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KAUPO TEESALU

Autoantibodies against desmin and transglutaminase 2 in celiac disease: diagnostic and functional significance

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Department of General and Molecular Pathology, University of Tartu, Estonia

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Supervisors: Raivo Uibo, MD, PhD, Professor of Immunology, University of Tartu, Estonia

Meeme Utt, PhD, Senior Researcher at the Department of General and Molecular Pathology, University of Tartu, Estonia

Reviewers: Martti Laan, MD, PhD, Senior Researcher at the Department of General and Molecular Pathology, University of Tartu, Estonia

Kalle Kilk, PhD, Senior Researcher at the Department of Biochemistry, University of Tartu, Estonia

Opponent: Ilma R. Korponay-Szabó, MD, PhD, Associate Professor in Paediatrics, University of Debrecen, Hungary

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To my sons Koit and Kaur

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ABBREVIATIONS

AdGA	anti-deamidated gliadin antibodies
AGA	anti-gliadin antibodies
ANOVA	analysis of variance
AP	alkaline phosphatase
ARA	anti-reticulin antibodies
ATP	adenosine triphosphate
AU	arbitrary units
AUC	area under the curve
BSA	bovine serum albumin
CD	celiac disease
CV	coefficient of variation
DC	dendritic cell
DGR	aspartic acid-glycine-arginine
DH	dermatitis herpetiformis
DMEM	Dulbecco's modified Eagle's medium
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EmA	anti-endomysium antibodies
ERK	extracellular-signal-regulated kinase
ESPGHAN	European Society of Pediatric Gastroenterology, Hepatology, and Nutrition
FN	fibronectin
Fmoc	9-fluorenylmethyloxycarbonyl
FoxP3	forkhead box P3
GDP	guanosine triphosphate
GFD	gluten-free diet
gpTG2	guinea pig transglutaminase 2
GTP	guanosine triphosphate
HLA	human leukocyte antigen
HPLC	high-performance liquid chromatography
hrTG2	human recombinant transglutaminase 2
HS	heparan sulphate
HSPG	heparan sulphate proteoglycans
HUC	human umbilical cord
IEL	intraepithelial lymphocytes
IFN	interferon
Ig	immunoglobulin
IIF	indirect immunofluorescence
IL	interleukin
IPTG	isopropyl-β-D-thiogalactoside
K_d	dissociation equilibrium constant

MALDI-TOF	matrix assisted laser desorption/ionization-time of flight
MICA	major histocombatibility complex class I-related chain A
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK-cell	natural killer cell
NKG2D	natural killer group 2 member D
OD	optical density
PBS	phosphate buffered saline
PDB	protein data bank
RGD	arginine-glycin-aspartic acid
RhoA	Ras homolog gene family, member A
ROC	receiver operating characteristic
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SMA	smooth muscle antibodies
SPR	surface plasmon resonance
TCR	T-cell receptor
TG	transglutaminase
TGF-β	transforming growth factor β
TNF-α	tumor necrosis factor α
uSMA	umbilical cord smooth muscle antibodies
WB	western blot

1. INTRODUCTION

Celiac disease (CD) is a condition of immunologic intolerance to proteins in the common food cereals wheat, rye and barley. CD is primarily a bowel disease, as the pathogenic events take place in the small intestinal mucosa resulting in inflammation, mucosal atrophy, malabsorption, and finally, development of clinical symptoms. The causal relationship between wheat gluten proteins and the development of CD was established in the 1940s; since then there has been huge progress in understanding the mechanisms of the disease pathogenesis. Meanwhile, the disease has changed too – from a relatively rare disease of early childhood with classical symptoms of diarrhoea, failure to thrive, and abdominal distension, to a widespread condition affecting up to 1% of the western population of all ages, and displaying a wide range of clinical symptoms.

The development of CD can be regarded as the loss of immunologic tolerance towards gluten proteins in wheat and similar proteins in rye and barley. The innate and adaptive immune response against gluten is accompanied by autoimmunity against a self protein, transglutaminase 2 (TG2), leading to inflammation and tissue lesion in the intestinal mucosa of CD patients. Detection of anti-TG2 IgA antibodies from blood sera has become a valuable tool for the serologic screening of CD, displaying high diagnostic accuracy. However, a few false positive results can still be found when screening for CD using anti-TG2 antibodies, prompting the need for further optimization of anti-TG2 antibody assays. Intestinal biopsy is still required to diagnose CD, but the role of accurate antibody assays is highly important for the efficient selection of patients for biopsy. In addition, finding new autoantibodies as markers to screen, monitor, and prognose disease progression more efficiently remains a challenging task for researchers.

