $\beta$ -barrel 1 and Lys173, Lys174 on the core domain), however, some additional amino acids on other domains can also be important for GDP/GTP-binding and GTP hydrolysis (Iismaa SE et al., 2000).

The  $\text{Ca}^{2+}$ -binding sites of TG2 were extensively investigated by Bergamini (Bergamini CM and Signorini M 1988) and recently by our group (Kiraly R et al., 2009). According to these data, TG2 binds six  $\text{Ca}^{2+}$ . Three of them (S1-S3) are homologous to the  $\text{Ca}^{2+}$ -binding sites of TG3 (Ahvazi B et al., 2002), while the other three were located by identification of negatively charged amino acids, which can act as a  $\text{Ca}^{2+}$ -binding site (Ambrus A et al., 2001). The relevance of these sites was proved by amino acid changes at the identified proposal areas and investigation of the mutant TGs by calcium-45 equilibrium dialysis and isothermal calorimetric titration. Each of the mutations resulted in decreased amount of bound  $\text{Ca}^{2+}$  and mutation at one side affected the binding of more  $\text{Ca}^{2+}$  suggesting cooperativity between the sites. S1 is a strong  $\text{Ca}^{2+}$ -binding site, while mutations at S4 and S5 sites have an effect on the antigenicity for celiac antibodies of TG2 (Kiraly R et al., 2009).

#### 2.2.2.2. Effects of celiac antibodies on the activity of transglutaminase 2

TG2 plays important role in the pathogenesis of CD by deamidation of gliadin peptides resulting in stronger immune response, moreover, TG2-specific autoantibodies bound to the enzyme can be found in the gut and in other organs (Korponay-Szabo IR et al., 2004, Salmi TT et al., 2006). Alteration of the enzymatic activity of TG2 by these antibodies can be an important factor in the disease development and progress.

It was shown as early as in 1977 that two antibody populations can be produced in rabbits by immunization with guinea pig TG2 and these either activate or inhibit the enzyme (Fesus L and Laki K 1977). Controversial data have been published on the effect of celiac autoantibodies on human TG2. IgA and IgG type celiac antibodies could inhibit the *in vitro* enzymatic activity of TG2 (Byrne G et al., 2010, Esposito C et al., 2002), while according to another report, total IgA fractions of celiac sera were inefficient to block the enzyme and affinity purified TG2-specific IgA population had partially inhibitory effect (Dieterich W et al., 2003). Similarly, only partial inhibitory effect was observed with membrane-associated TG2 in fibroblast cell culture (Barone MV et al., 2007). However, previous results demonstrated that celiac antibodies enhance the transamidation of large substrates in solid phase and when the enzyme is bound to fibronectin while they inhibit the G protein function of TG2 (Kiraly R et al., 2006). We also showed in an earlier report that celiac antibodies can bind to TG2 even when the active site is occupied by an inhibitor (Myrsky E et al., 2009a) and HUVEC cell culture studies of our Finnish collaborators found cellular changes that were consistent with increased TG2 enzymatic activity (Caja S et al., 2010).

The exact process how the antibodies affect the enzyme-activity is unclear. TG2 has other enzymatic and non-enzymatic functions as well, but the antibody-effect on these functions has not been addressed yet.

#### 2.2.2.3. Epitope mapping studies

TG2 epitopes were investigated by several groups, with various methods, but no such unique epitope has been found so far and it is not clear either whether only one or several such epitopes exist. In most of the studies truncated forms of the TG2 were created and the antibody-binding was examined either by radioimmunoassay (Nakachi K et al., 2004, Seissler J et al., 2001) or ELISA (Sblattero D et al., 2002). The major conclusions from these measurements were (i) neither the C-terminal nor the N-terminal domains are essential for antigenicity, but if both are missing, the celiac antibody binding is lost; (ii) the N-terminal region of the molecule may harbor one epitope; (iii) the C-terminus can be another important site; (iv) the core domain can also harbor one epitope; (v) truncation of the core domain is not tolerated, which indicates that the conformation of this part is important. Serum samples from the same patients sometimes recognized several complementary fragments. Based on these results it was suggested that the antibody response may be dispersed and variable according to

the subjects. Further, a difference was noted between young female celiac patients and those with other clinical manifestations (Tiberti C et al., 2003). In another study, mutant TG2 containing mutations of the catalytic triad had severely reduced antibody binding capacity (Byrne G et al., 2007). However, these authors did not confirm with any kind of activity measurements or structural studies that the conformation of the mutant protein was stable, so it cannot be excluded that the decreased antibody binding is due to the improper folding of the molecule.

Further studies conducted by TG2 fragments displayed on the surface of phages also indicated that the conformation of the protein might have a high importance to form a functional binding site for the celiac antibodies, as it was not possible to identify the epitopes of celiac patient derived monoclonal antibodies by these means either (Di Niro R et al., 2005).

### **2.3. AIMS OF THE STUDY**

1. After critical evaluation of available epitope mapping studies we aimed to search for the exact location of the binding sites of celiac anti-TG2 antibodies by molecular modeling and site directed mutagenesis. We also aimed to establish whether a celiac disease-specific binding site exists.
2. A second aim was to compare the binding properties of antibodies from different patient groups, from different ages and from different tissue sources in order to see which antibody specificities are clinically relevant.
3. We also aimed to establish whether the found TG2 epitopes are involved in the biological effects of celiac disease antibodies, which are the downstream consequences of the binding and whether any of the effects could be inhibited by a site-specific inhibitor.
4. We also wished to investigate a possible molecular mimicry between TG2 and gliadin peptides that could shape antibody specificities and could have a role in disease pathogenesis.

### **3. MATERIALS AND METHODS**

#### **3.1. Materials**

All materials were purchased from Sigma, otherwise indicated.

#### **3.2. Patients**

Serum samples from altogether 216 untreated and 22 treated celiac disease patients (aged 11 months-78 years) having Marsh grade III villous atrophy at diagnosis were used for the epitope mapping of TG2. Eleven CD subjects initially had preserved small bowel villous architecture, thus the diagnosis of celiac disease could not immediately be made (latent celiac disease). Six of these subjects were symptom-free family members of known celiac disease patients and were picked by family screening. During a follow-up of 0.8-3 years, nine of these subjects developed moderate to severe villous atrophy in the small bowel with local antibody deposition. The serum samples were collected at the latent stage of the disease. Included non-celiac controls had normal small bowel villous architecture.

Further 74 CD patients (median age, 7 years; range, 1.6–39.7 years) and 65 EMA negative nonceliac controls with normal small bowel villous architecture (median age, 13 years; range, 0.9–42.4 years) took part in the diagnostic evaluation of DGP peptides.

#### **3.3. Autoimmune sera**

Eleven serum samples with non-celiac TG2 antibodies from adult patients with established other disease diagnoses were selected from routine clinical samples investigated in 2008. These patients had systemic lupus erythematosus (SLE, n=3), rheumatoid arthritis (n=2), Sjögren's syndrome (n=6). None of these had serum endomysial antibodies or antibodies against deamidated gliadin peptides. The reactivity with human recombinant TG2 was confirmed by using two different recombinant constructs. Small bowel biopsy was performed in 3 patients with negative histology results (Marsh 0). HLA-DQ results were not available.

#### **3.4. Monoclonal antibodies**

Commercial anti-TG2 antibodies were purchased from NeoMarkers, Fremont, USA and Upstate; research antibodies with linear and conformation epitopes were obtained from collaborating groups. In the studies the following monoclonal antibodies were used:



Clone name	Immunogen	TG2 epitope
CUB7402	Guinea pig TG2	aa 447-478
TG100	Guinea pig TG2	aa 447-538
G92.1.2	Guinea pig TG2	aa 1-14
H23.1.2	Human red blood cell TG2	aa 433-438
885	Human recombinant TG2	aa 153
890	Human recombinant TG2	not identified
895	Human recombinant TG2	aa 649-687
896	Human recombinant TG2	aa 649-687
901	Human recombinant TG2	core domain
925	Human recombinant TG2	core domain
3.3.1	Human recombinant TG2	I. domain
4E1	Human recombinant TG2	aa 637-648
6B9	cell extract of human T lymphocytes	I. domain
4G3	Human recombinant TG2	aa 1-165

**Table 3.** Source and further characteristics of these monoclonal antibodies are described in Supplement 2.

### 3.5. Expression and purification of recombinant TG2 proteins

Full-length human recombinant N-terminally His-tagged TG2 (aa 1-687) was expressed in Rosetta 2 cells (Novagen, Darmstadt, Germany). pGEX-2T-TG2 DNA (Ambrus A *et al.*, 2001) was amplified with specific primers (primer 1, 5'-gac gacgacaagatgagaatt cag accatggcc gag gagctg g – 3', and primer 2, 5'- gag gagaag ccc ggttgaattcggttaggcggggccaat gat gac – 3'), and subcloned into pET-30 Ek/LIC Vector. TG2 mutations were generated according to the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Agilent Technologies, Inc., Santa Clara, CA, USA). Domain deletion mutant TG2s were created by the same technique with a template pcDNA 3.1/myc-His mammalian expression vector (Invitrogen, Carlsbad, CA, USA) containing the following combinations of TG2 structural domains: A/ I+II+III (aa 1-584), B/ I+II+IV (aa 1-471 and 585-687), C/ II+III+IV (aa 139-687), D/ I+III+IV (aa 1-138 and 472-687), and E/ His-tag only, without the sequence of the enzyme. The templates were kind gifts of Professor Soichi Kojima (Molecular Cellular Pathology Research Unit, RIKEN, Wako, Saitama, Japan). The sequence of the mutants was confirmed by DNA sequencing using the ABI PRISM® 3100-Avant Genetic Analyzer.

Rosetta 2 cells (Novagen, Darmstadt, Germany) were transformed with the expression vectors containing wild type or mutant TG2s and grown in LB at 37°C to an OD600 0.6-0.8. To induce the expression of His-tagged proteins, the cultures were grown for 5h at 20°C in the presence of 0.3 mM isopropyl  $\beta$ -D-thiogalactoside, and cells were harvested by centrifugation at 4°C. The cells were resuspended in binding buffer + 10% (v/v) glycerol, 1% (v/v) Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (PMSF) (binding buffer: 50 mM sodium phosphate (pH 7.8), 500 mM NaCl, 20 mM imidazole, 20 mM  $\beta$ -mercaptoethanol). After cell lysis by sonication the lysate was centrifuged for 25 minutes 20000g. Supernatants were diluted 2 times with binding buffer and applied to ProBond Ni-NTA resin (Invitrogene). The column was washed with 40 column volume binding buffer + 800 mM NaCl and 20 mM imidazole and with 15 column volume binding buffer + 500 mM NaCl and 30 mM imidazole. The proteins were eluted with 10 column volume binding buffer containing 250 mM imidazole, concentrated with AmiconCentricon-YM 50 MW (Millipore, Billerica, MA, USA) and the buffer was exchanged to 20 mM Tris-HCl, pH 7.2, with 150 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA) and 10% (v/v) glycerol. All purification steps were performed on ice or at 4°C. The protein concentration was determined by Bradford method (Bio-Rad, Philadelphia, PA, USA).

The following constructs were prepared and used in the studies:

Mutant name	Applied mutations
S1	N229S/N231S/D232N/D233N
S4	D151N/E153Q/E154Q/E155Q/E158Q
S4.1/4/5	D151N/E154Q/E155Q
S5	D434N/E435Q/E437Q/D438N
433	R433S/E435S
R	R19S
E	E153S
M	M659S
RE	R19S/E153S
RM	R19S/M659S
EM	E153S/M659S
REM	R19S/E153S/M659S
154K	E154K
RKM	R19S/E154K/M659S

**Table 4.**

Mutant name	Amino acid composition
A (domains I+II+III)	aa 1-584
B (domains I+II+IV)	aa 1-471 and aa 585-687
C (domains II+III+IV)	aa 139-687
D (domains I+III+IV)	aa 1-138 and aa 472-687
truncated TG2	aa 1-648

**Table 5.**

### 3.6. Western Blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) was performed according to standard techniques. TG2 proteins were separated by SDS/PAGE and transferred to Polyvinylidene Fluoride (PVDF) membrane (Millipore). After blocking the membrane it was incubated with goat polyclonal anti-TG2 antibody (Upstate, Millipore) diluted 1:20000 in TTBS or with mouse monoclonal anti-TG2 antibody TG100 (NeoMarkers, Lab Vision Products, Thermo Fisher Scientific, Fremont, CA, USA) diluted 1:15000, followed by extensive washing and incubation with anti-goat antibody or with anti-mouse antibody conjugated with horseradish peroxidase (HRP), 1:30000 in TTBS. The bands were revealed by Chemiluminescent ECL Detection System (Millipore).

### 3.7. Measurement of transglutaminase and GTP-ase activity

Transglutaminase activity was measured with microtiter plate assay based on the incorporation 5-(biotinamido) pentylamine (Invitrogen) into immobilized *N,N*-dimethylated casein (Kiraly R et al., 2006) with the following modifications. The reaction mixture (in a total volume of 200  $\mu$ L 100 mM Tris-HCl pH 8.0) contained 10 mM dithiothreitol, 1 mM *N*-(5-aminopentyl) biotinamide and 5 mM CaCl<sub>2</sub>. The reaction was performed at 37°C for 30 minutes, starting with the addition of 0.5  $\mu$ g TG2 enzyme diluted in 50  $\mu$ L Tris-HCl buffer. Reaction blanks contained 10 mM EDTA and no added CaCl<sub>2</sub>. Amine incorporation was detected by streptavidine-alkaline phosphatase then with 200  $\mu$ L 25 mM p-nitrophenyl phosphate and the absorbance was measured at 405 nm; enzyme activity was calculated from  $\Delta A$ /min of colour development between 10 and 30 minutes.

GTPase activity was measured with charcoal method (Kiraly R et al., 2006) with the following modifications. The 100  $\mu$ L reaction mixture contained 2  $\mu$ g of recombinant wild type or mutant TG2 in 50 mM Tris-HCl, pH 7.5, 4 mM MgCl<sub>2</sub>, 1mM DTT, 10% (v/v) glycerol, 9.9  $\mu$ M GTP and 0.1  $\mu$ M [ $\gamma$ -<sup>32</sup>P]GTP (3000 Ci/mmol, Institute of Isotopes Ltd.).

The reaction was performed at 37°C for 30 min and was stopped with 700 µL of 6% (w/v) activated charcoal in ice-cold 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.5. The mixture was centrifuged and released [<sup>32</sup>P] Pi was determined by counting of 150 µL samples of the supernatant. Blank was determined without the enzyme. Transglutaminase and GTPase activities of the mutants were calculated as percentages compared to WT TG2 and are presented as means from two separate measurements done in triplicate.

### **3.8. Molecular modeling**

Crystal structures of human TG2 (PDB code: 1KV3) and TG3 (PDB code: 1VJJ) were used for modeling the full length TG2. Residues 1-14, 44-55 and 123-132 were missing in the crystal structure of TG2, however, the corresponding regions were visible in the TG3 structure, which had highly similar sequence and three-dimensional structure. Homologous model was built by Modeller (Sali A and Blundell TL 1993) using the multiple template option of the program. Graphical analysis was made on Silicon Graphics Fuel workstation using Sybyl program package (Tripos International, St. Louis, MO, USA) and VMD (Humphrey W et al., 1996) with the help of Péter Bagossi.

### **3.9. Single Chain Variable Fragments (scFv)**

Total antibody libraries and ScFvs were selected and prepared by Daniele Sblattero and colleagues as described (Marzari R et al., 2001). Briefly, Total RNA was prepared from intestinal biopsy sample from three previously untreated CD adult patients. cDNA was synthesized and Ig variable (V) regions were amplified by using specific V region primers and assembled into scFv. Anti-TG2 specific scFvs were selected by phage display and scFv were used directly as supernatants of induced bacterial cultures.

### **3.10. Anti-TG2 ELISA**

Microtiter plates (ImmunoPlateMaxisorp, Nunc, Thermo Fisher Scientific, Fremont, CA, USA) were coated with 0.6 µg TG2 in 100 µL of tris-buffered saline (TBS) containing 5 mM CaCl<sub>2</sub> (pH 7.4) (Ca-TBS). When denatured TG2 was investigated, TBS contained 8 M urea or 6 M Guanidine-HCl during the coating. The plates were washed 3 times with TBS containing 0.1% (v/v) Tween20 and 10 mM EDTA (TTBS+EDTA) to block unoccupied binding sites. All antibodies were diluted in TTBS+EDTA. Serum samples (diluted 1:200), ScFvs used as bacterial culture supernatants (1:15-1:2.5) or monoclonal antibodies (TG100, 1:500, NeoMarkers; CUB7402, 1:1000, Neomarkers; 895, 4G3; G92; H23) were incubated for 1 h at

room temperature. Plates were washed and incubated either with HRP-conjugated rabbit anti-human IgA or IgG (1:5000; DAKO AS, Glostrup, Denmark) or with monoclonal antibody (MAb) recognizing SV5 tag at the C-terminus of the ScFv, followed by HRP-conjugated anti-mouse IgG (1:5000) for 1h at room temperature. The color reaction was developed by adding 100  $\mu$ L 3,3',5,5'-tetramethylbenzidine substrate and then stopped with 50  $\mu$ L 1 N H<sub>2</sub>SO<sub>4</sub>. The absorbance was read at 450 nm.

Standard curves were prepared using dilutions of TG100 monoclonal anti-TG2 antibody (1:500, 1:1000 and 1:6250) for each mutant and the binding of other antibodies was calculated by 4-parameter fit if binding to wild-type TG2 was 100%.

### **3.11. Fibronectin-TG2 ELISA**

Microtiter plates were coated with 0.3  $\mu$ g human fibronectin (FBN) diluted in bicarbonate buffer pH 9.6 for 1h at room temperature. The plates were incubated with 0.8  $\mu$ g TG2 in TBS containing 5 mM CaCl<sub>2</sub> and 0.1% (v/v) Tween 20 and the assay continued as above.

### **3.12. Liquid phase competition assay**

Microtiter plates (Maxisorp) were coated with 0.6  $\mu$ g wild type TG2. Celiac sera were preincubated with different amounts of Wt or mutant TG2 in Ca-TBS containing 0.1% Tween 20 for 10 min at room temperature. This mixture was added to the wells and incubated for 1h at room temperature. After extensive washing steps the coated Wt TG2-bound IgA antibodies were detected with HRP-conjugated rabbit anti-human IgA (1:5000) for 1h at room temperature. The color reaction was developed by adding 100  $\mu$ L 3,3',5,5'-tetramethylbenzidine substrate and then stopped with 50  $\mu$ L 1 N H<sub>2</sub>SO<sub>4</sub>. The absorbance was read at 450 nm.