Apart from being a good disease marker, anti-TG2 antibodies could have a role in CD pathogenesis. TG2 is expressed in the small intestine, and in patients with CD, IgA antibodies co-localise with extracellular TG2 in the intestinal mucosa. The transamidase activity of TG2 can modify proteins post-translationally, which in patients with CD could lead to the modification of gluten peptides and enhanced T- and B-cell responses towards gluten and TG2 itself. TG2 is also involved in many biological processes like cell proliferation, apoptosis, adhesion, endocytosis, and the formation of the extracellular matrix (ECM). Therefore, the question arises as to whether autoantibodies can affect any of the biological or pathological activities of TG2. Previous studies show that anti-TG2 antibodies from CD patients interfere with the transamidation activity of TG2, as well as with various cellular functions of TG2, while the exact mechanisms of the antibody action have remained elusive.

The aims of this thesis were to identify new autoantibody markers for CD and to develop advanced antibody assays for the detection of anti-TG2 antibodies for CD screening. The thesis also investigated whether anti-TG2 antibodies could affect the cell adhesion function of TG2 through inhibiting TG2 binding to heparan sulphate/heparin molecules. Finally, a preliminary identification of the heparin-binding regions of TG2 was undertaken, using synthetic candidate peptides.

2. BACKGROUND TO THE STUDY

2.1. Celiac disease

Celiac disease, also known as celiac sprue or gluten-sensitive enteropathy, is a chronic inflammatory intestinal disease, which is induced by the ingestion of proline- and glutamine-rich proteins of wheat (gluten), rye and barley in genetically susceptible individuals (Lundin *et al.* 2005). Samuel Gee first described the classical features of CD in 1888 (Gee 1888), but it was not until the 1940s that the Dutch paediatrician, Willem-Karel Dicke, established a causal relationship between cereal consumption and intestinal symptoms, and suggested a gluten-free diet (GFD) as the primary cure for CD (van Berge-Henegouwen and Mulder 1993). CD was considered to be a condition mainly affecting young children when they encountered cereals in their diet. The classical disease form is characterized by malabsorbtion syndrome with diarrhoea, failure to thrive, and abdominal distension as the main intestinal symptoms (Lundin *et al.* 2005). Nowadays the clinical spectrum of CD is more varied, encompassing an increasing number of mild disease forms, extra-intestinal manifestations and even subclinical cases (Green and Jabri 2003; Di Sabatino and Corazza 2009). Also the disease can be found and diagnosed in all age groups (Volta and Villanacci 2011).

Villous atrophy and crypt hyperplasia of the intestinal mucosa in the upper-middle part of the jejunum are the histological hallmarks of CD. Marsh (1992), graded the extent of tissue alterations from intraepithelial lymphocytosis to partial, subtotal, and total villous atrophy; minor intestinal changes, like intraepithelial lymphocytosis, are less specific for CD (Volta and Villanacci 2011). The small intestinal biopsy with a characteristic CD histology has remained a gold standard for CD diagnosis, along with clinical or histological improvement after a period on a gluten-free diet (Walker-Smith *et al.* 1990). The strong association of CD with certain class II human leukocyte antigen (HLA) haplotypes and the presence of serum antibodies against TG2 as reliable disease markers, has led to growing support for undertaking CD diagnosis without performing a biopsy in a subgroup of patients with high autoantibody levels (Volta and Villanacci 2011; Husby *et al.* 2012).

CD occurs mainly in countries where the disease inducing cereals are consumed – that is all over the world except for parts of Asia and Africa. The highest prevalence is in Europe where CD affects up to 1% of the population (Dubé *et al.* 2005; Mustalahti *et al.* 2010). The prevalence of CD does vary significantly between countries, as from 0.3% in Germany to over 1% prevalence in Sweden and Finland (Mäki *et al.* 2003; Mustalahti *et al.* 2010; Walker *et al.* 2010). The incidence and prevalence of CD has been increasing for some decades; the wide use of serologic assays for CD screening and an increased awareness among physicians have been suggested as the main reasons for this (Green and Jabri 2003; Di Sabatino and Corazza 2009). On the other hand, the

incidence of many allergic and autoimmune diseases has increased in developed countries as a consequence of changing environments and lifestyles (Borchers *et al.* 2010). For example, the incidence of CD among children increased tenfold in Estonia, to 1 per 2,700 live births in a year, after clinical screening in 1990–1994, whereas no CD cases were found by screening for antibodies in general population (Uibo *et al.* 1996). Ten years later, after a profound socioeconomic change in the country, the serological screening for CD among schoolchildren revealed a disease prevalence of 0.34% in Estonia (Ress *et al.* 2007).

CD is associated with several, mainly autoimmune diseases, which also comprise potential risk groups for CD screening. The prevalence of CD is higher than in the general population among patients with IgA deficiency (Collin *et al.* 1992), type 1 diabetes (Holmes 2001), autoimmune thyreoiditis (Sategna-Guidetti *et al.* 1998), autoimmune hepatitis (Volta *et al.* 1998), Down's syndrome (George *et al.* 1996), Turner syndrome (Ivarsson *et al.* 1999) and in some other conditions (Volta and Villanacci 2011).

Dermatitis herpetiformis (DH), a blistering skin disease with IgA deposits in the dermis, is considered to be a skin manifestation of gluten enteropathy, as almost all patients have intestinal lesions and antibody markers characteristic of CD (Sárdy *et al.* 2002). Refractory sprue or refractory CD is a rare CD-related condition with a poor prognosis, not responsive to the GFD, and leading frequently to development of T-cell lymphoma (Cellier *et al.* 2000). The risk of lymphoproliferative malignancies is also higher for untreated CD (Di Sabatino and Corazza 2009). Furthermore, the overall mortality rate for symptomatic CD is increased, but varies in different studies, and is found to be associated with the level of gluten consumption in the population (Biagi and Corazza 2010). A gluten-free diet remains the only approved and effective therapy for CD, but many initiatives are being undertaken to develop novel therapies for CD, directed at modifying the immunogenic potential of gluten or the immune response to it (Schuppan *et al.* 2009).

2.2. Immunopathogenesis of CD

2.2.1. Environmental triggers

The early observations in 1940s, that wheat consumption is harmful to celiac patients, have initiated the sudies in the following decades to explore the disease inducing role of wheat gluten and homologous proteins in rye and barley (Heyman *et al.* 2011). Although the mechanisms of CD pathogenesis have been described at molecular and cellular level, the primary events leading to disease development in the genetically predisposed subjects still remain unknown.

Gluten consists of water-insoluble storage proteins of wheat grains, which can be separated to alcohol-soluble and non-soluble gliadin and glutenin fractions, respectively (Wieser 1995). Both fractions contain many closely related proteins, altogether coded by at least 100 genes, and characterized by a

high content of glutamine (approximately 35 mol%), proline (15 mol%), and hydrophobic amino acids (19 mol%). Prolamin is a common term for alcohol-soluble proteins in cereals, including proteins similar to gliadin in rye (secalin), barley (hordein), and oats (avenin). Based on sequence similarity and their biochemical properties, gliadin proteins are divided into α-, γ- and ω-gliadins (Wieser 1995).