### **3.13. Competition ELISA assays**

Microtiter plates were coated with 0.6  $\mu$ g WT TG2 in 100  $\mu$ L of TBS containing 5 mM CaCl<sub>2</sub> (pH 7.4). Mixture of M13 phage-conjugated ScFvs and increasing amounts of soluble ScFvs were simultaneously added and bound phage-ScFvs were detected with anti-M13 phage antibody conjugated with HRP. In other assays, wells were incubated with celiac serum (1:800) and increasing amounts of purified total celiac IgG antibodies (dilution 1:200-1:50) and binding of IgA and IgG antibodies were detected. Competition assays were also carried out with celiac serum added to the plate together with increasing amounts (up to 18  $\mu$ g) of monoclonal mouse antibodies (885, CUB7402, H23) and bound IgA was measured.

### **3.14. Displacement ELISA measuring serum IgG anti-TG2 in IgA deficient samples**

Serum samples from 56 untreated celiac disease patients aged 11 months-73 years (median 10.5) with selective IgA deficiency (total serum IgA <0.05 g/l), from 23 non-celiac IgA deficient and 22 normal serum IgA controls with normal small bowel architecture (age of the controls 1-18 years, median 5.5) were measured in ELISA with wild-type TG2 antigen. Investigated serum samples were diluted 1:100 with assay buffer containing a celiac IgA tracer antibody diluted 1:500 and the binding of the IgA antibody was measured by anti-IgA peroxidase-conjugated secondary antibodies. The optical density values (OD) obtained with blank were defined as 100% inhibition and the signal with the IgA tracer antibody without added IgA deficient samples was defined as 0% inhibition. The inhibition obtained with the investigated samples was calculated as  $100 - (OD_{\text{tracer}} - OD_{\text{sample}} / OD_{\text{tracer}})$ .

### **3.15. IgG and IgA purification**

For competition assays IgG antibodies were purified from serum samples of CD, autoimmune patients and healthy control subjects with Protein G conjugated Sepharose beads (AP Biotech) according to the manufacturers' instructions. IgG fractions were eluted with 0.1 M glycine-HCl, pH 2.8 and neutralized by 1 M Tris-HCl buffer, pH 8.0 to prevent denaturation. Flow-through were collected and diluted with 75 mM Tris-HCl, pH 8.0 for IgA purification by anti-human IgA ( $\alpha$ -chain specific)-agarose. After washing with 0.5 M NaCl, the antibody fractions were eluted with 0.1 M glycine-HCl + 0.5 M NaCl, pH 2.5 and neutralized in the same way as mentioned above.

The antibody fractions were concentrated using Centricon tubes (Millipore) and the buffer was changed to 0.1 M Tris-HCl, pH 7.5. The protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad) with human immunoglobulin IgA and IgG (Calbiochem, Merck, Darmstadt, Germany) as standard. The TG2-specific antibody contents were determined by anti-TG2 ELISA.

### **3.16. Immunofluorescent studies**

Unfixed 5 $\mu$ m thick frozen sections of patient tissues were investigated for deposited immunoglobulins by direct immunofluorescence. Sections were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-human IgA or IgG (DAKO) at dilution of 1:40 in phosphate buffered saline (PBS), pH 7.4. These stainings were also combined with double labeling for TG2 by anti-TG2 MAbs (CUB7402 and 885), diluted 1:200 in PBS. For

secondary antibody, Alexa 594-conjugated anti-mouse antibody (Invitrogen, Carlsbad, CA, USA) was used. For detecting competition, TG2-specific MAbs were added to the tissue for 30 minutes in PBS, and the incubation solutions were recollected and tested for patient IgA by ELISA after a purification step removing MAbs by protein-G conjugated magnetic beads (Dynabeads, Invitrogen).

### **3.17. Elution of IgA from tissue sections**

Unfixed cryosections were washed twice with PBS to remove blood and non-bound antibodies. Elution buffer containing 0.25% chloroacetic acid (Fluka Chemic AG, Buchs, Switzerland) with 0.2 M/L NaCl, pH 2.7 was applied to the sections for 20 minutes. The eluates were neutralized with 1 M/L imidazole, concentrated on 10K membranes (Microsep, Pall Corporation, Ann Harbor, Michigan, USA) and the buffer was changed to PBS. These concentrated samples were used in anti-TG2 ELISA measurements.

### **3.18. Human umbilical vein endothelial cells (HUVEC) preparation and cell culture experiments**

HUVEC cells were prepared and cultured using standard techniques (Palatka K et al., 2006). HUVEC cells were mixed with Matrigel (BD Biosciences Franklin Lakes, NJ, USA) and grown in 24-well dishes (50.000 cells/well) with complete endothelial medium EGM1 (Lonza, Basel, Switzerland) containing 2% fetal bovine serum with or without 1 ug/well MAb885. After 4 hours, 1 ug/well purified patient or control IgAs were added. After further 24 hours ten images were taken per well and the length of formed endothelial tubules was analyzed by Image J software. Results were expressed as % median tubule length of basal values with 95% confidence intervals using as reference the geometric mean of tube length in wells without antibodies.

In some experiments HUVECs were plated together with the investigated antibodies on collagen I and grown for 24 hours (Myrsky E et al., 2009a). Cell lengths were evaluated by Image J software in similar ways.

### **3.19. DGP-ELISA**

DGP-reactive antibodies were measured from patient serum samples by Quanta Lite™ Celiac DGP Screen test kit (Inova Diagnostics, San Diego, CA, USA) according to the manufacturer's instructions. This kit contains ELISA plate coated with synthetic peptides corresponding to deamidated gliadin peptides and a combined HRP conjugate that recognizes

both IgA and IgG class human antibodies. The same plates were also probed with mouse monoclonal TG2-specific antibodies (Table 3.) using the buffers provided in the kit. The mouse antibodies were recognized by HRP-conjugated anti-mouse antibodies (DAKO) diluted 1:4000 and the reaction was revealed as written in anti-TG2 ELISA.

### **3.20. Competition studies with DGP ELISA**

Monoclonal TG2-specific antibodies were added to the commercial DGP-ELISA plates in the presence of increasing amounts of human recombinant TG2 constructs or celiac patient serum samples. The His-tagged construct devoid of the sequence of the enzyme was used as negative control. The OD450 signal of the blank was defined as 100% inhibition, and the signal without added TG2 or celiac serum was defined as 0% inhibition.

### **3.21. Statistical analysis**

ELISA results were analyzed using GraphPad Prism Software and STATISTICA. For comparison of antibody binding to mutant TG2, data were analyzed using repeated measures ANOVA followed with Dunnett's Multiple post-test, one way ANOVA followed by Tukey's post-test, or Kruskal-Wallis test followed by Dunn's multiple comparison test as appropriate. A  $p$  value  $< 0.05$  was considered significant.

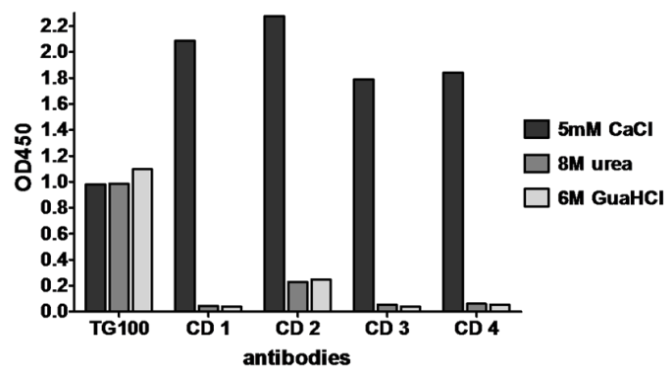


## 4. RESULTS

### 4.1. The celiac transglutaminase 2 epitopes are conformational

Several previous attempts were made to identify the celiac epitopes of TG2, mainly by using truncated TG2 mutants. In overall, the results suggested an important binding site on the C-terminal  $\beta$ -barrel and in the case of some patients also on the N-terminal  $\beta$ -sandwich domain, while in another study the mutation of the catalytic triad on the core domain led to altered celiac antibody binding. These show that the bindings of CD antibodies are dispersed and several parts of the molecule can take part in the formation of the epitope(s). However, it was also possible that the extended truncation of TG2 altered the three-dimensional structure of the molecule and this caused the diminished binding of the CD antibodies. In our initial approach we also considered that CD antibodies can not recognize TG2 in Western-blot experiments after usual SDS/PAGE which destroys the three-dimensional structure of the molecule and if the bindings of CD antibodies depend on the intact fold of TG2, the CD epitope can be conformational.

To establish which proportion of celiac antibodies in polyclonal patient serum samples bind to linear epitopes or to conformational binding sites we used guanidine-hydrochloride and urea, which agents have chaotropic properties and denature the enzyme. We coated the ELISA plates with TG2 diluted in buffers containing these agents and wells coated with TG2 in  $\text{CaCl}_2$  were applied as controls. TG100 monoclonal anti-TG2 antibody with a linear binding epitope was used to prove that denatured TG2 was present in equal amounts on the plate.

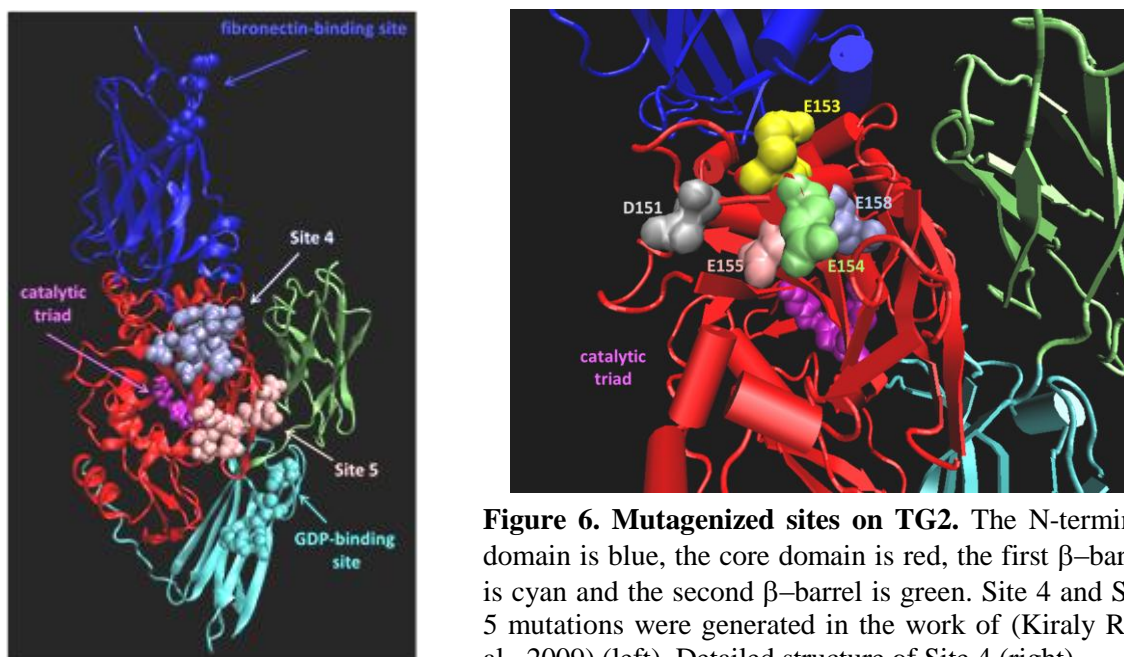


**Figure 5. Binding of celiac antibodies to TG2 is conformation dependent.** Binding of monoclonal anti-TG2 antibodies TG100 (NeoMarkers) targeting a linear epitope at amino acids 447-538 and of serum IgA antibodies from 4 celiac patients (CD1-CD4) to wild type TG2 in ELISA. The antigens were coated in the presence of 5 mM  $\text{CaCl}_2$ , 8 M urea or 6 M Guanidine hydrochloride. Representative values of two independent experiments.

The binding abilities of celiac IgAs to TG2 were abolished when the enzyme was denatured (Fig. 5.), while the binding was unaltered when the enzyme remained intact in the presence of  $\text{CaCl}_2$ . This result suggests that the investigated celiac autoantibodies are conformational and they practically do not bind to linear epitopes.

#### 4.2 The celiac epitope is related to $\text{Ca}^{2+}$ -binding sites of TG2 but $\text{Ca}^{2+}$ ions do not form part of the epitope

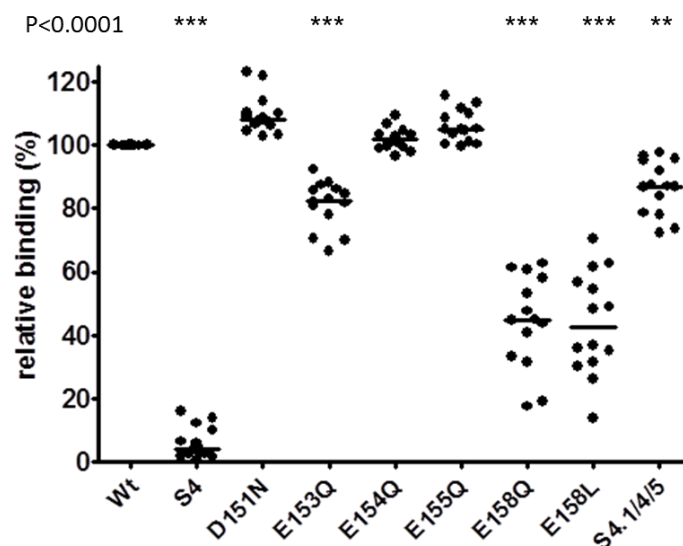
During previous work performed in our laboratory (Kiraly R et al., 2009) two  $\text{Ca}^{2+}$ -binding sites on the core domain, Site 4 (S4, aa 151-158) and Site 5 (S5, aa 433-438), were identified which may play a role in the binding of celiac antibodies. These areas were considered as  $\text{Ca}^{2+}$ -binding sites based on the high density of negatively charged glutamate and aspartate residues, which were changed to neutral glutamine and asparagine during the creation of mutant TG2 constructs. The overall structures of the mutant proteins were maintained shown by circular dichroism spectroscopy. The S4 and S5 mutants have decreased  $\text{Ca}^{2+}$ -binding (3 bound  $\text{Ca}^{2+}$  ions per TG2 molecule, while Wt TG2 binds 6) and celiac antibody-binding properties in ELISA (Kiraly R et al., 2009). S4 is located at the first alpha helix of the core domain, relatively close to S5 (Fig. 6.). In the case of S4 the binding of CD IgAs was diminished to  $11.6 \pm 8.5\%$  (if binding to Wt TG2 is 100%), so we decided to search for the anchor points of the celiac epitope in this area.



**Figure 6. Mutagenized sites on TG2.** The N-terminal domain is blue, the core domain is red, the first  $\beta$ -barrel is cyan and the second  $\beta$ -barrel is green. Site 4 and Site 5 mutations were generated in the work of (Kiraly R et al., 2009) (left). Detailed structure of Site 4 (right).

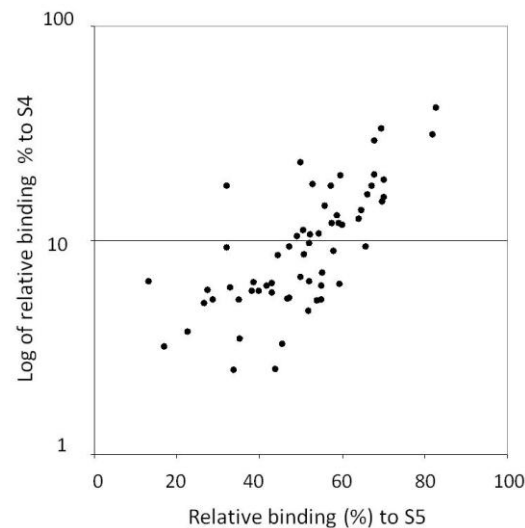
During this work we prepared TG2 point mutants holding D151N, E153Q, E154Q, E155Q, E158Q and E158L amino acid changes separately (Fig. 6.) and the binding of serum IgA from celiac patients was measured in ELISA. Only changes at residues 153 or 158 decreased the binding of celiac antibodies significantly (Fig. 7.;  $p < 0.0001$ , median 82.6%, 44.9% and 42.6% remaining binding for E153Q, E158Q and E158L, respectively if binding to Wt TG2 is 100%). However, the point mutation of Glu153 to Gln was not sufficient to abolish patient antibody binding completely, and residue 158 is not surface exposed, since it is situated inside a helix formed by the S4 site amino acids. Analyzing the crystal structure of TG2 (1KV3)(Liu S et al., 2002), we found that loss of the negative charge at 158 probably influences antigenicity of TG2 indirectly, by changing the relative position of the helix to other surface amino acids. This could explain that there was no difference between a bulky substitution (E158Q) and a small side-chain (E158L). Based on these results, Glu153 seemed to be one potential anchor point for the binding of celiac antibodies, but it might need cooperativity of other surface parts to form an epitope.

We combined the three amino acid changes D151N/E154Q/E155Q creating mutant S4.1/4/5, to see whether these surface-exposed residues determine the celiac epitope, but the decrease of celiac antibody binding was even less (median 86.6% remaining binding), than in the case of single mutant E153Q (median 82.6%).



**Figure 7. Binding of celiac IgA serum antibodies (n=14) in ELISA to wild-type (Wt) and mutant TG2s.** S4 contains the combined mutations D151N-E153Q-E154Q-E155Q-E158Q (Kiraly R et al., 2009). Dash indicates median. \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$  compared to Wt, analyzed by one way ANOVA followed by Tukey's post-test.  $P < 0.0001$  represents significant differences between groups by ANOVA test.

Decreased celiac antibody-binding properties were found also for mutant S5 ( $51.3 \pm 16.0\%$  if binding to Wt enzyme is 100%, as measured with 62 serum samples of CD patients) and these binding values were used in correlation analysis together with binding values for S4. The values for S5 correlated with the logarithmic values of S4, indicating that the main anchor residues can be found in S4, while the positions of the determining side chains may be affected by the nearby S5 as well.

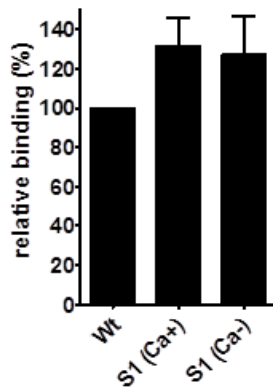


**Figure 8. Correlation of relative bindings of celiac antibodies (n=62) to Site 4 and Site 5 mutants if binding to wild type TG2 is 100%.**

During the analysis of the S5 site two charged amino acids (Arg433 and Glu 435) were identified as possible anchor points and these were also situated at the lowest distance from Site 4. When these were substituted with Ser (mutant 433), no decrease in celiac antibody binding was observed, but we found that a non-celiac mouse monoclonal anti-TG2 antibody (H23) is having its binding site in this region and it is unable to recognize mutant 433. H23, however, did not compete with celiac antibodies for the binding to Wt TG2 and it does not bind to endomysial structures indicating that this binding site does not have primary importance for celiac disease.

Since none of the S4 and S5 site amino acids alone could be held responsible for the celiac antibody binding and S4 is a  $\text{Ca}^{2+}$ -binding site, we explored next if the  $\text{Ca}^{2+}$ -ions themselves would take part in the formation of the epitope. This possibility arose from the fact that in earlier studies (Sulkanen S et al., 1998) celiac antibodies bound to the autoantigen preferably in the presence of  $\text{Ca}^{2+}$ -ions. The previous study of Kiraly et al. (Kiraly R et al., 2009)

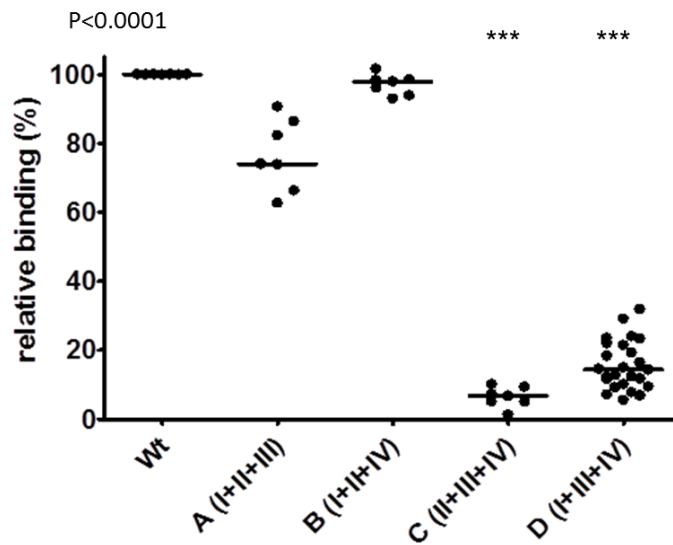
revealed that Wt TG2 binds 0.5 mol  $\text{Ca}^{2+}$  per mol enzyme even after extended dialysis in EDTA-containing buffer. This strongly bound  $\text{Ca}^{2+}$  can only be eliminated if the high-affinity  $\text{Ca}^{2+}$ -binding site is destroyed by mutating the S1 site (amino acids 229-233). The S1 mutant (after dialysis in EDTA-containing buffer) could bind celiac antibodies equally well ( $\geq 100\%$ ) in ELISA assay both in the presence and absence of  $\text{Ca}^{2+}$ -ions during the coating procedure (Fig. 9.). This result excludes the structural role of  $\text{Ca}^{2+}$  ions in the potential epitope(s).



**Figure 9. Binding of celiac antibodies to wild-type TG2 and to mutant of the S1 (strong)  $\text{Ca}^{2+}$ -binding site of TG2.** The S1 mutant was extensively dialyzed with EDTA and then coated to the plate in the presence or absence of 5 mM  $\text{Ca}^{2+}$ . Values represent means  $\pm$  SD. Representative values of two independent experiments with n=5 patient samples.

#### 4.3 Identification of anchor points outside of the core domain

We further investigated the additional anchor points of the celiac epitope on the other domains of TG2 and we expressed TG2 mutants each lacking one structural domain of TG2. In previous studies it was proved that the N-terminal domain (I) expressed alone (Hang J et al., 2005) and also domains III and IV would adopt a functional conformation also without the presence of the core (II) domain (Pinkas DM et al., 2007). When the domain mutants were applied in equimolar concentrations in immunoassays, both domain I (aa 1-139) and II (aa 147-460) turned out to be important for autoantibody binding (Fig. 10., median 6.7% and 14.4% for remaining binding of mutant C and D, respectively). The binding was also influenced to some extent also by the loss of domain IV (aa 585-687; median 74.0% for remaining binding of mutant A). In contrast, antibody binding proceeded normally even in the complete absence of domain III (aa 472-584; median 97.9% for remaining binding of mutant B). Based on these results and in line with some previous data (Nakachi K et al., 2004, Sblattero D et al., 2002, Seissler J et al., 2001), it can be suggested that parts of celiac epitopes are found both on the N-terminal and C-terminal domains. However, patient antibodies showed lack of binding to the mutant containing only domains I-III-IV but not domain II, indicating that the presence of the core domain anchor residues is needed for efficient binding, and so in their absence the other epitope parts were not able to form functional binding sites.

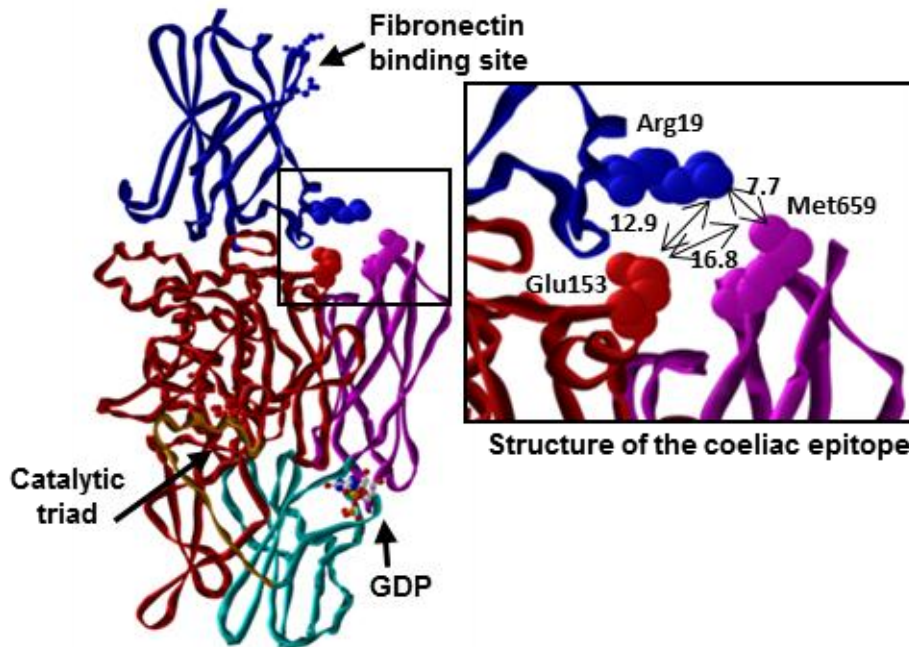


**Figure 10. Binding of celiac IgA serum antibodies (n=7-25) in ELISA to wild-type (Wt) and mutant TG2s.** Dash indicates median. \*\*\*, p<0.001; compared to Wt, analyzed by one way ANOVA followed by Tukey's post-test. P<0.0001 represents significant differences between groups by ANOVA test.

#### 4.4 Three anchor points determine a composite epitope formed by three distinct domains and two of these are sufficient for binding of celiac antibodies

Next, we used molecular modeling to evaluate whether cooperation of core domain Glu153 together with other amino acids on the N-terminal and/or C-terminal domains might form a conformational and functional epitope or epitopes. The X-ray crystallographic model of TG2 with bound GDP was used (1KV3, 2.8-Å) (Liu S et al., 2002) and to increase the accuracy of the determination, the conformation of residues missing in the crystal structure of TG2 (amino acids 1-14, 44-55 and 123-132) were allocated by homolog modeling according to the crystal structure of transglutaminase 3 (TG3; 1VJJ, 2.1-Å) (Ahvazi B et al., 2002). The N- and C-terminus of the molecule are close to each other and also to the surface of the core domain. Glu153 on core domain is in the closest proximity to Arg19 on the N-terminal domain (12.9 Å) and to Met659 on the C-terminal domain (16.8 Å). Besides these, Arg19 is also close to Met659 (7.7 Å), so this three amino acids possibly form a common conformational epitope (Fig. 11.). Therefore, mutant TG2 molecules containing changes of these amino acids to serine as single point mutations (R, R19S; E, E153S; M, M659S) or in combination (RE, EM,

RM, and REM) were created. Serine was used for replacement instead of the more conventional alanine (Ala) because all investigated positions were located at the surface of the molecule, and hydrophobic portion introduced by mutagenesis using Ala may cause folding problems.

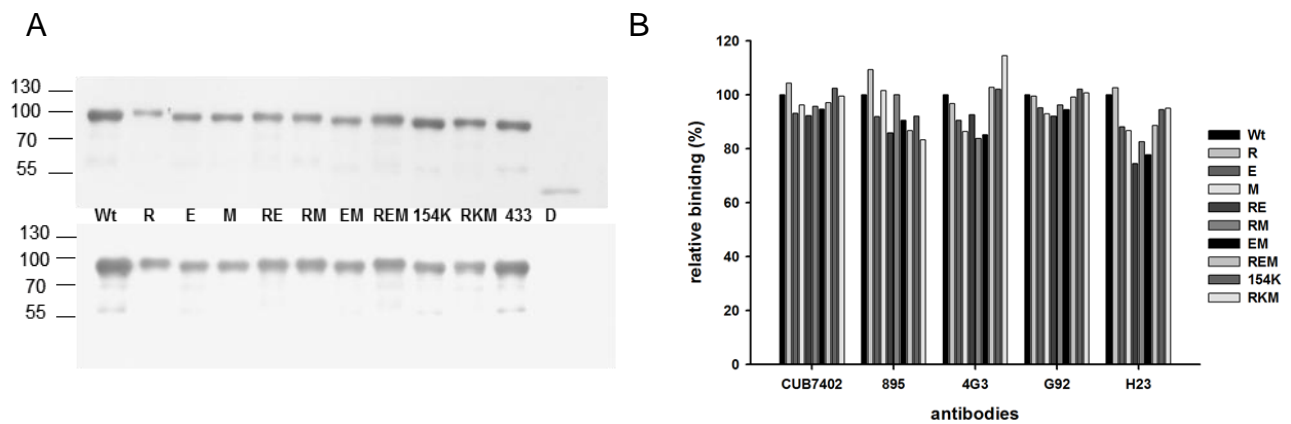


**Figure 11. Identification of the celiac epitope.** Three-dimensional view of TG2 in the closed conformation with the N-terminal  $\beta$ -sandwich shown in blue, catalytic (core) domain in red,  $\beta$ -barrel 1 in cyan and  $\beta$ -barrel 2 in pink. The bound GDP, fibronectin binding site (Asp94, Asp97) and catalytic triad (Cys277, His335, Asp358) are represented as ball-and-stick side chains. The amino acids of the putative celiac epitope with their lowest distances in Ångström (in the frame) are illustrated as surface representation.

Recombinant TG2s were expressed in histidine-tagged forms and purified with nickel affinity chromatography. The protein fractions were checked by SDS/PAGE and Western blotting. Goat polyclonal anti-TG2 antibody and TG100 anti-TG2 monoclonal antibody (MAb TG100, epitope aa 447-538) were used for the blotting analysis.

As expected, both antibodies recognized the TG2 mutants (Fig. 12.A) except for the recognition of mutant D by MAb TG100, which antibody has an epitope on the core domain. All of the constructs harbor this site except for mutant D, where the core domain is lacking. The protein bands showed little proteolytic degradation; however, they indicated slight differences in the intensity compared to the wild type enzyme. This phenomenon is probably due to the different properties of the mutants, which could affect the expression or purification

efficiency. The Coomassie brilliant blue-staining of the SDS gel showed purity greater than 80% (data not shown).



**Figure 12. Characterization of the mutant transglutaminase 2 (TG2) proteins I.** A. Western blot. Upper panel: primary antibody: goat polyclonal anti-TG2, secondary antibody: anti-goat-HRP; lower panel: primary antibody: mouse monoclonal anti-TG2 antibody TG100, secondary antibody: anti-mouse-HRP. B. Binding properties of a set of mouse monoclonal (MAb) anti-TG2 antibodies to the mutants measured by anti-TG2 ELISA. Representative values of two independent experiments. The changed amino acids are listed in Table 4.

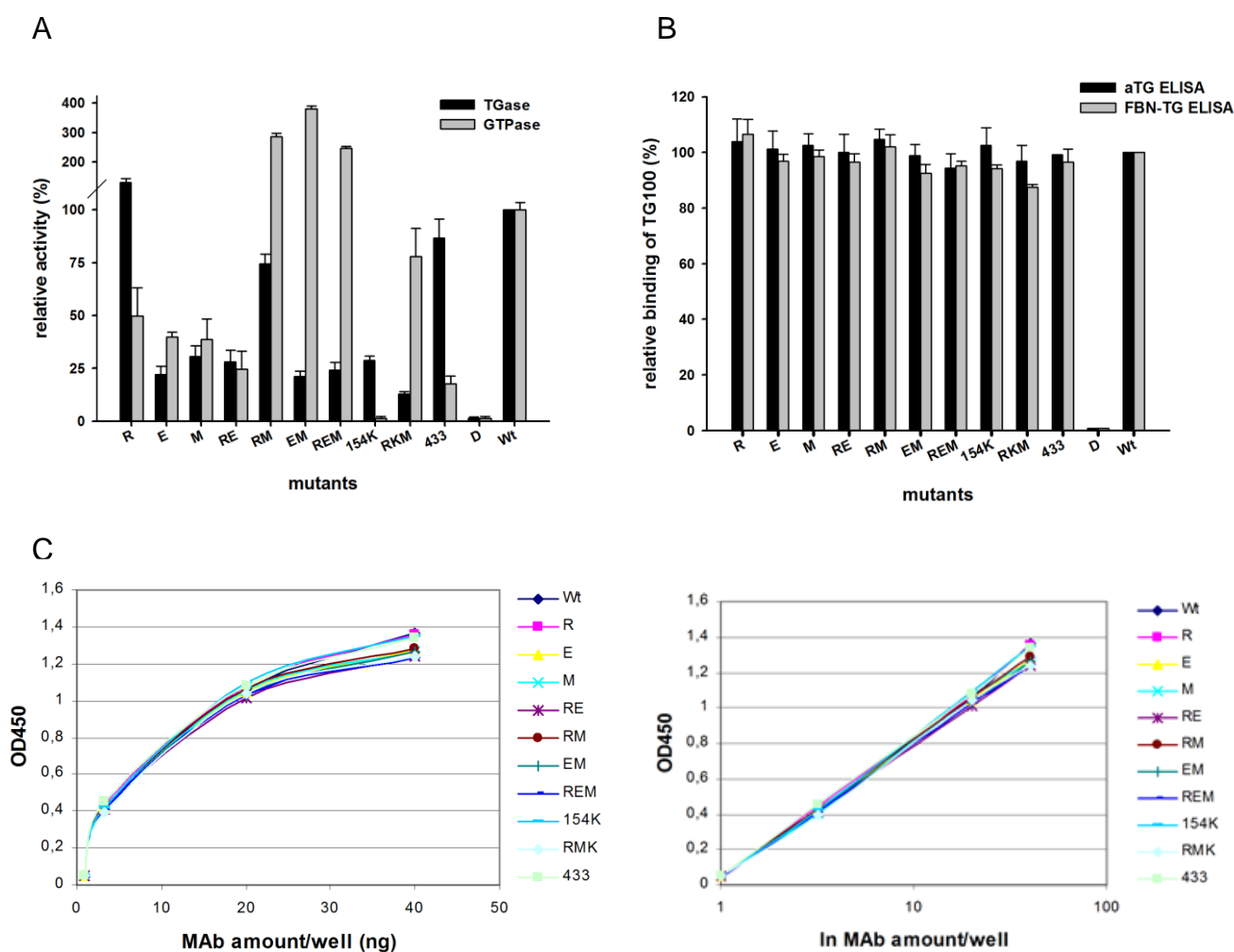
Binding properties of mutant TG2 proteins were tested with a large set of mouse monoclonal anti-TG2 antibodies (CUB7402 with epitope aa 447-478; 895 with epitope aa 649-687 (Table 3.); 4G3 with epitope aa 1-165 (Akimov SS and Belkin AM 2001); G92 with epitope aa 1-14 (Trejo-Skalli AV et al., 1995) and H23 with epitope aa 433-438). All mutants could bind effectively the monoclonal antibodies with different TG2-epitopes (Fig. 12.B), which suggests that the mutated enzymes harbor these epitopes and may have proper conformational properties. The folded structure of the key mutants was also shown by circular dichroism spectroscopy.

In order to determine whether transglutaminase activities were affected by the mutations crosslinking activities of the mutants were tested with microtiter plate assay. Mutant R showed  $\text{Ca}^{2+}$ -dependent TGase activity (Fig. 13.A) ~30% higher than the wild type (Wt) transglutaminase. All of the other mutants showed decreased TGase activity ranging from ~21% (EM mutant) to ~86% (433 mutant) if the activity of the Wt is 100%. Mutant D, as expected, did not show TGase activity.

GTPase activities of the mutants were measured by the charcoal method. The specific activity (cleaved pmol GTP/min/mg enzyme) of the Wt enzyme was set to 100%. The GTPase activities of mutant RM, EM and REM were more than two fold higher than the activity of the Wt (Fig.13.A).The single mutants and double mutants RE and 433 showed only 24-50%.



To confirm that the amino acid changes did not affect the FBN-binding capacity of the mutants 0.3  $\mu\text{g}$  FBN was coated on the microtiter plate incubated with TG and the bound TGs were recognized by MAb TG100. The mutants bound monoclonal antibody in the same extent as Wt (Fig. 13.B). In order to confirm that the mutants cover the plate in equivalent amounts binding of monoclonal anti-TG2 antibody to the mutant TG2s were tested with anti-TG2 ELISA. As expected, all of the mutants were recognized by TG100 and they bound to the plate in the same amount as Wt TG2. MAb TG100 was unable to recognize mutant D.