The gliadin fraction, especially α-gliadin, is regarded as the most potent gluten component to induce CD, retaining its toxicity after digestion with pepsin and trypsin (Heyman *et al.* 2011). Although initially hypothesized, no inherited inability to degrade gliadin proteins by digestive enzymes in gut lumen has been found in CD (Matysiak-Budnik *et al.* 2003). However, the relative resistance of gliadin proteins to hydrolysis by intestinal proteases and peptidases, compared with other food proteins, has been established as a factor for increased immunogenicity (Hausch *et al.* 2002). Based on numerous *in vivo* and *ex vivo* studies, and also using intestinal biopsies from CD patients in culture, gliadin toxicity for CD has been mostly confined to α-gliadin amino acid sequences 31–55 and 57–89 (de Ritis *et al.* 1988; Wieser 1995; Shan *et al.* 2002). The immune response against gliadin is considered to be the crucial event in the immunopathogenesis of CD (Jabri and Sollid 2006). Also the direct effects of gliadin, especially α-gliadin peptide 31–55, on intestinal epithelial cells have been described in cellular model systems. Gliadin peptides increase cellular oxidative stress (Rivabene *et al.* 1999; Luciani *et al.* 2010) and the rate of apoptosis (Giovannini *et al.* 2000), induce actin cytoskeleton rearrangements (Clemente *et al.* 2003), and exhibit growth factor-like activity on intestinal epithelial cells (Barone *et al.* 2007b). The binding of the gliadin peptides to the chemokine receptor, CXCR3, with the subsequent release of zonulin, a regulatory protein of enterocyte tight junctions, and an increase in intestinal permeability, has been reported (Lammers *et al.* 2008).

The transport of gliadin peptides into the lamina propria of small intestinal mucosa occurs both by paracellular and transcellular pathways in CD (Heyman *et al.* 2011). Paracellular permeability is controlled by tight junctions between intestinal epithelial cells which are permeable to ions and small molecules in normal conditions. The intestinal barrier function is impaired in active CD and this can be mediated by the direct gliadin effect on the intestinal epithelium (Sander *et al.* 2005), or by ongoing inflammation with interferon (IFN)-γ production in the lamina propria (Schumann *et al.* 2008). The transcellular passage of non-degraded gliadin peptides is also increased in the small intestinal mucosa of CD patients (Schumann *et al.* 2008), and in a macaque model of CD (Bethune *et al.* 2008). An abnormal retrotransport of gliadin-IgA complexes by the CD71 receptor on the apical part of enterocytes has been suggested as an additional mechanism promoting gliadin translocation to the lamina propria of intestinal mucosa in CD patients (Matysiak-Budnik *et al.* 2008).

Intestinal infections are considered to be additional factors for gluten intolerance in CD, but only limited data are available to support this hypothesis

(Forsberg *et al.* 2004). An epidemiological study found that rotavirus infection increased the risk of CD in genetically predisposed children (Stene *et al.* 2006).

2.2.2. Genetic susceptibility

CD has strong familial clustering, indicated by a disease concordance rate of 75% in monozygotic twins, compared with 11% in dizygotic twins (Greco *et al.* 2002). The major genetic determinants of CD are class II HLA genes encoding the HLA-DQ2 heterodimer in more than 90% of CD patients and the DQ8 heterodimer in most of the remaining CD patients (Sollid *et al.* 1989; Spurkland *et al.* 1992). The alleles *HLA-DQA1*05* and *HLA-DQB1*02*, encoding for HLA-DQ2 α and β subunits, are carried either by a DR3 haplotype in the *cis* position or by DR5/DR7 haplotypes in the *trans* position, with a gene dosage effect on CD risk (Sollid *et al.* 1989; Ploski *et al.* 1993). However, the expression of HLA-DQ2 or HLA-DQ8 molecules is necessary but insufficient to develop CD, since the corresponding HLA haplotypes occur in about 30% of the European population, indicating the involvement of other genes in CD heritability (Dubois and van Heel 2008). Linkage and association studies have revealed some additional candidate chromosomal regions and genes to be associated with CD (Djilali-Saiah *et al.* 1998; Greco *et al.* 1998). The recent application of genome-wide association studies has led to the identification of a number of new CD common variant risk loci with more candidate genes (van Heel *et al.* 2007; Hunt *et al.* 2008; Dubois *et al.* 2010; Trynka *et al.* 2011). Twenty eight of the 39 non-HLA susceptibility loci for CD identified so far were found to be related to the genes of the immune system involving pathways of immune cell signalling, T- and B-cell differentiation, and innate immunity. Moreover, 26 CD genetic risk regions are also associated with other auto-immune and inflammatory conditions, suggesting a role for common pathways in the immunopathology of these diseases (Trynka *et al.* 2010). The individual risk values of associated loci are moderate for CD (highest odds ratio 1.7), and the current set of non-HLA genetic markers has been estimated to describe about 14% of the genetic variability of CD, compared with a contribution of about 40% of *HLA-DQ* (Trynka *et al.* 2011).

2.2.3. Adaptive immune response

The isolation and characterization of gluten-specific HLA-DQ2 restricted CD4+ T-cells from the intestinal mucosa of CD patients has established a causal link between HLA association and the induction of a gluten-specific T-cell response as an essential disease mechanism in CD (Lundin *et al.* 1993). The following studies have dissected the nature of gluten peptides presented by HLA-DQ2 and HLA-DQ8 to T-cells, as well as the type of the T-cell response triggered (Sollid 2002).

The deamidation of certain glutamine residues to glutamate in gliadin peptides by TG2, a CD autoantigen, has been found to enhance the gliadin-specific CD4+ T-cell response in CD (Molberg *et al.* 1998), substantiated by the higher affinity of modified peptides for binding to disease-associated HLA-DQ molecules (Sjöström *et al.* 1998; Arentz-Hansen *et al.* 2000). Subsequent studies identify immunodominant, TG2-dependent or -independent epitopes in α- and γ-gliadins and glutenins (van de Wal *et al.* 1998; Anderson *et al.* 2000; Arentz-Hansen *et al.* 2000; Tollefsen *et al.* 2006). A 33 amino acid long peptide (33-mer) resistant to intestinal proteases was identified in an α2-gliadin sequence (amino acids 57–89), inducing potent T-cell responses in all studied CD patients, and thus forming the structural basis for gluten intolerance in CD (Shan *et al.* 2002). The 33-mer peptide contains multiple immunodominant epitopes of α-gliadins, and is similar to the sequences in other gliadins as well as in hordeins and secalins (Shan *et al.* 2002; Shan *et al.* 2005). There is also some evidence of the presence of gliadin-specific CD8+ T-cells restricted to HLA-A2 in the small intestinal mucosa of CD patients (Gianfrani *et al.* 2003; Mazzarella *et al.* 2008).

Gluten-specific CD4+ T-cells from the intestinal biopsies of CD patients are producing cytokines characteristic of type 1 helper T-cells or of mixed type response with predominant production of IFN-γ (Nilsen *et al.* 1995) and IL-21 (Bodd *et al.* 2010), which are also increased at the tissue level (Nilsen *et al.* 1995; Fina *et al.* 2008). Research suggests that helper T-cell differentiation in the small intestinal mucosa of CD patients is promoted by overproduction of IFN-α, IL-18, and IL-21 (Monteleone *et al.* 2001; Salvati *et al.* 2002; Fina *et al.* 2008). Also IL-17A producing T-cells have been found in CD (Castellanos-Rubio *et al.* 2009; Monteleone *et al.* 2010), but their specificity, and the actual increase of IL-17A in the CD lesion are not yet clear (Bodd *et al.* 2010). The pathogenic T-cell response in CD is accompanied by a relative increase of regulatory T-cells in the intestinal mucosa, both of IL-10 producing T-cells (Gianfrani *et al.* 2006), and FoxP3+/CD25+ T-cells (Tiittanen *et al.* 2008; Vorobjova *et al.* 2009), although the ability of FoxP3+/CD25+ T-cells to suppress effector T-cell proliferation and IFN-γ production is impaired in CD by an IL-15 dependent mechanism (Zanzi *et al.* 2011; Hmida *et al.* 2011). IFN-γ and other proinflammatory cytokines, produced by gluten-specific T-helper cells, have direct pathogenic effects on the intestinal epithelial cells as well indirect effects on other immune cells for promoting inflammation in the intestinal mucosa (Przemioslo *et al.* 1995; Jabri and Sollid 2009).

The number of plasma cells in the lamina propria of CD patients' small intestinal mucosa is higher than in healthy people, consisting mainly of IgA+ plasma cells of both IgA1 and IgA2 subclasses in about equal amounts (Savilahti 1972; Kett *et al.* 1990). Approximately 5–10% of intestinal plasma cells are estimated to produce anti-gliadin antibodies with a predominance of the IgA class (Lycke *et al.* 1989; Brandtzaeg 2006). Still, the pathogenic potential of IgG antibodies, especially of IgG1 subclass, has been considered

higher due to complement activation and other proinflammatory effector functions (Brandtzaeg 2006). High concentrations of secreted IgA and IgM anti-gliadin antibodies have been detected in the jejunal fluid of untreated CD patients (Volta *et al.* 1988). Moreover, intestinal IgA/IgM antibodies against gliadin and TG2 appear in CD patients before the development of small intestinal atrophy (O'Mahony *et al.* 1990; Salmi *et al.* 2006b), reflecting early homeostatic immune activation (Brandtzaeg 2006). Also, the majority of serum IgA antibodies against gliadin and TG2 have been suggested to have mucosal origins (Brandtzaeg 2006; Kett *et al.* 1990).