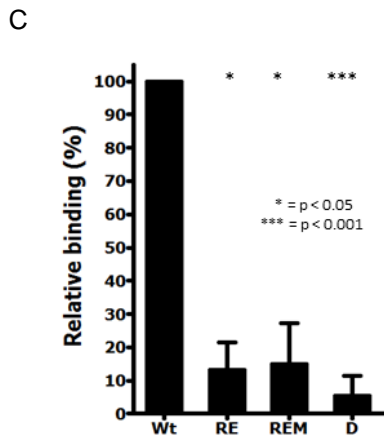
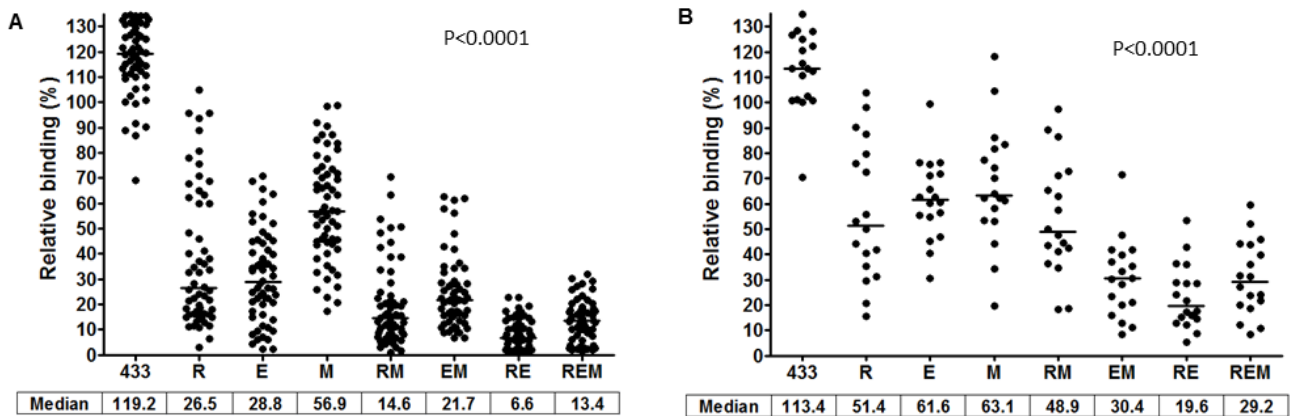
**Figure 13. Characterization of the mutant transglutaminase 2 (TG2) proteins II.**

Transglutaminase and GTPase activities of the mutants (A). Mutations that include E (Glu153) are affecting the S4  $\text{Ca}^{2+}$  binding site required for the transglutaminase catalytic activity. Binding of the mutants to fibronectin in ELISA (B). TG2 was recognized by MAb TG100 diluted 1:500 and this value was set to 100. Calibration curves representing the binding of increasing concentrations of MAb TG100 to the mutants by anti-TG2 ELISA (C). These curves were used for the calculation of relative amounts of bound antibodies during ELISA measurements.

Standard curves were also prepared from dilutions of MAb TG100 for each mutant and were used in later ELISA tests to determine the concentration of the TG2-bound celiac antibodies (Fig. 13.C).

The bindings of autoantibodies from consecutively diagnosed 58 childhood (Fig. 14.A) and 18 adult celiac patients (Fig. 14.B) were tested with the R, E and M mutants. Each single mutation resulted in significant decrease in celiac antibody binding (median 26.5%, 28.8% and 56.9% remaining binding for R, E and M, respectively; Fig. 14.A) and double and triple mutations caused proportionally greater changes. The EM mutant still showed 21.7% binding capacity, RM had 14.6%, while triple mutant REM showed roughly the same 13.4%. Surprisingly, the lowest binding capacity belongs to double mutant RE with median 6.6%, ranging from 1.1%-22.7% in the case of celiac children. Mutation at Ca<sup>2+</sup>-binding Site 5 (double mutant 433) did not have effect on the binding of celiac IgAs. Antibodies of celiac adults showed similar binding pattern, but slightly higher binding rates compared to children's antibodies.

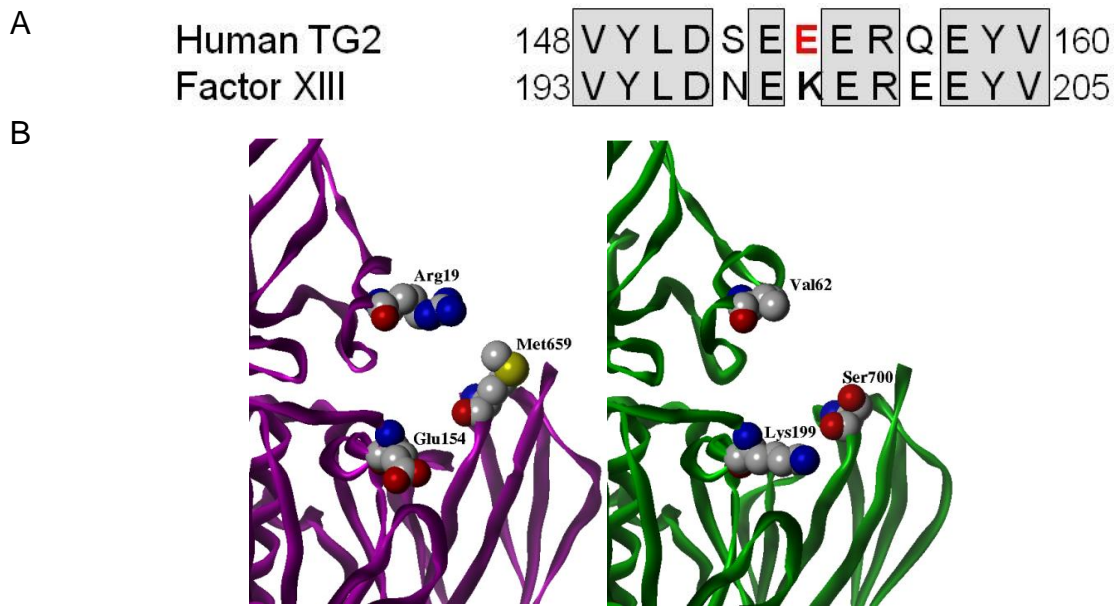
Although the investigated TG2-specific serum autoantibodies represent polyclonal immunoglobulin molecules it seems that the majority bind to the identified celiac epitope. To further investigate whether additional immunoglobulin clones with different binding site(s) exist, we obtained single chain variable fragments (ScFv) from our Italian collaborator partner (Daniele Sblattero, University of Trieste, Trieste, Italy). These ScFvs were selected against recombinant human TG2 from a total antibody library created from DNA isolated from lymphocytes of celiac intestinal biopsy samples. This ScFv library represents all of the antibodies that the patient produces during life and can be screened for any type of antigens (e.g. TG2) with phage display method. These ScFvs are monospecific, bearing only one binding site on the enzyme and represent an independent patient group compared to the Hungarian celiacs. Binding of 8 ScFvs were tested and all were abolished when mutants RE, REM and D were applied as antigens (mean 13.0%, 14.0% and 5.4% remaining bindings) (Fig. 14.C).



**Figure 14.** Binding of serum IgA from 58 celiac children (A), from 18 celiac adults (B) and single chain variable fragment monoclonal antibodies (n=8) isolated from celiac patients (C) to TG2 mutants in ELISA. Bound antibody concentrations were calculated from a calibrator curve (Fig. 13.C) Binding to wild-type (Wt) is 100%. All samples were examined in duplicates. Dash indicates median. P<0.0001 represents significant differences between groups by ANOVA test. Values represent means  $\pm$  SD \*, p<0.05; \*\*\*, p<0,001, compared to Wt.

#### 4.5 Investigation of mutants based on homology with factor XIII

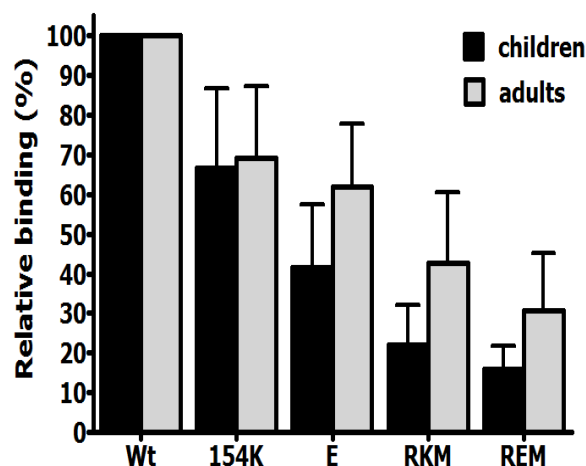
Further aim was to determine one single amino acid which plays a key role in the built up of the celiac epitope. Based on the homology with blood coagulation fXIII and the phenomenon that the occurrence of high titer IgA-antibodies against fXIII is infrequent in CD (Sjober K et al., 2002), we have compared the primary amino acid sequence (Fig. 15.A) and structure (Fig. 15.B) of the identified site in the core domain with fXIII to identify one amino acid difference, which could be responsible for the low antigenicity of fXIII. Amino acids Glu154 in TG2 and the corresponding Lys199 in fXIII have been identified, which show a notable difference in the polarity and in the length of the side chains of these amino acids. Amino acid Glu154 in TG2 was changed to lysine (mutant 154K) to create a homologous structure to fXIII, and this replacement was combined with amino acid changes in the other domains (combination of E154K with R19S and M659S changes: mutant RKM, Table 4.).



**Figure 15.** Structure and amino acids of the celiac epitope in TG2 (left) and the corresponding site in Factor XIII (right).

These mutants were also analyzed by Western blot (Fig.12.A) and their enzymatic activities were also measured (Fig.13.A). Both mutants had decreased TGase activity; the GTPase activity of mutant 154K was abolished, while mutant RKM showed 78% compared to the Wt. The mutants could bind to FBN in the same extent as Wt (Fig. 13.B).

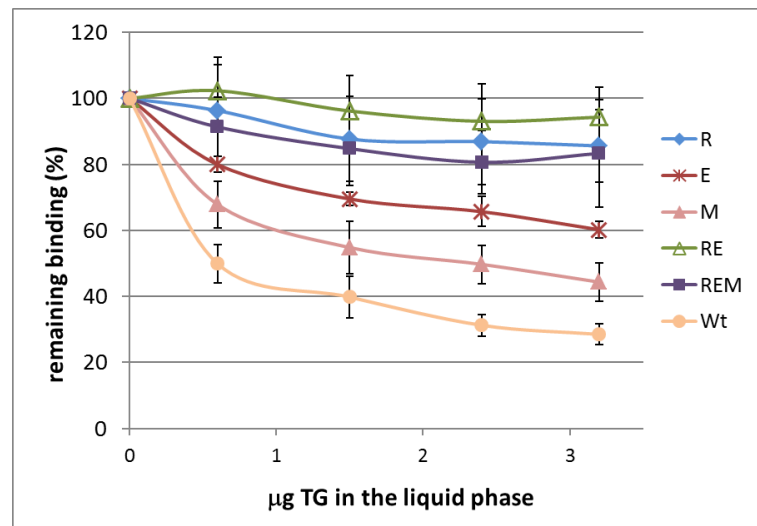
One amino acid change to lysine (154K) decreased the binding of celiac sera in anti-TG2 ELISA (mean 66.6% remaining binding, Fig.16.). Comparing these with single mutant E, the binding pattern is similar, since both contain the mutation in this site of the core domain. Triple mutant RKM showed similarly decreased binding as mutant REM. Again, adult CD samples had slightly higher binding compared to those from children.



**Figure 16.** Binding of celiac serum IgA antibodies from childhood (n=20) and adult (n=18) celiac patients to Factor XIII-homologue TG2 mutants. Values represent means  $\pm$  SD, respectively.

#### 4.6. Liquid phase competition measurements with TG2 mutants

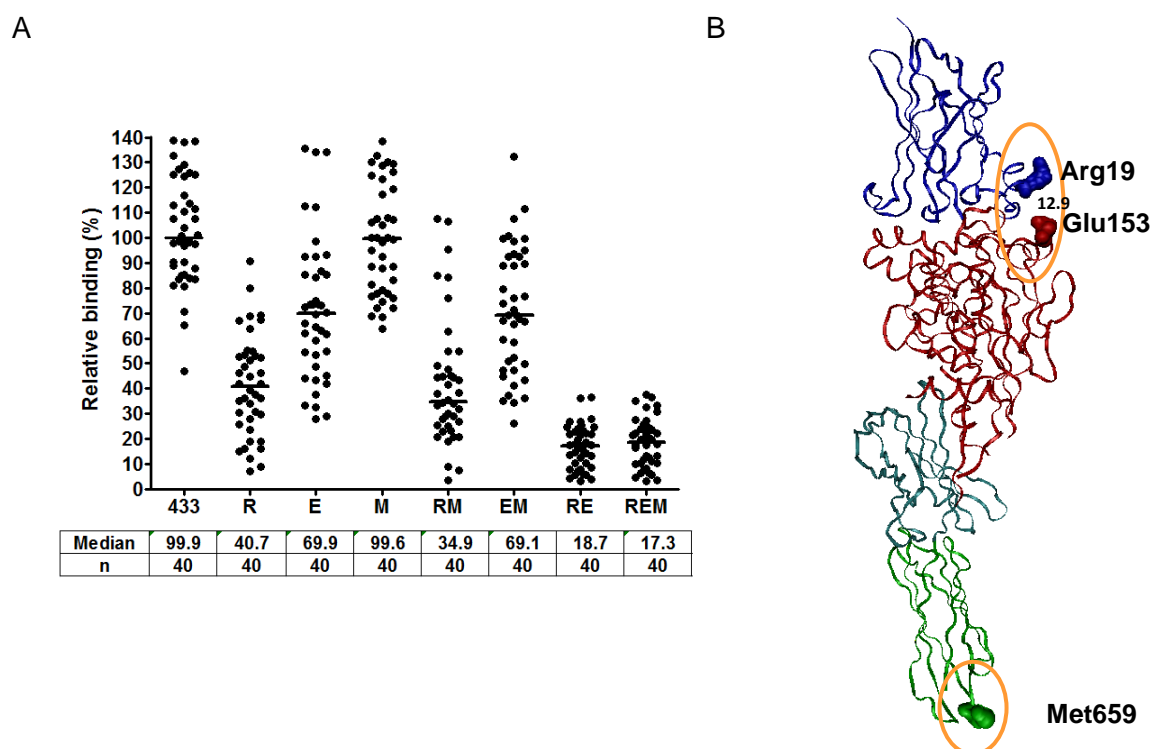
Liquid phase competition measurements were used to confirm that the bindings of the antibodies are independent from attaching the enzyme to the surface of the plate. Antibodies, which did not bind to the investigated mutants added in solution, can be detected in the assay by their attachment to the plate-coated Wt TG2. Mutant RE and REM could bind negligible amount of the antibodies even in the liquid phase and did not compete with the binding of celiac antibodies to the plate-coated Wt TG2 (Fig. 17.; remaining bindings to coated Wt TG2 are above 80%, if binding without TG2 in the liquid phase is 100%). In Fig. 14.A and B it can be observed, that the reaction of investigated serum IgAs showed some variability in the case of the single mutant R (with mutation R19S). Some of the samples seemed to have good binding properties to this mutant (with remaining binding above 60%), while the others (the majority) showed highly decreased binding to mutant R (with remaining binding below 50%). Serum samples used in the liquid phase measurements belonged to the second group, they are unable to bind to mutant R neither in solid nor in liquid phase. Mutant E could bind slightly more antibodies in solution than in solid phase (remaining binding to coated Wt TG2 is above 60%), while mutation on the IV. domain of TG2 (M659S) did not alter the binding of celiac antibodies in large extent. These data suggest that Arg19 and Glu153 have major role in antibody binding.



**Figure 17. Liquid phase competition ELISA.** Measurements were done with 3 celiac serum samples, representative values of two independent experiments.

#### 4.7. Binding of celiac antibodies to fibronectin-bound transglutaminase 2 as model of epitope exposure in the extracellular matrix

The reactivities of celiac antibodies to different TG2 mutants were also tested with fibronectin-TG2 ELISA. TG2 appears on the cell surface complexed with FBN and integrins that is why FBN-TG2 ELISA was also used to mimic these conditions. Beside this, FBN-TG2 ELISA measures only the FBN-bound TG2, so it is a further purification step of the enzyme on the plate (contaminations or degradation products are washed away during the washing steps of the ELISA) and with this coating the possible unfolding of the TG2 on the plastic can be excluded. The celiac antibodies showed similar binding pattern to the FBN-bound mutants as to the enzymes plated directly on the surface of the ELISA plate (Fig. 18.A). The binding to the double mutant 433 and single mutant M was slightly decreased or slightly elevated (47.1%-140.8%, median 99.9% and 99.6% respectively, if the binding to Wt is 100%). Decreased binding was observed with mutant E and EM (median 69.9% and 69.1%, respectively), while the reduction was more striking when the mutant contained amino acid change R19S. It was just 40.7% in the case of single mutant R and under 20% with double mutant RE and triple mutant REM.

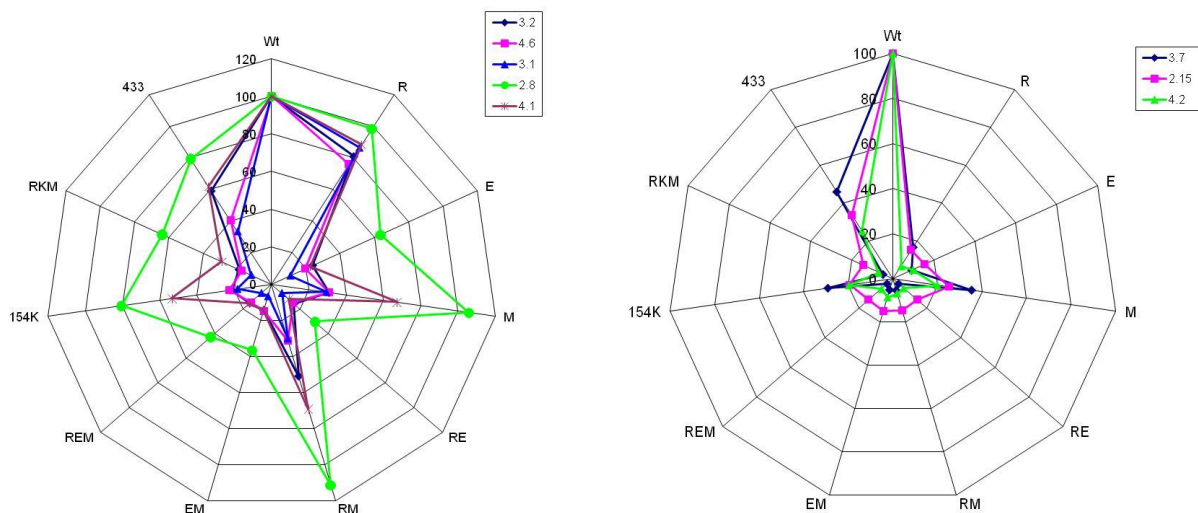


**Figure 18. Binding of celiac serum IgA to fibronectin-coated TG2 (A)** from pediatric (n=25) and adult (n=15) samples. All mutants bound to fibronectin similarly well as wild-type TG2 (Fig. 13.B). Structure of the opened form of TG2 (B; PDB code: 2Q3Z), the anchor points for celiac antibody binding are indicated on the model as surface representation (in the circles).