2.2.4. Innate immune response

Studies over the last decade have shown that both innate and adaptive types of immunity are necessary to induce the cascade of immunopathogenic events that lead to CD in subjects with genetic susceptibility (Jabri and Sollid 2006). In Figure 1, the major immunopathogenetic mechanisms of CD are presented.

By testing gliadin peptides on *ex vivo* intestinal biopsies from CD patients, early immune activation with increased IL-15 production and enterocyte apoptosis was observed with α-gliadin peptide 31–43, but not with CD4+ T-cell immunodominant gliadin peptides (Maiuri *et al.* 2003). The number of intraepithelial lymphocytes (IEL), consisting mainly of T-cell receptor (TCR) αβ bearing CD8+ T-cells with some TCRγδ+ , mainly CD8– T-cells, is known to be increased in CD (Halstensen *et al.* 1989; Kutlu *et al.* 1993). TCRαβ+ IELs are correlated with gluten consumption and the degree of villous atrophy and their cytolytic activity has been related to enterocyte damage in CD (Kutlu *et al.* 1993; Ciccocioppo *et al.* 2000). Increased production of IL-15 in the intestinal mucosa of CD patients (Maiuri *et al.* 2000; Mention *et al.* 2003) is considered crucial to differentiate IELs into natural killer (NK)-cell type phenotype of CD8+ IEL with expression of NKG2D and other activating receptors (Hüe *et al.* 2004; Meresse *et al.* 2004; Meresse *et al.* 2006). Simultaneously, stressed intestinal epithelial cells are expressing the non-classical HLA class I receptor MICA as a ligand for NKG2D, leading to the destruction of enterocytes by IELs independently of TCR engagement (Hüe *et al.* 2004; Meresse *et al.* 2004). Furthermore, IL-15 and other cytokines delivered by stressed enterocytes and activated IELs could change tolerogenic dendritic cells into an inflammatory type, and thereby promote a DQ2/DQ8 dependent gliadin-specific T-cell response in the small intestinal mucosa (Jabri and Sollid 2009). Activated IELs also produce arachidonic acid and leukotrienes, which could promote local inflammation and recruitment of granulocytes into the lesion (Tang *et al.* 2009). However, the mechanisms by which gliadin and possible other factors induce intestinal epithelial cell stress, IL-15 production, and loss of tolerance to gluten in CD, have still not been resolved (Jabri and Sollid 2009).

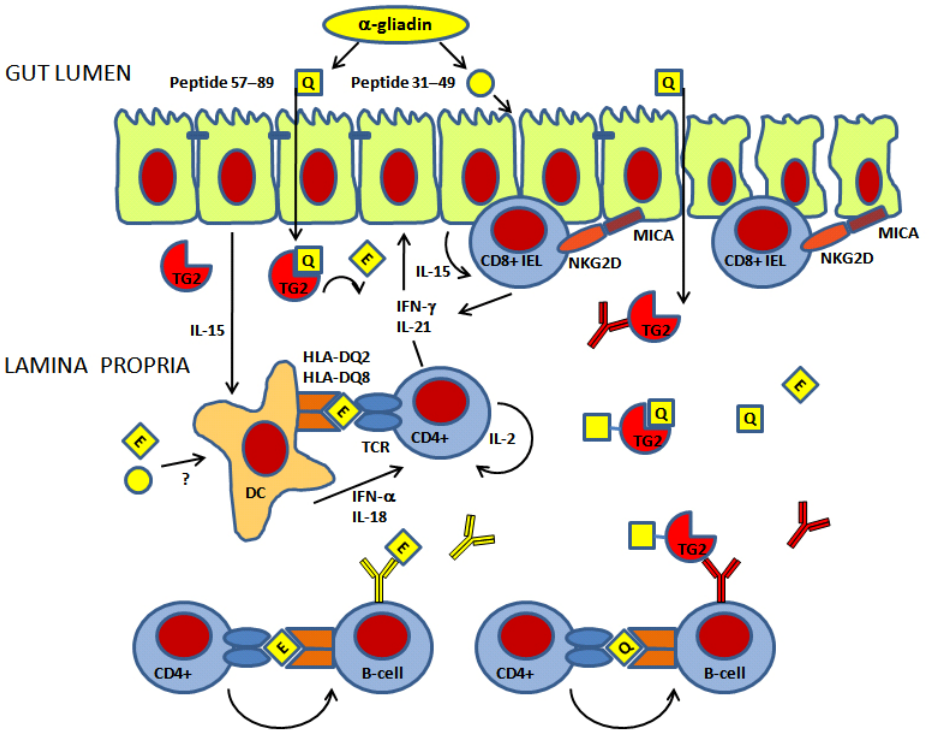

Figure 1. The model of CD immunopathogenesis in the small intestinal mucosa. Gliadin peptides in the gut lumen enter lamina propria of intestinal mucosa through both the transcellular and paracelluar pathways. Peptides corresponding to α-gliadin amino acid sequence 57–89 trigger the CD4+ T-cell response when presented in the complex with HLA-DQ2 or -DQ8 molecules by dendritic cells (DC). TG2 converts glutamine (Q) residues in native gliadin peptides to glutamate (E), resulting in a more vigorous T-cell response. Activated gliadin-specific T-helper cells produce cytokines (IFN-γ, IL-21) and provide help to B-cells for producing antibodies against gliadin and TG2. Innate immune mechanisms are activated by the effects of α-gliadin amino acid sequence 31–49, leading to IL-15 production by enterocytes and subsequent licensing of intraepithelial lymphocytes (IEL) to lyse enterocytes through engagement of NKG2D/MIC receptor system. TCR, T-cell receptor; NKG2D, natural killer group 2 member D; MICA, major histocombatibility complex class I-related chain A.

2.3. Transglutaminase 2

2.3.1. Structure and biological functions

Transglutaminase 2 (TG2; tissue transglutaminase, transglutaminase C) belongs to the transglutaminase protein family encoded by eight genes in the human genome. The common feature of all transglutaminases is their ability to catalyze

the post-translational modification of proteins either by forming N^{ε}-(γ-glutamyl) lysine isopeptide bonds between certain glutamine and lysine side chains, incorporating polyamines into proteins, or by deamidating glutamine residues to glutamate in the absence of amine donors (Lorand and Graham 2003). Compared to other transglutaminases expressed in the limited tissues or cell types, TG2 is expressed ubiquitously in tissues and it is carrying diverse functions in multicellular organisms. TG2 is involved in the cellular processes of apoptosis, endocytosis, signal transduction, adhesion, and extracellular matrix formation (Griffin *et al.* 2002; Iismaa *et al.* 2009). Apart from its Ca^{2+}-dependent transamidation activity, TG2 binds GTP/GDP and acts as a GTPase in intracellular G protein signalling (Nakaoka *et al.* 1994). The protein disulfide isomerase (Hasegawa *et al.* 2003) and serin/threonin kinase (Mishra and Murphy 2004) activities of TG2 have also been described, although they have been studied less.

TG2 is encoded by the *TGM2* gene on the human chromosome 20q11–12 and contains 687 amino acid residues in a polypeptide chain with a molecular weight of 77 kDa. Human TG2 consists of four protein domains characteristic of all transglutaminases: N-terminal β-sandwich domain (amino acids 1–139), catalytic or core domain with mainly α-helical secondary structure (147–460), and two C-terminal β-barrel domains (472–583 and 591–687) (Liu *et al.* 2002). A schematic representation of the molecular structure of TG2 and its domain composition are shown in Figure 2. The loop between the catalytic domain and the first β-barrel domain acts as a hinge region to enable the switch between two conformational states of TG2: an "open" conformation with transamidation activity (Pinkas *et al.* 2007) and a "closed" conformation with GTPase activity (Liu *et al.* 2002). These two conformational states are reciprocally regulated by binding Ca^{2+} ions or GTP/GDP, respectively, and stabilized by intracellular disulphide bonds between cysteine residues (Cys-230, Cys-370, Cys-371) (Pinkas *et al.* 2007; Han *et al.* 2010). The catalytic site in the open conformation of TG2 is accessible for binding first with acyl-donor and then with acyl-acceptor substrates for transamidation, and the catalytic triad residues of Cys-277, His-355,Asp-358 are critical in this reaction (Liu *et al.* 2002; Pinkas *et al.* 2007).