Based on the results of FBN-TG2 ELISA it seems that mutation of Met659 alone did not alter celiac antibody binding. This anchor point on the C-terminal (IV) domain is less important and that is why celiac antibodies may bind to TG2 also in its catalytically active form when TG2 adopts an open-extended conformation where domain IV with Met659 swings out (Pinkas DM et al., 2007). Measurements on the position of Arg19 and Glu153 in this open form crystal structure (2Q3Z) did not show a difference in distance as compared to the closed conformation (1KV3; Fig. 18.B).

#### 4.8. Antibodies of different celiac patients recognize parts of the same epitope

All of the investigated celiac patient serum samples displayed a severely reduced reaction with REM, RKM triple and RE double mutants, but – as earlier mentioned – their reaction showed some variability with the single mutant where only the N-terminal anchor point (Arg19) had been changed (Fig. 14.A). Celiac patient-derived monoclonal ScFvs expressed from phage libraries (Marzari R et al., 2001) belonged to two groups as well, one reacting with R19S and one not (Fig. 19.).

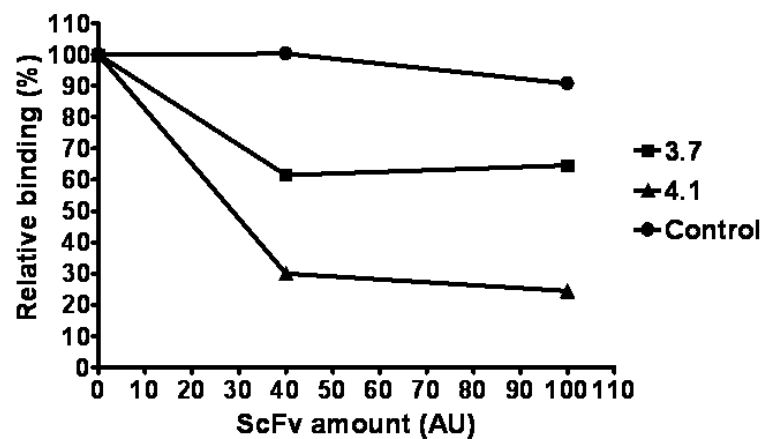


**Figure 19. Epitope mapping of soluble single chain variable fragments (ScFvs) by anti-TG2 ELISA divides them into two groups.** Group I (left, five clones) reacts with mutant TG2 R19S (mutant R), Group II (right, three clones) does not react with mutant R. ScFvs were used as bacterial supernatants in 1:15-1:2.5 dilutions. Relative amounts of bound antibodies were calculated by comparison to a calibrator curve constructed with mouse monoclonal antibody TG100; binding to wild-type (Wt) TG2 is 100%.

However, both these groups showed greatly reduced binding to Glu153 (E) or Glu154 (154K) mutants, which raised the question whether they recognize separate epitopes or simply bind differently to a common surface area. We addressed this question in competition studies

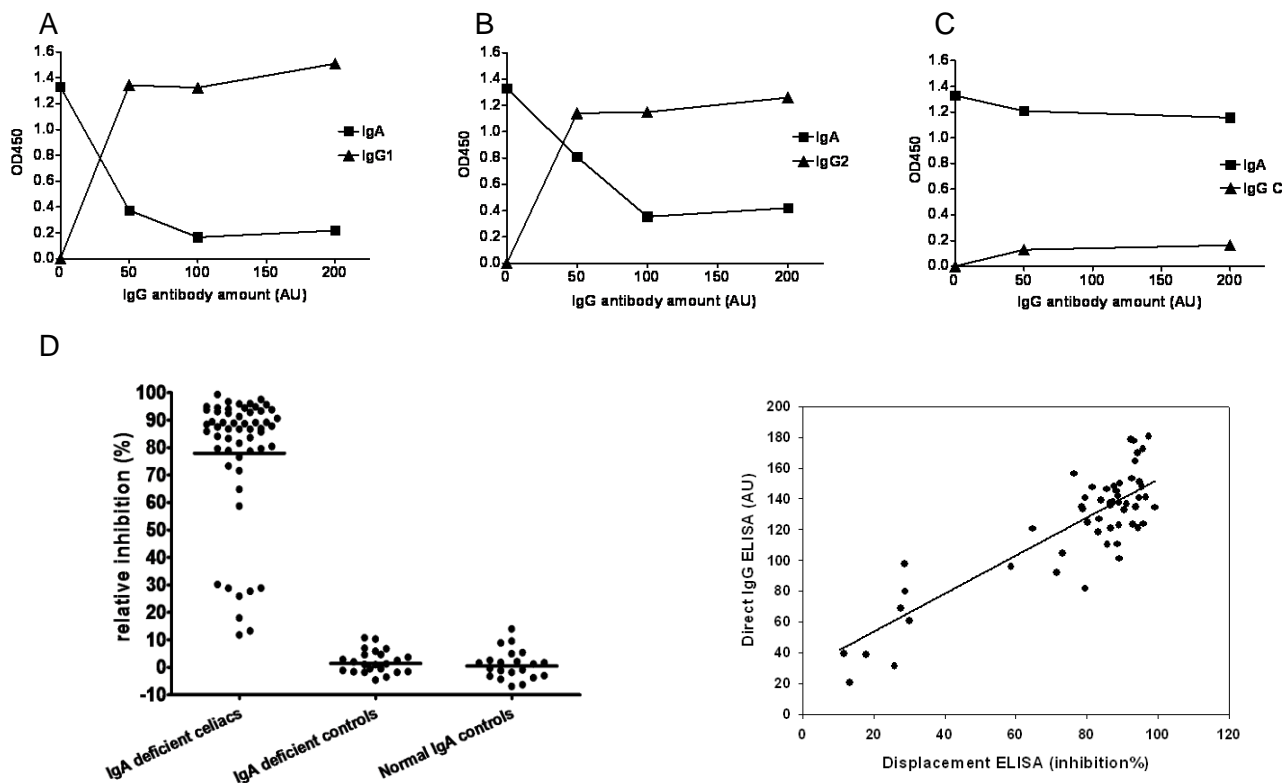
measuring the binding to wild-type TG2 of a phage-linked scFv (reacting with R19S) with a recognizable M13 phage tag and increasing amounts of soluble ScFvs representing the above groups were added. Soluble ScFvs reacting with R19S (clone 4.1) inhibited the binding of phage-linked scFv by 76%, but soluble ScFv not reacting with R19S (clone 3.7) also competed effectively with the binding of the phage antibody reacting with R19S causing 40% inhibition (Fig. 20.). Using phage-linked ScFv not reacting with R19S, 30% inhibition could be obtained with both groups of soluble ScFvs (data not shown).

For another approach, natural IgA and IgG antibodies purified from sera of celiac patients were used by adding increasing amounts of IgG and constant amounts of IgA to the plate covered with Wt TG2 and the bound IgA and IgG were measured in parallel wells. Celiac IgA and celiac IgG1 were reactive with R19S, while celiac IgG2 was not reactive with R19S; purified IgG fraction of a healthy person was used as control. The celiac IgA and IgG antibodies from different groups could compete with each other (Fig. 21.A and B), while the control IgG did not have any inhibitory capacity (Fig. 21.C).



**Figure 20. Competition measurement I.** Inhibition of binding of phage-conjugated Group I ScFv to WT TG2 in the presence of increasing amounts of soluble ScFvs of Group I (4.1), Group II (3.7) or irrelevant (control) ScFv. The degree of binding was calculated using a calibrator curve of diluted phage-conjugated ScFv; binding of phage-conjugated ScFv without soluble ScFvs is 100%. Representative values of two independent experiments.





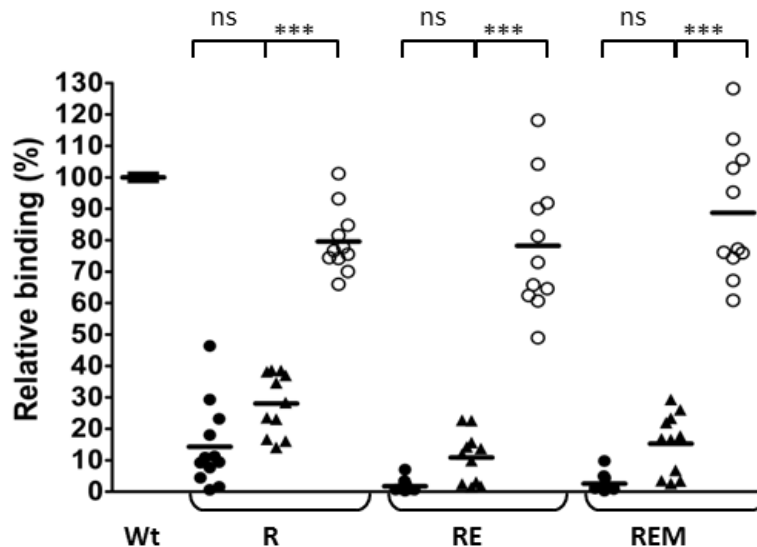
**Figure 21. Competition measurements II.** Celiac IgA was incubated with wild-type TG2 in presence of increasing amounts of purified IgG fractions from celiac patients and binding of IgA and IgGs were measured in parallel wells by ELISA. Celiac IgA and celiac IgG1 are reactive with R19S, celiac IgG2 is not reactive with R19S. (A, B) Purified IgG fraction from a healthy person was used as control (C). Data are representative of three independent experiments. D. Measurement of serum IgG class anti-transglutaminase 2 (TG2) antibodies in IgA deficient patients by displacement of an IgA celiac patient antibody. Correlation of the displacement effect with the serum concentration of IgG anti-TG2 antibodies measured by direct recognition of human IgG by monoclonal mouse anti-human IgG (Phadia) and anti-mouse secondary HRP-conjugated antibodies (DAKO);  $r=0.88$ .

Further, we were able to develop a diagnostic ELISA with 98,2% sensitivity and 95,6% specificity for measuring IgG class celiac antibodies in subjects with selective humoral IgA deficiency by their concentration-dependent displacement effect ( $r=0.88$ ) on a known celiac IgA tracer antibody (Fig. 21.D)

#### 4.9. Disease specificity of the composite epitope

We compared the binding pattern of celiac disease serum samples with that of serum samples from patients with anti-TG2 antibodies due to other autoimmune diseases (SLE, Sjögren's syndrome, rheumatoid arthritis). The non-celiac group showed a clearly different binding pattern to the celiac epitope (Fig. 22.); and interestingly, these subjects were also negative in antigliadin-antibody ELISA test and in endomysial assay (EMA) that detects anti-TG2 antibodies with tissue sections in a celiac-specific manner. In order to investigate, if the

targeting of the celiac epitope already exists in the early preclinical stage of CD, serum samples from patients without villous atrophy but with circulating anti-TG2 antibodies (latent cases) were obtained. These serum IgAs showed low reactivity to Glu153 and Arg19 mutants similarly to those with overt disease (Fig. 22.). These data collectively suggest that celiac disease results in a particular and directed immune response towards TG2 and this pattern of epitope recognition has diagnostic predictive value.

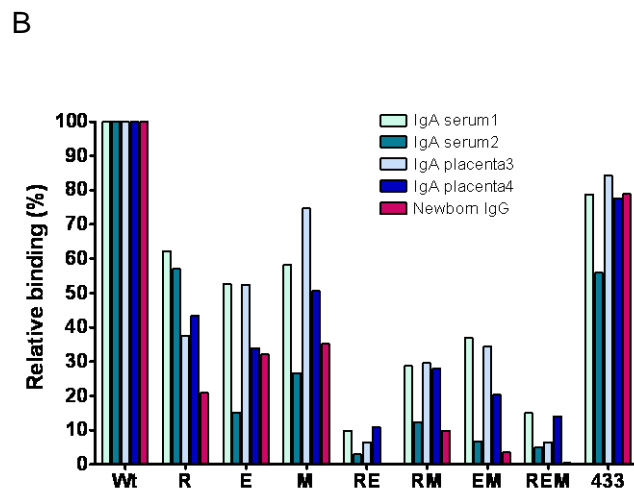
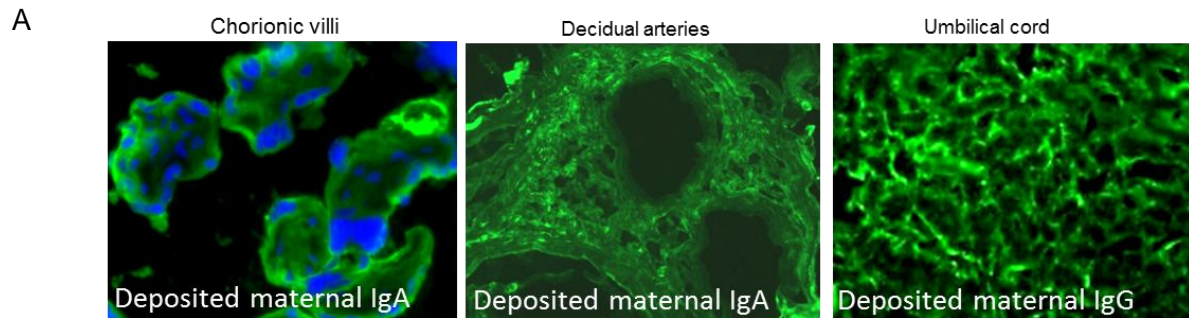


**Figure 22.** Binding pattern to mutant TG2 proteins of serum IgA antibodies from patients with early stage celiac disease (●) without villous atrophy, manifest celiac disease (▲) with small bowel villous atrophy (n=11) and from patients with other autoimmune diseases (n=11) (○), measured by anti-TG2 ELISA. The binding to wild-type (Wt) was set to 100%. Dash indicates median. \*\*\*, p<0.001, ns, non-significant.

#### 4.10. Similar TG2 epitope specificity of circulating antibodies and of those bound to patient tissues *in vivo*

High avidity antibodies might be trapped in the tissues explaining seronegative celiac cases in the minority of patients (Salmi TT et al., 2006), but these might be more important for inducing pathology. We eluted patient IgA antibodies from tissue sections and after purification we tested their binding epitopes with the panel of relevant mutant TG2 proteins. In order to avoid contamination by nonspecific IgA contained within plasma cells or epithelial cells, we chose instead of gut biopsy samples an extraintestinal organ without local production of IgA. We obtained placenta samples from two seropositive celiac mothers, and both contained high amounts of TG2-bound maternal antibodies in the decidual parts and on the surface of the chorionic villous structures (Fig. 23.A). This IgA was eluted with chloroacetic acid and used

in ELISA measurement. One additional celiac mother was IgA deficient with high serum levels of IgG class antibodies. IgG (but not IgA) class anti-TG2 antibodies deposited in the umbilical cord and appeared in all three infants' serum. These antibodies (newborn IgG) and IgAs eluted from placentas (IgA placenta) showed similar epitope specificity pattern as serum antibodies of the mothers (IgA serum) (Fig. 23.B).

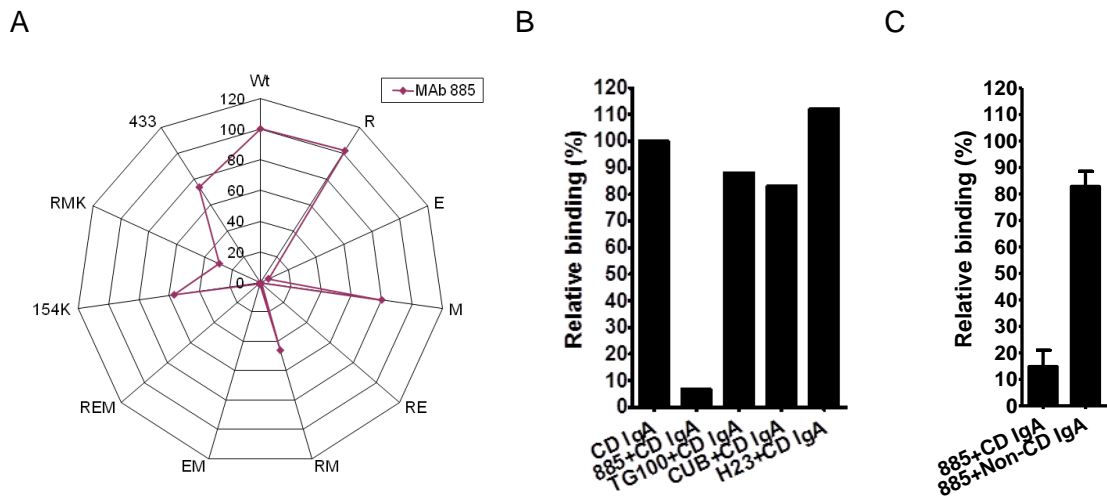


**Figure 23. In vivo-bound celiac antibodies in the placenta and newborn tissues** A. Frozen sections of placenta and umbilical cord stained with FITC-conjugated anti-IgA or IgG from a pregnancy with celiac mother with active disease. Maternal IgA bound *in vivo* to chorionic villi (left panel) and to the vessels of the placenta (middle panel) is shown in green, the nuclei of the fetal villous structures are shown in blue by DAPI. Celiac IgG is bound to the umbilical cord in the endomysial pattern (right panel). B Epitope specificity of serum IgA (1,2), of IgA eluted from celiac tissues (IgA placenta 3,4) and of passively transferred maternal IgG from newborn serum measured using mutant TG2 proteins in ELISA.

#### 4.11. Monoclonal antibody targeting Glu153 interferes with celiac antibody binding *in vitro*, displaces them from patient tissues and prevents their biologic effects

A mouse monoclonal anti-TG2 antibody clone (MAb 885, Phadia, Uppsala, Sweden, Table 3.) was found to target Glu153, thus a part of the celiac epitope (Fig. 24.A). When wild-type human TG2 was incubated with three different celiac patient serum in the presence of MAb 885, only 6.9-10.1% of natural polyclonal celiac IgA could bind whereas similar amounts of anti-TG2 MAb TG100, CUB7402 or H23 having an irrelevant epitope did not have significant effect (Fig. 24.B). In order to prove further that the found epitope is disease-specific, we compared the anti-TG2 autoantibodies of celiac and non-celiac autoimmune patients whether they can compete with MAb 885 (Fig. 24.C). Six celiac and six non-celiac serum samples were investigated in the presence of excess amounts of MAb 885. Celiac IgA could effectively compete with MAb 885 (remaining binding 14.8%, if the binding without

MAB 885 is 100%), while non-celiac IgA could bind to Wt TG2 also in the presence of MAB 885 (remaining binding 82.8%).



**Figure 24. Biochemical characterization of anti-TG2 monoclonal antibodies 885.** A. Epitope mapping by anti-TG2 ELISA. Relative amounts of bound MAB 885 were calculated by calibrator curve constructed from the concentration dependent binding of anti-TG2 MAB TG100; binding to wild-type (WT) is 100%. B. Comparison of the displacing effect of anti-TG2 MABs 885, CUB7402, TG100 or H23 (18  $\mu$ g/well) using one of the celiac serum samples. Representative values from three independently performed experiments. C. Competition effect of MAB 885 on the recognition of wild-type TG2 in ELISA by antibodies from celiac disease patients (n=6) and from non-celiac autoimmune patients with anti-TG2 antibodies (n=6). Remaining IgA binding in the presence of 18  $\mu$ g/well MAB 885 if the binding without MAB 885 was 100%. Values represent means  $\pm$  SD.

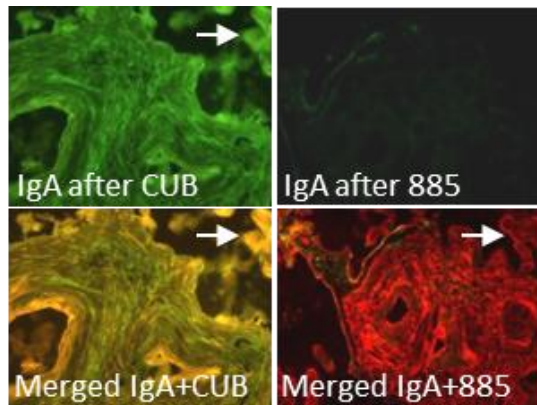
Further, competition studies were performed with MAB 885 and control MABs on the two frozen celiac patient placenta sections containing deposited IgA *in vivo*-bound to intrinsic TG2 of the patient. MAB 885 was able to bind to tissue sections and could be recognized along extracellular TG2 by secondary anti-mouse antibodies labeled with red fluorescent dye. In parallel tissue-bound IgA completely disappeared from the tissue. Incubation with buffer only or with isotype control mouse antibodies did not alter the binding of *in vivo* tissue-bound anti-TG2 IgA antibodies.

In order to evaluate whether MAB 885 caused the lack of IgA staining by simply interfering with the binding of secondary anti-IgA green fluorescent dye or by displacing celiac IgA and removing it from the binding to TG2, the solution of 885 antibodies used for the staining was carefully aspirated from the tissue sections after the incubation time and tested for the presence of IgA in ELISA after the removal of mouse antibodies by magnetic beads conjugated with protein G (Fig. 25.A). This solution contained TG2-specific IgA antibodies whereas control drops with buffer only or isotype control were negative after the same length

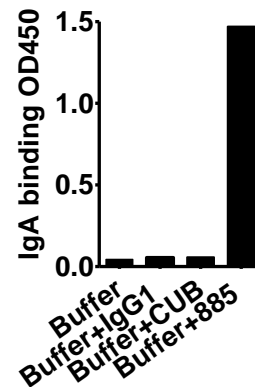
of incubation time with tissues, indicating that celiac IgA was not released spontaneously from the tissue, only in the presence of competing mouse MAb 885.

Celiac IgA remained unchanged and did not appear in the buffer if celiac tissues were incubated with CUB7402 anti-TG2 MAb with a different TG2 epitope-specificity (Fig. 25.B).

A



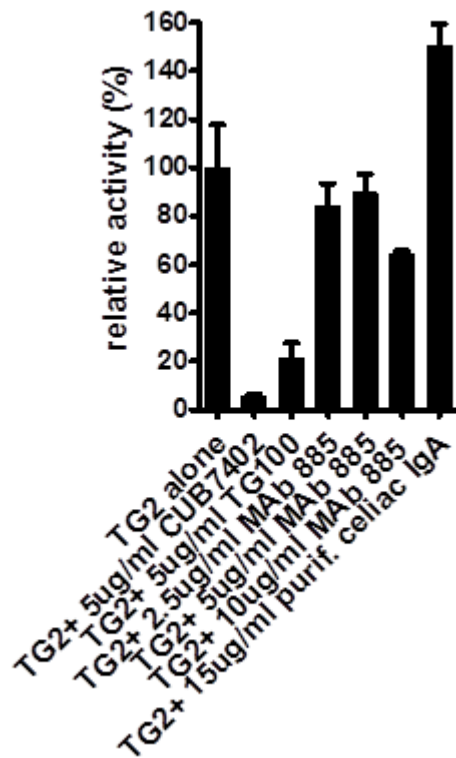
B



**Figure 25. Tissue binding of celiac antibodies mediated by the celiac TG2 epitope.** Celiac IgA (green) *in vivo* deposited in the placenta and on the surface of chorionic villi (arrows) merge to yellow after incubation of the tissue sections with CUB7402 anti-TG2 MAb and double-stained (red) by anti-mouse antibodies. After incubation with MAb 885 the IgA signal is no more visible and anti-TG2 IgA is detected in the buffer by ELISA (representative values of one from two independent experiments).

No IgA was released by incubation with isotype control MAb (IgG1) or CUB.

Given this competition MAb 885 might have a therapeutic potential in celiac patients by interfering with celiac IgA binding and its downstream pathogenic effects. For this, MAb 885 themselves should not have the same biological effects as patient antibodies, and we tested this by measuring the transamidating activity of TG2 with microtiter plate method. In line with our earlier findings, celiac antibodies caused an increment (150.4% if activity of Wt TG2 without antibodies is 100%), while MAb CUB7402 and TG100 blocked catalytic activity of the enzyme reaction (5.6% and 21.6%, respectively), MAb 885 did not have any modulating effect (89.6%) when the same antibody amount was used (5  $\mu$ g/ml) as used in the case of the other MAbs (Fig. 26).

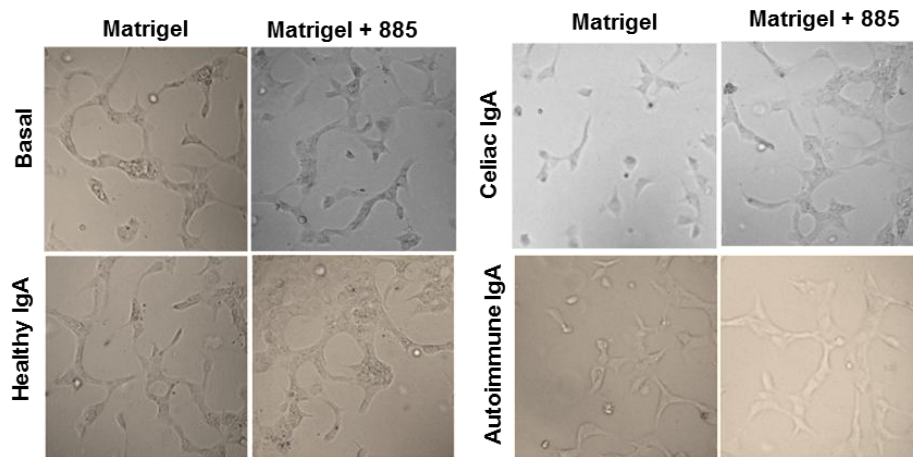


**Figure 26. Effect of MAb 885 on the catalytic activity of TG2 in comparison with other anti-TG2 MAbs by microtiter plate method.** Representative values from two independent experiments done in triplicates. Values represent means of triplicates  $\pm$  SD.

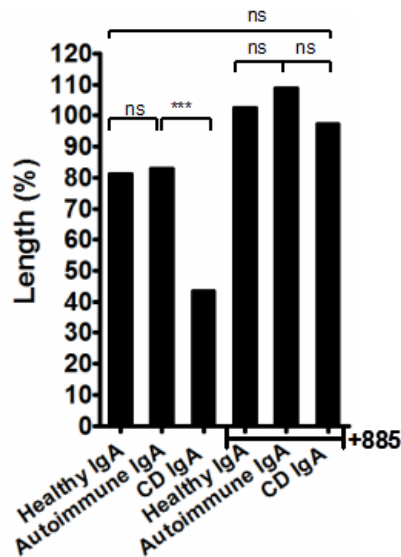
#### 4.12. Monoclonal 885 antibodies may prevent the effects of celiac antibodies

According to earlier studies disorganized mucosal vasculature can be observed in celiac patients and celiac antibodies can be found bound to TG2 below the epithelial basement membrane and around capillaries in the small intestinal mucosa (Korponay-Szabo IR et al., 2004). Moreover, defective angiogenesis was suggested to contribute to the architectural changes eventually leading to mucosal flattening (Myrsky E et al., 2009b), that is why we also investigated the differentiation of commercial HUVEC as a standard angiogenesis assay. These cellular experiments were performed by our collaborator Sergio Caja in Tampere, Finland.

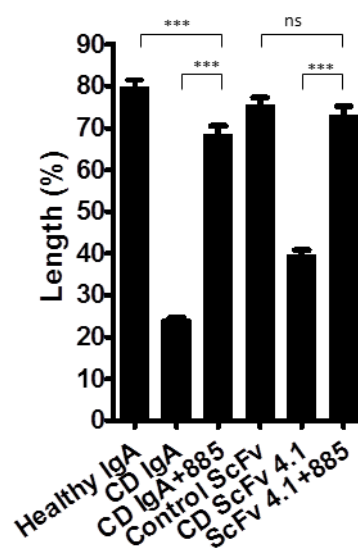
A



B



C



**Figure 27. Endothelial tubule formation of normal human umbilical cord endothelial cells in matrigel.** A. IgA from celiac (CD) patients (n=5), autoimmune patients with non-celiac TG2 antibodies (n=6) and biopsied antibody-negative controls (n=3) were administered alone or together with mAb 885 to normal HUVECs in matrigel and B. vessel formation was measured. Median lengths of endothelial tubules with 95% confidence intervals are shown compared to wells without antibodies set to 100%. ns, not significant; \*\*\*, p<0.001. C. Endothelial cell length on collagen I in the presence of celiac patient antibodies, celiac patient-derived single chain variable fragments (ScFv 4.1) and corresponding controls in the absence and presence of MAb 885.

Purified IgA fractions of celiac, non-celiac (from autoimmune patients) or healthy control serum samples were added to HUVEC cells cultured in matrigel in the presence or absence of 885 antibodies. Purified IgA from autoimmune patients with non-celiac TG2 antibodies and control IgA from antibody-negative healthy persons caused only slight non-specific decreases in endothelial tubule formation (median 83.1% and 81.3%, if tubule length without antibodies is 100%) but celiac IgA significantly decreased tubule length (median 43.7%, p<0.001

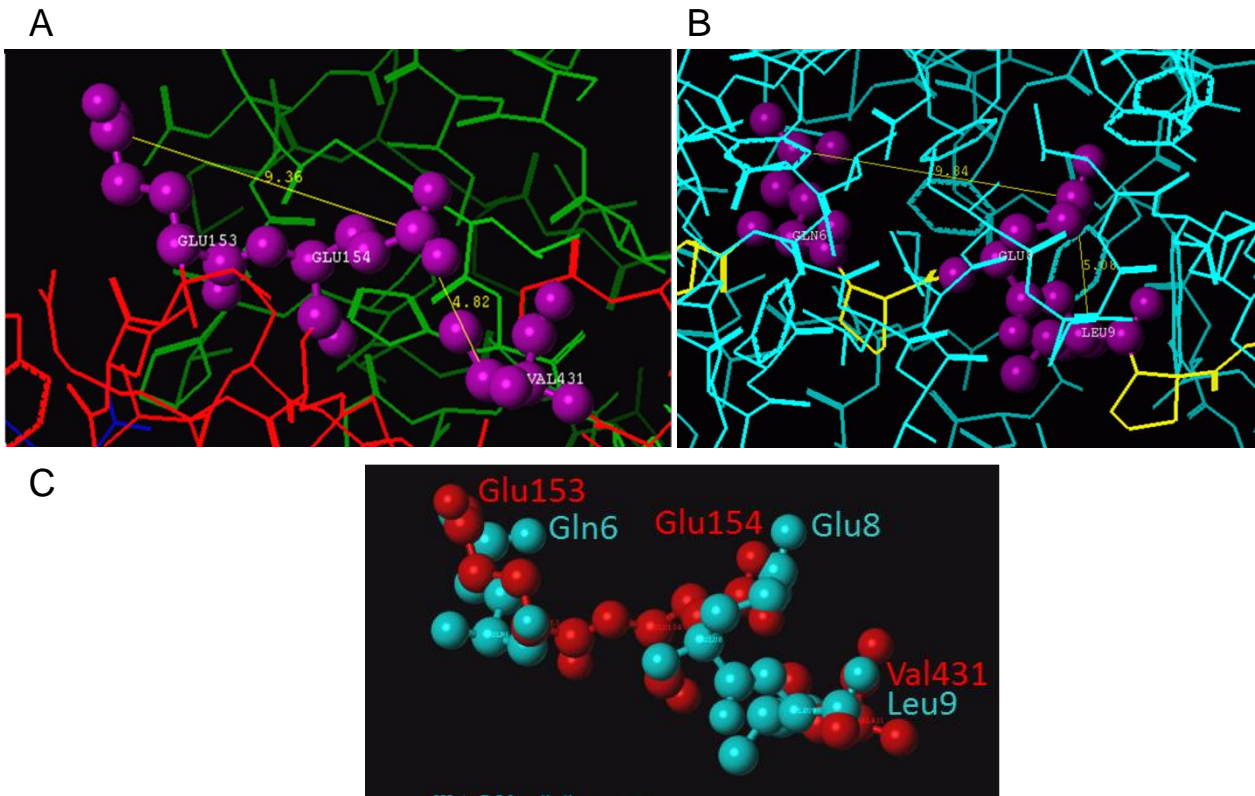
compared to autoimmune IgA) and formation (Fig. 27.B). This effect was prevented if MAb885 were also present (there is no statistical difference between tubule length in the presence of healthy control IgA and tubule length in the presence of celiac IgA co-administered with MAb 885). Similar effect was observed on cell lengths when MAb 885 antibodies were applied together with purified monospecific celiac ScFvs to HUVEC cells grown on collagen I. (Fig. 27.C). These results indicate that MAb 885 can antagonize also biological effects of celiac antibodies and show that these effects are mainly caused by TG2-specific antibodies in the total IgA fraction.

#### **4.13. Structural similarities of the TG2 epitope and HLA DQ-docked gliadin peptide**

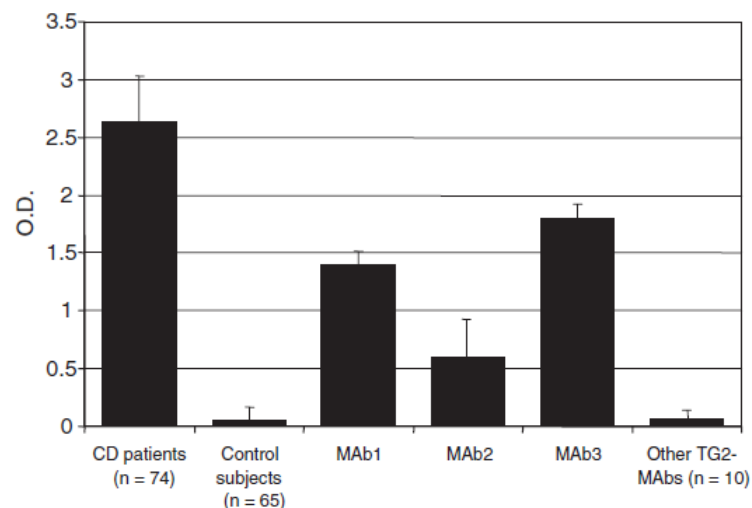
Since our studies show that the majority of celiac antibodies prefer one specific epitope, one should speculate how this knowledge helps us to understand the pathogenesis of the disease. Considering the features of CD it is well known that the trigger of the disease is gliadin, which provokes the patients' immune system to produce autoantibodies against a self-antigen TG2 at the end. So we investigated whether there are some similarities between the structures of these two molecules: gliadin and TG2.

Although short gliadin peptides in solution likely have unordered secondary structures, the crystal structure of an immunogenic gliadin peptide (LQPFQPELPY) is available showing it as docked into the antigen binding groove of HLA-DQ2 (pdb: 1S9V). According to the model, amino acids Gln6, Glu8 and Leu9 of the gliadin peptide seem to interact with the residues in the antigen binding groove of HLA-DQ2. The distance of side-chain carbon atoms of Gln 6 and Glu8 (9.84 Å) and their spatial arrangement is similar to the distance measurable between the side-chains of Glu153 and Glu154 forming the celiac epitope (Fig. 11.) in the TG2 structure (9.36 Å), and the distance of Glu8 to Leu9 in the gliadin peptide is similar to that between Glu154 and Val431 of TG2 with a good overlap of these side-chains when the two structures are superimposed (Fig. 28.). Surprisingly, Val431 is located in the investigated Ca<sup>2+</sup>-binding Site 5 of TG2, which site also takes part to some extent in the celiac antibody-binding or in the maintenance of the main epitope.





**Figure 28. Comparison of the crystal structures of transglutaminase 2 (TG2, pdb:1KV3) and deamidated gliadin peptide LQPFQPELPY docked in the antigen-binding groove of HLA-DQ2 (pdb: 1S9V).** A (left). The core (II) domain of TG2 is shown in red with the amino acids Glu153, Glu154 and Val431 highlighted as ball-and-stick structures in purple. The C-terminal (IV) domain is visible in green in the background. B (right). HLA-DQ2 is shown in cyan. The Gln6, Glu8 and Leu9 residues of the gliadin peptide are shown as purple ball-and-sticks and the flanking prolines as yellow rings. Distances of the side-chain carbon atoms are shown in Å. C (lower panel). Superimposition of the similar side-chains of the TG2 epitope amino acids (red) and the gliadin peptide (cyan).



**Figure 29. Reaction of celiac disease (CD) patient sera, non-celiac controls and monoclonal TG2-specific mouse antibodies (MAB) with deamidated gliadin peptides in ELISA.** Error bars for MAb1-3 show variations in three independent experiments.

#### **4.14. Investigation of the DGP antigen by non celiac monoclonal TG2-specific antibodies**

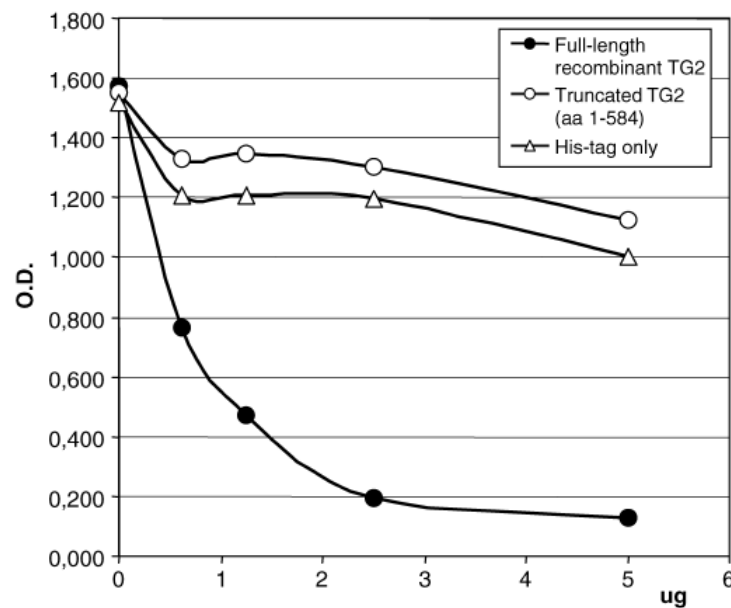
To further investigate the potential similarity between TG2 and gliadin epitopes (explained above), 74 EMA-positive untreated celiac disease patients were tested by the Celikey (Phadia, Freiburg, Germany; measures anti-TG2 antibodies) and the Celiac DGP Screen (Inova Diagnostics, San Diego, CA; using deamidated gliadin peptide (DGP) as antigens) tests. All of the samples were positive for both TG2 antibodies and DGP antibodies as well.

Next we explored whether some antigens in the DGP test could be similar to TG2 and would be recognized also by other non celiac TG2-specific antibodies. We obtained 13 different monoclonal mouse TG2-specific antibodies with different binding sites as characterized by earlier studies (Table 3. and unpublished data), spanning all 4 domains of TG2. We applied them in the DGP test and three of them could recognize DGP on the ELISA plate: clone 925, clone 4E1, and clone 895, which were referred to as MAb1, MAb2, and MAb3 hereafter (Fig. 29.). According to earlier studies (Di Niro R et al., 2005) and measurements with truncated recombinant TG2 constructs MAb1 has a linear binding site in the core domain, whereas the epitopes of MAb2 (aa 637–648) and MAb3 (aa 649–687) are in the C-terminal domain of TG2. The epitope for MAb3 is sensitive to chaotropic agents and probably is conformational. MAb1 and MAb3 give EMA-type staining patterns on tissues, while MAb2 is unable to bind to TG2 when it is exposed in the endomysium and only recognizes TG2 in ELISA. The applied DGP constructs were synthetic peptides, thus contamination by natural transglutaminase was unlikely. Mab 885 did not react with the commercial DGP plate. Additionally, commercial TG2-MAb CUB7402 (with binding site aa447-478 on TG2), and TG100 antibody (with binding site aa 447-538), were unable to recognize DGP peptides.

#### **4.15. Competition of transglutaminase and DGP for antibody binding**

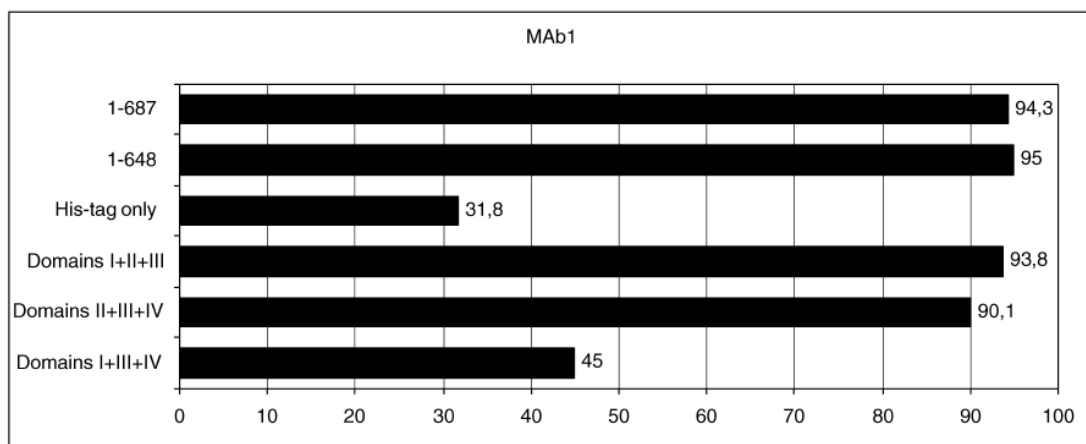
Next, we investigated whether the binding of the TG2-MAbs to DGP was specific and could be inhibited by TG2 or its fragments. In the presence of soluble full-length recombinant human TG2 the bindings of MAb1, MAb2, and MAb3 to DGP were completely inhibited and the inhibition was dose dependent (Fig. 30.). The empty His-construct and TG2 constructs without the binding domains for the MAbs did not inhibit the binding, which indicates that the binding was specific for the specific TG2-binding sequence of the MAbs. Although MAb2 did not recognize truncated TG2 containing aa 1-648 in an earlier study in ELISA, its binding to DGP was effectively inhibited by this construct, but not by truncated TG2 with aa 1-584, which contains only domains I+II+III (Fig. 31.). This result confirms that the binding sites of

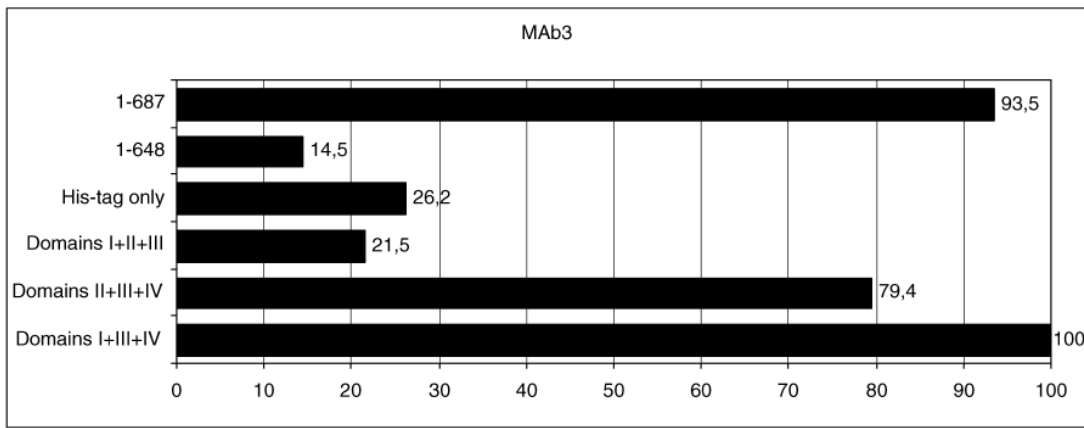
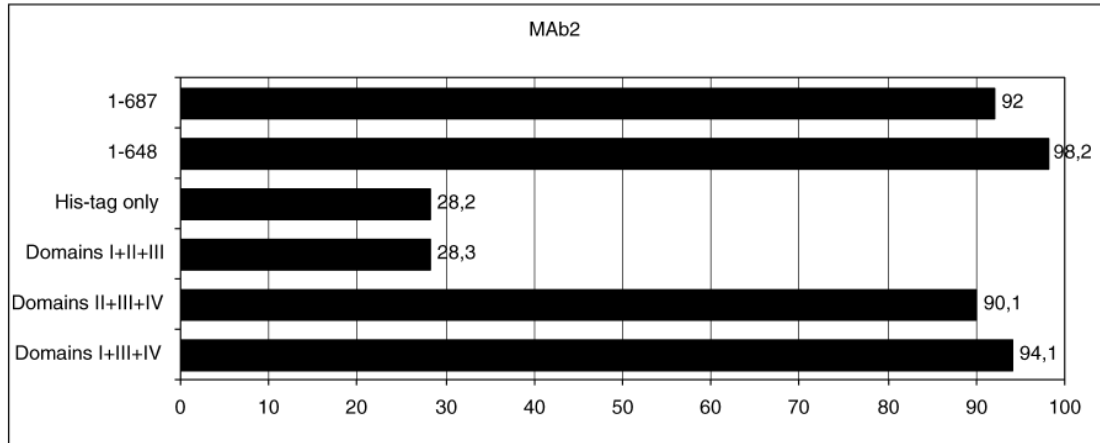
MAb2 and MAb3 are distinct. Together these results suggest the presence of multiple epitopes in DGP that can resemble to TG2.



**Figure 30. Competition measurement III.** His-tagged full-length recombinant human TG2 inhibits the binding of transglutaminase-specific monoclonal antibody MAb3 to deamidated gliadin peptides in a dose dependent manner (filled circles). His-tagged construct consisting of amino acids 1-584 of TG2 but not containing the binding epitope of MAb3 (open circles) and His-tag alone (open triangles) do not interfere with the binding. Representative values from three independent experiments.

Full length recombinant TG2 or any of the MABs could not inhibit the binding of celiac patient samples to DGP, which indicates that celiac IgA also contain additional antibody clones to DGP epitopes that are different from TG2.





**Figure 31. Competition measurements IV.** Inhibition (%) of the binding of transglutaminase 2 (TG2)-specific monoclonal mouse antibodies to deamidated gliadin peptides with full-length recombinant TG2 (1-687) and its fragments. TG2 domains are designated as I-IV. Domain deficient mutant I+II+IV inhibited the binding of all three MABs (not shown).

## 5. DISCUSSION

### 5.1. Characteristics of the main celiac epitope of TG2

The main result of this study is the identification of a previously unknown conformational and composite epitope on TG2 as the target of celiac disease antibodies. We proved that circulating serum antibodies and those derived from tissues or cloned from the small intestinal biopsies from celiac disease patients recognized parts of the same composite epitope and previous results suggesting individual variability in antigen recognition (mapping studies) can mostly be attributed by the different recognition of the same surface part of the autoantigen. This celiac TG2 epitope is composed of three adjacent domains of the enzyme and it is specific for celiac disease, since patients with other autoimmune diseases produce antibodies that prefer other binding site(s).

TG2 plays a key role in the pathogenesis of celiac disease as the main autoantigen, it can catalyze the deamidation of gliadin peptides and thus strengthen the immune response. Growing evidence confirm that the autoantibodies can have a pathogenetic role. Celiac IgA antibodies can enhance the transamidating activity of TG2 (Kiraly R et al., 2006), alter epithelial transports (Rauhavirta T et al., 2011), disturb angiogenesis (Myrsky E et al., 2008), induce morphologic changes and affect the cell cycle in fibroblasts *in vitro* (Barone MV et al., 2007). Identification of the main epitope allowed us to find a specific competitor antibody which offers a research tool to further dissect these pathophysiologic processes and also to explore whether interference with these processes at the level of antibody binding could be of therapeutic potential.

In this study three amino acids were identified by molecular modeling as the constituents of the main celiac epitope. The main anchor point of the epitope is Glu153, which is situated in the first  $\alpha$ -helix of the catalytic core (II.) domain and can cooperate with Arg19 in the first  $\alpha$ -helix of the  $\beta$ -sandwich (I.) domain. Arginin is a good candidate anchor residue in protein-protein interactions in general, and the binding site involving these two above mentioned helices results in the largest solvent-accessible surface area reduction upon binding as described by Rajamani et al. (Rajamani D et al., 2004). Also polar or negatively charged residues often serve as anchors, so cooperation of Glu153 with Arg19 could be predicted as a favorable binding site. The third residue which can take part in the formation of the epitope is

Met659 and it is located in the second  $\beta$ -barrel (IV.) domain. Based on this analysis, mutant TG2s were generated and the binding properties of celiac antibodies of different origins were investigated. Binding of both celiac IgA antibodies (Fig. 14.A and B) and celiac patients-derived monoclonal antibodies (Fig. 14.C) to double mutant RE was significantly reduced (remaining binding 1.1%-22.9% in anti-TG ELISA;  $p < 0.0001$ , ANOVA), while further amino acid changes (triple mutants) did not diminish the binding further, suggesting that changes in two of these amino acids are enough to abrogate the binding of the autoantibodies. This finding is supported also by liquid phase ELISA measurements (Fig. 17.) and when TG2 was applied in FBN-bound form (Fig. 18.A), where the mutation M659S did not alter the antibody binding significantly. In the activated, open conformation of the enzyme (Pinkas DM et al., 2007) the C-terminal residues (both  $\beta$ -barrels) are displaced by 120 Å, while the positions of amino acids Arg19 and Glu153 do not change during the process (Fig. 18.B). This suggests that the identified epitope is independent from the conformational shift occurring upon activation and can be supported by the observation that celiac antibodies could bind to the  $\text{Ca}^{2+}$ -bound TG2, to TG2 without  $\text{Ca}^{2+}$  (Fig. 9.) (ez itt nem kellene) Sulkanen S et al., 1998) and to the inhibitor-bound TG2 as well (Myrsky E et al., 2009a).

TG2 shows ubiquitous distribution in the extracellular matrix and on the cell surface complexed with FBN,  $\beta$ -integrins and - in focal adhesions - with heparan sulphate proteoglycans such as syndecan-4 (Verderio EA et al., 2009). The resting conformation of this extracellularly located form is hypothetically closed (Siegel M et al., 2008) and thus the three identified amino acids can build up a single epitope, which can be recognized by the coeliac antibodies as they form depositions in the jejunum and in extraintestinal sites (Korponay-Szabo IR et al., 2004). Mimicking *in vivo* conditions, binding rates of celiac samples from children and adults were measured with FBN-TG2 ELISA (Fig. 18.A). The binding to mutant M659S (94%) in these experiments was different from the values obtained with TG2 directly coated to the ELISA plate. This indicates that the FBN-binding may influence the exposure of the epitope and thus the binding of celiac autoantibodies, may favor antibody binding and thus offer more exact results than the conventional anti-TG2 ELISA.

According to our findings Glu153 and Arg19 effectively determine the epitope; however, only Glu153 seems to be crucial for antibody-binding. Some celiac serum IgAs and a part of the monoclonal scFvs could effectively bind to mutant R19S. This is in line with earlier findings,

where proper binding was observed if either the N- or the C-terminal part of TG2 were truncated, but the binding disappeared if both of these or the core domain were lacking. We also showed that the domain mutant lacking the core domain but containing both the N- and C-terminal domains was not a recognizable antigen for celiac antibodies and thus the anchor residue on the core domain is essential for binding.

In another study, where point mutations were applied instead of truncating the protein, the catalytic triad was shown to play a role in the celiac epitope (Byrne G et al., 2007). The catalytic triad is situated inside the enzyme, and it is exposed on the surface only when  $\text{Ca}^{2+}$  and substrates are available and the open conformation is adopted, however, the antibodies can recognize the open and closed form of the molecule equally well, and they also can bind to the enzyme when its active site is occupied by an inhibitor (Myrsky E et al., 2009a). These results cast doubt on the direct targeting of the catalytic triad and the fact that the autoantibodies have an enhancing effect on the transamidating activity of TG2 shows that they should bind to another site of the molecule than the catalytic triad. It is, however, possible that the mutagenesis of the catalytic triad influences the antigenicity by altering the protein's conformation. A similar situation was encountered during our study when celiac antibody binding was lost by the mutations of the not surface-exposed Glu158 (E158L and E158Q). This amino acid is situated at the basis of the first  $\alpha$ -helix of the core domain and these mutations altered the relative position of this helix to other surface parts, including Arg19 on the first  $\alpha$ -helix of the N-terminal domain.

To confirm that the conformations of our other mutants were not affected by the mutagenesis, activity measurements were performed. The TGase activity of mutant R was 30% higher than the Wt, which proves that the mutant is properly folded (Fig. 13.A). Such increased crosslinking activity could be found in the case of factor XIII for SNP V34L (de Lange M et al., 2006). The activities of the other mutants were variably decreased, some mutations (E and 154K mutations) colocalize with one of the putative  $\text{Ca}^{2+}$ -binding regions of the molecule and this may explain by itself the diminished activity. Three of the mutants (RM, EM, REM) could hydrolyze GTP in a higher extent than the Wt (Fig. 13.A). All of these mutants contain the M659S amino acid change, which residue is situated in the C-terminal domain of the molecule. According to earlier data (Lai TS et al., 1996) the removal of the last 52 amino acids from the C-terminal increases GTP hydrolysis activity. It is possible, that mutation at this site (M659) of the C-terminal domain, when it is combined with further mutations (R19S

or E153S) can enhance the GTPase activity of the enzyme. However, the mutation of this residue alone (mutants M) and combined with R19S-E154K (mutant RKM) resulted in a 58% and 73% reduction of the GTPase activity, respectively. Beside this, the FBN-binding capacities of all of the mutants are the same as the Wt enzyme's capacity (Fig. 13.B) suggesting the proper folding of the mutants and CD spectra of the key mutants do not show unordered segments.

To further prove that the core domain surface around Glu153 is directly involved in celiac antibody binding, we created mutant 154K with amino acid change E154K based on the homology between TG2 and factor XIII. This change led to similar effect as in the case of mutant E, while the earlier mutation E154Q did not alter the antibody binding. Taken together it is likely that the conformation of E154Q is intact and performing amino acid change in the same site to lysine does not influence the folding of the side chains, because with this change TG2 resembles to another naturally properly folded structure, fXIII. Combination of E154K with mutations in the I. and IV. domain (mutant RKM), however, resulted in similar decreased binding as mutant REM.

The identification of several possible anchor points (Arg19, Glu153, Glu154, Met659) of the celiac epitope raises the question whether these amino acids are able to form one joint epitope or these residues represent distinct epitopes. Interestingly, these amino acid changes were important for all natural serum antibodies representing polyclonal antibody populations and intact Arg19 and Met659 in the core domain deficient mutant were not sufficient to ensure binding in the absence of Glu153. Glu153 was also important for all monospecific scFv antibodies, but they showed variability in the binding properties to mutant R and could be divided into two groups: R-reactive and R-non-reactive antibodies. They could effectively compete with each other from the same, as well as from the other group (Fig. 20.), which indicates that they bind to the same common epitope. This was true also for the celiac IgA and IgG type antibodies (Fig. 21.). Moreover, in the beginning of our experiments two possible binding sites were suggested, which are connected to the Ca<sup>2+</sup>-binding sites of TG2. Mutations in Site 4 and Site 5 on the core domain affected the binding of celiac antibodies and there is a correlation between the effects of these two sites (Fig. 8.). As the surface mutations in Site 5 (mutant 433) did not alter the binding, it is more likely that the more extended Site 5 mutant had some influence rather indirectly on the position of the helix of Site 4.



In general, the binding pattern of pediatric and adult samples did not differ, although the remaining binding to the mutants in the latter group was slightly higher. Nonetheless, all adult samples showed a clearly reduced binding to RE and REM, indicating that their major epitope specificity is still the same. These samples did not have higher antibody content nor was any indication for an increased avidity. As patient sera contain polyclonal antibodies, during a long-standing disease for decades, also other specificities may arise through epitope spreading. Compared to other autoimmune diseases like type I diabetes, autoimmune hepatitis, bullous pemphigoid (Hashimoto T et al., 2011, Hintermann E et al., 2011, Sohnlein P et al., 2000), where epitope is prominent, our patients followed up into adulthood without treatment showed little changes in the binding patterns (Simon-Vecsei et al., 2011 supporting material).

## **5.2. Clinical significance and implementation**

Recombinant human TG2 is widely used in the diagnosis of CD in clinical ELISA kits with good sensitivity and specificity. However several studies on TG2-based tests described false positive anti-TG2 IgA results in the absence of IgA endomysium positivity and false negative IgA TG2 results with IgA endomysium antibody positivity (Wong RC et al., 2002). False positive TG2 antibody results are relatively common in the clinical settings (Green PH and Cellier C 2007) and this severely restricts the use of TG2 antibody positivity as the sole diagnostic test. Patients with other autoimmune diseases, tumors, cardiac failure, neurological disorders, psoriasis and liver diseases may exhibit low levels of antibodies reacting with TG2 (Damasiewicz-Bodzek A and Wielkoszynski T 2008, Peracchi M *et al.*, 2002, Rumbo C et al., 2002, Sardy M et al., 2007, Shamaly H et al., 2007, Villalta D et al., 2005). This can be explained with the fact that relatively high amounts of TG2 can be found in tissues intracellularly, which can be liberated by cell damage and can provoke antibody production independent from CD.

For our experiments, we obtained non-celiac serum samples from patients with other autoimmune diseases: systemic lupus erythematosus, rheumatoid arthritis, Sjögren's syndrome and systemic sclerosis based on their anti-TG2 antibody content. These samples, however, did not show EMA staining or positivity for DGP antibodies. In line with this, they showed markedly different binding pattern to TG2-mutants, and the alterations at Arg19, Glu153 and M659 did not influence their binding, nor could they be competed out by a TG2 epitope specific monoclonal (MAb 885). It is thus possible to design a new diagnostic kit

including wild type TG2 protein in which the celiac epitope is intact and a test TG2 in which the celiac epitope is impaired. As celiac autoantibodies cannot bind to the test molecule or bind only at a reduced level, this type of test can distinguish real CD antibodies from anti-TG2 antibodies in other diseases. Such a kit could be designed on several platforms like ELISA, radioimmunoassay (RIA), immunoprecipitation, or label-free binding assays, e.g. BIACORE for clinical use. Currently, small bowel biopsy is applied as a verification test for finalizing the diagnosis of CD after a positive TG2 antibody result. A more simple verification tool like the epitope-specific antibody test shown here could reduce the number of unnecessary invasive procedures and could enhance patient comfort. The more specific tests would be important also for the implementation of the new paediatric ESPGHAN diagnostic guidelines, which will allow the omission of small bowel biopsy in selected cases with high and technically reliable TG2 antibody positive serology result (Husby S, et al., 2011). Currently, it is an additional clinical problem that not all TG2 antibody kits work equally well and numeric results may differ. There is thus a need for standardization. MAb 885 can be utilized for example as a future interchangeable calibrator, useful for the measurement of the available antigenic TG2 determinants in different clinical test kits.

The finding that celiac antibodies recognize the same main TG2 epitope and compete with each other also has clinical implications. IgG class anti-TG2 antibodies are difficult to measure: both the immunofluorescent tests and ELISAs have high backgrounds, mainly because it is difficult to obtain specific and reliable secondary antibodies against human IgG. This is, however, clinically very important for the evaluation of IgA deficient patients. The prototype displacement ELISA presented here allows the detection of IgG TG2 antibodies with high sensitivity and specificity by the measurement of an IgA indicator antibody in IgA deficient patients. The detection of IgA is technically more easy and reliable and the test can be combined on the same plate with usual IgA class antibody measurement in an affordable manner.

Utilization of the epitope specific ELISA also may help the diagnosis in the preclinical stage of the disease (latent celiac cases), because these patients had antibodies with the same epitope specificity as manifest cases. This subgroup of the disease often called as 'potential' CD also, since patients may present villous atrophy and crypt hyperplasia after several years, whilst other organs can be severely damaged (gluten ataxia, cardiomyopathy, diabetes

mellitus, hypothyreosis). Thus, the early diagnosis of these patients is highly important to avoid these associated diseases.

### **5.3. New insights into celiac disease pathogenesis**

The existence of a single disease-specific autoantigenic epitope also may change our knowledge on celiac disease pathogenesis. Interestingly, even several decades of active disease did not shift the main epitope specificity in CD adults, although epitope spreading is normally a regular feature in many autoimmune disorders (Schlosser M et al., 2005). In this respect, peculiar properties of celiac antibodies have been already noted by other groups: all CD antibodies are characterized by the VH<sub>5</sub> usage (Marzari R et al., 2001) and the rate of somatic hypermutation is very low for celiac antibodies. We also experienced the stability of the epitope targeting pattern in long-term followed non-compliant patients. These results collectively suggest that gliadin peptides induce a specific reaction toward a particular surface of TG2. Our epitope modeling studies and further experiments with DGP and monoclonal TG2 antibodies show that some gliadin peptides and TG2 can have similar surface properties and suggest that molecular mimicry could take part in the presentation of some peptides. Similar for soluble antibodies, these structures may be mimotopes also for the B cell receptor mediating a more efficient internalization of some gliadin peptides or gliadin-TG2 complexes by TG2-specific B cells as non-professional APCs. These cells can then get a preferential help from gliadin-specific CD4<sup>+</sup> T cells resulting in production of TG2-specific antibodies that recognize this epitope. This possibility does not exclude the hapten-carrier mechanism, rather complements it and can be a triggering initial feature in the disease process.

However, our experimental results show that binding of serum celiac antibodies to DGP can not be inhibited by full length transglutaminase, which indicates that the patients' sera contain IgA antibodies against other epitopes on DGP that are not cross-reactive with TG2. Further, the 885 monoclonal antibodies recognizing part of the celiac TG2 epitope did not react with the gliadin peptides similarly as celiac antibodies. Taken together it seems that a simple mimicry between gliadin peptides and TG2 would not explain the entire disease process and the cooperation of gliadin-specific T and TG2-specific B cells needs further investigations.

#### 5.4. Therapeutic implications

In the complex immunopathology of celiac disease apparently orchestrated by gliadin-specific T lymphocytes (Sollid LM 2002), the role of anti-TG2 antibodies is still undefined and often debated. The pathogenic effect of some other autoantibodies has already been clarified, e.g. in rheumatoid arthritis (Kuhn KA *et al.*, 2006, Petkova SB *et al.*, 2006), pemphigus vulgaris (Ding X *et al.*, 1999), or myasthenia gravis (Lennon VA and Lambert EH 1981). Several lines of new evidence suggest that autoantibodies against TG2 also might have pathogenic role in celiac disease manifestations. Anti-TG2 antibodies are present virtually in all celiac patients and if not detectable in the circulation, these antibodies can be found *in vivo* deposited on extracellular TG2 (Salmi TT *et al.*, 2006) in various damaged tissues. Small bowel, liver, muscles, kidney (Korponay-Szabo IR *et al.*, 2004), brain (Hadjivassiliou M *et al.*, 2006b) are known examples and now, binding to the surface of chorionic villous structures of the infant and in the maternal parts of the placenta was proven (Fig. 23.A). Here we showed that antibodies also appear in the neonate's umbilical cord and circulation if the mother has active CD. The antibodies in the placenta may affect the activity of TG2 and cause impaired nutrient import through the syncytiotrophoblast microvillus membrane (Anjum N *et al.*, 2009), lower birth weight and clinically known altered pregnancy outcome in celiac disease.

Celiac sprue is considered as a life-long disease and unless it is treated increased morbidity and mortality prevails (Green PH and Cellier C 2007). In fact, compliance with the strict gluten free diet is difficult and additional effective treatment option may be of help to neutralize the consequences of dietary transgressions. The common target epitope of CD-specific autoantibodies raises the possibility that antibody interference could be used in future as an adjuvant therapeutic modality. Here we showed that tissue-deposited celiac autoantibodies, which could be associated with the multi-organ manifestation of the disease (Korponay-Szabo IR *et al.*, 2004) can be displaced by a monoclonal antibody recognizing part of the main celiac epitope characterized in this study. Furthermore, this displacing antibody did not have the typical pathologic effects elicited by celiac antibodies in cell culture experiments and could even antagonize the latter. Monoclonal antibody therapy interfering with a key cytokine, growth factor or cell receptors is today a common therapeutic strategy in chronic inflammatory, immune and malignant disorders, like rheumatoid arthritis, Crohn's disease and some tumors. Our results give initial tools to explore such a treatment possibility also in celiac disease.

## 6. SUMMARY

Celiac disease is a pathologic immune reaction to ingested gluten and it is characterized by gliadin-specific T lymphocytes and the production of disease-specific autoantibodies against the enzyme type-2 transglutaminase (TG2).

In our work we identified the main celiac epitope of TG2, which is conformational and located at the surface of the enzyme where amino acids from three domains of TG2 are close to each other. The epitope is related to one of the  $\text{Ca}^{2+}$ -binding site of the enzyme, but the  $\text{Ca}^{2+}$  itself is not involved in the composition of the epitope and in the binding of celiac serum antibodies. Core domain Glu153 and N-terminal domain Arg19 determine the celiac epitope which is accessible both in the closed and open conformation of the protein. Met659 on the C-terminal domain and Glu154 can cooperate in antibody binding. The epitope is relevant and accessible also under tissue conditions when TG2 is bound to fibronectin and was crucial also for celiac antibodies isolated from tissues or passively transferred to newborns from celiac mothers. Using patient-derived monoclonal single chain antibody fragments we proved in competition experiments that the identified amino acids form one common main epitope. Serum samples from patients with other autoimmune diseases (systemic lupus erythematosus, Sjögren's syndrome, rheumatoid arthritis) contained anti-TG2 antibodies for which this epitope was not important and showed a clearly different binding pattern to mutants with altered celiac epitope. This indicates that the epitope characterized in this study is celiac disease specific. Our findings also show that the main epitope of TG2 is an antigen already in the early (latent) stage of the disease. Monoclonal mouse antibodies with partially overlapping epitope specificity released celiac antibodies from patient tissues but did not alter the enzymatic activity of TG2 and did not cause similar harmful biological effects in cell culture experiments as celiac antibodies. Such antibodies might have a therapeutic potential.

We found that there is a possible structural homology between deamidated gliadin peptides (DGP) and TG2; moreover, non-celiac monoclonal TG2-specific antibodies can recognize DGP. However, according to competition studies celiac IgA also contain additional antibody clones to DGP epitopes that are different from TG2.

Identification of the binding site celiac antibodies allows the development of even more specific diagnostic tests and interference with celiac antibody binding might offer some additional therapeutic benefit. The three dimensional homology of DGP and TG2 suggests that molecular mimicry can be an important factor in celiac disease pathogenesis.

## MAGYAR NYELVŰ ÖSSZEFOGLALÓ

A coeliakia a vékonybél leggyakoribb krónikus autoimmun betegsége, amely a klinikailag fogékony egyéneknél változatos módon manifesztálódhat. A betegség folyamán autoantitestek termelődnek a transzglutamináz 2 (TG2) enzim ellen.

Munkánk során azonosítottuk a TG2 fő coeliákiás epitópját, amely konformációs, és az enzim három különböző doménjén, térben egymáshoz közel elhelyezkedő felszíni aminosavai alkotják. Az epitóp kapcsolatban áll az enzim  $\text{Ca}^{2+}$ -kötő helyeivel, de maga a  $\text{Ca}^{2+}$  ion nem része az epitópnak. A core doménon található Glu153 és az N-terminális doménon elhelyezkedő Arg19 határozzák meg kötőhelyet, amely az enzim “csukott” és “nyitott” konformációjában is elérhető az antitestek számára. Az C-terminális doménon lokalizálódó Met659 és Glu154 szintén részt vehet az antitest-kötésben. Az epitóp fontosnak bizonyult attól függetlenül, milyen környezetben jön létre az antigén-antitest kötődés: az enzim szilárd fázishoz kötött, liquid fázisban található vagy fibronectinhez kapcsolt. Az általunk vizsgált szérum eredetű poliklonális, vékonybél biopsziából klónozott monoklonális, szövetekhez kötött és anyából magzatba átjutó coeliákiás antitestek esetében is ez az epitóp volt meghatározó. A betegekből klónozott egyláncú variábilis antitest fragmentek kompetíciós vizsgálatával igazoltuk, hogy a fenti aminosavak egy közös epitópot alkotnak. Különböző más autoimmun betegségekben (szisztémás lupus erythematosus, Sjögren szindróma, rheumatoid arthritis) szenvedő nem coeliákiás betegek antitestei eltérő kötődési mintázatot mutattak a megváltoztatott kötőhelyű mutáns enzimekhez, mint a coeliákiás antitestek, amely arra enged következtetni, hogy az epitóp betegség-specifikus. Ezenkívül bizonyítottuk, hogy ugyanerre az epitóra kötődnek a betegség kezdetkor a látens fázisban lévő betegek antitestei is. Az epitóp egyik részéhez kötődő TG2-specifikus egér monoklonális antitestek képesek leszorítani a szövetekben megkötődött coeliákiás antitesteket in vitro körülmények között, ugyanakkor a TG2 transzamidáló aktivitását nem befolyásolják. Sejtkultúrákban végzett vizsgálatok alapján az is elmondható, hogy ezen monoklonális antitesteknek nincs a coeliákiás antitestekhez hasonló káros hatása, így ezen antitestek, mint specifikus kompetitorok terápiás alkalmazása is felmerül.

Vizsgálataink során lehetséges szerkezeti homológiát találtunk a TG2 enzim és a deamidált gliadin peptidek (DGP) között, valamint TG2-specifikus monoklonális antitestek képesek a DGP felismerésére is. Azonban kompetíciós mérések alapján megállapítható, hogy a coeliákiás antitestek a TG2-től eltérő DGP epitópokat is felismernek.

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Candidate: Zsófia Simon-Vecsei

Neptun ID: BERGU1

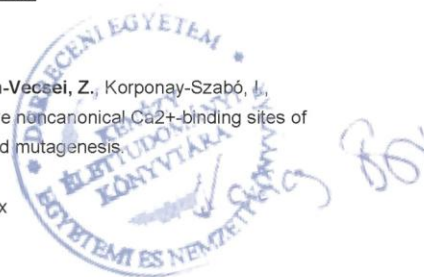
Doctoral School: Doctoral School of Molecular Cell and Immune Biology

### List of publications related to the dissertation

1. **Simon-Vecsei, Z.**, Király, R., Bagossi, P., Tóth, B., Dahlbom, I., Caja, S., Csósz, É., Lindfors, K., Sblattero, D., Nemes, É., Mäki, M., Fésüs, L., Korponay-Szabó, I.: A single conformational transglutaminase 2 epitope contributed by three domains is critical for celiac antibody binding and effects.  
*PNAS USA* - "accepted by publisher", 2011.  
IF:9.771 (2010)
2. Korponay-Szabó, I., **Vecsei, Z.**, Király, R., Dahlbom, I., Chirido, F., Nemes, É., Fésüs, L., Mäki, M.: Deamidated Gliadin Peptides Form Epitopes That Transglutaminase Antibodies Recognize.  
*J. Pediatr. Gastroenterol. Nutr.* 46 (3), 253-261, 2008.  
DOI: <http://dx.doi.org/10.1097/MPG.0b013e31815ee555>  
IF:2.132

### List of other publications

3. Király, R., Csósz, É., Kurtán, T., Antus, S., Szigeti, K., **Simon-Vecsei, Z.**, Korponay-Szabó, I., Keresztessy, Z., Fésüs, L.: Functional significance of five noncanonical Ca<sup>2+</sup>-binding sites of human transglutaminase 2 characterized by site-directed mutagenesis.  
*FEBS J.* 276 (23), 7083-7096, 2009.  
DOI: <http://dx.doi.org/10.1111/j.1742-4658.2009.07420.x>  
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4. Myrsky, E., Caja, S., **Simon-Vecsei, Z.**, Korponay-Szabó, I., Nadalutti, C., Collighan, R., Mongeot, A., Griffin, M., Mäki, M., Kaukinen, K., Lindfors, K.: Celiac disease IgA modulates vascular permeability in vitro through the activity of transglutaminase 2 and RhoA.  
*Cell. Mol. Life Sci.* 66 (20), 3375-3385, 2009.  
DOI: <http://dx.doi.org/10.1007/s00018-009-0116-1>  
IF:6.09
5. Király, R., **Simon-Vecsei, Z.**, Deményi, T., Korponay-Szabó, I., Fésüs, L.: Coeliac autoantibodies can enhance transamidating and inhibit GTPase activity of tissue transglutaminase: dependence on reaction environment and enzyme fitness.  
*J. Autoimmun.* 26 (4), 278-287, 2006.  
DOI: <http://dx.doi.org/10.1016/j.jaut.2006.03.002>  
IF:2.154

**Total IF: 23,189**

**Total IF (publications related to the dissertation): 11,903**

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

28 November, 2011



## **PATENT APPLICATIONS**

Korponay-Szabó IR, Fésüs L, Bagossi P, Csősz É, Király R, Simon-Vecsei Z, Mäki M.  
Diagnosis and treatment of gluten-induced autoimmune diseases. PCT WO 2010/116196 A2

Korponay-Szabó IR, Fésüs L, Bagossi P, Csősz É, Király R, Simon-Vecsei Z, Mäki M.  
Diagnosis of gluten-induced autoimmune diseases. PCT WO 2010/113025 A2

## **8. KEYWORDS**

Celiac disease, transglutaminase 2, autoantibody, epitope, antibody-binding, deamidated gliadin peptide, single chain variable fragment, monoclonal antibody

## **TÁRGYSZAVAK**

Coeliakia, transzglutamináz 2, autoantitest, epitope, antitest-kötődés, deamidált gliadin peptid, egyláncú variábilis fragment, monoklonális antitest

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