TG2 expression at the transcriptional level can be up-regulated by cytokines (TGF-β, TNF-α, IL-1 and IL-6), retinoids, vitamin D, and steroid hormones (Mehta *et al.* 2010). TGF-β and retinoid response elements are located in the promotor region of the *TGM2* gene (Ritter and Davies 1998). Intracellular TG2 is normally in the "closed" conformation, due to low Ca^{2+} and high GTP concentrations being the main regulators of TG2 activity (Monsonego *et al.* 1998; Zhang *et al.* 1998). Various stress stimuli, accompanied by the increase of intracellular Ca^{2+} levels, can switch on the transamidation activity of TG2 leading to the cross-linking of multiple proteins as part of the stress response or

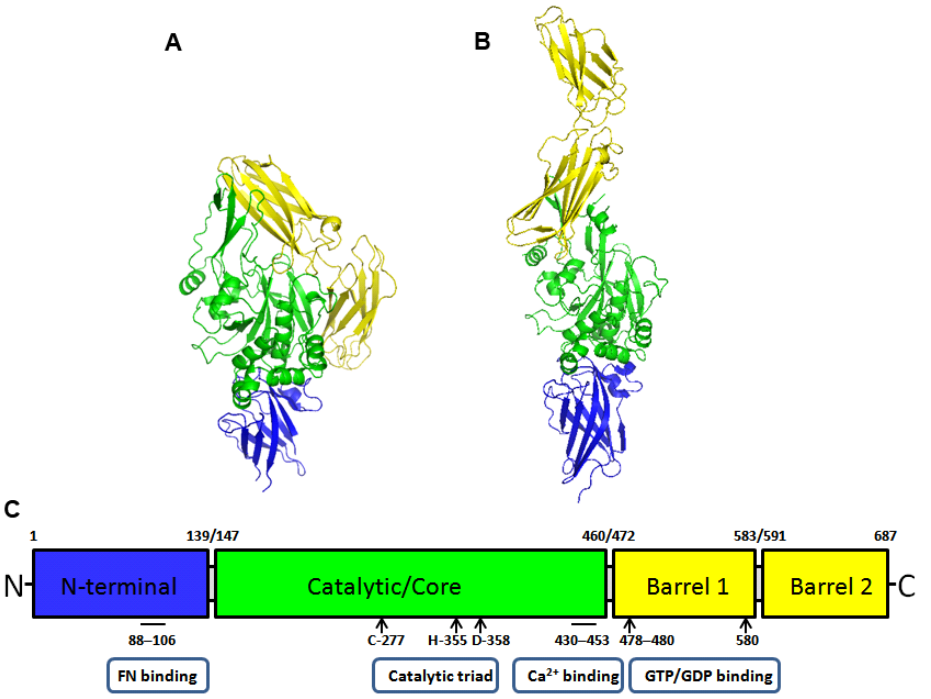

Figure 2. Schematic representation of the molecular and domain structure of TG2. (A) The GDP-bound form of TG2, representing the "closed" conformation with no transamidase activity (Liu *et al.* 2002; PDB code: 1KV3). (B) Extended, the "open" conformation of TG2 with transamidase activity (Pinkas *et al.* 2007; PDB code: 2Q3Z). A substrate binding site in the catalytic domain of TG2 is opened by a large Ca^{2+}-dependent conformational change. (C) The domain structure of TG2 and the main functional and binding sites.

apoptotic process. Both the pro- and anti-apoptotic effects of TG2 have been described, and the types of apoptotic stimuli, and the intracellular localization of TG2 are perceived as important determinants of its effect on apoptosis (Milakovic *et al.* 2004; Fésüs and Szondy 2005). The majority of intracellular TG2 is expressed in the cytosol, but TG2 is also found in the nucleus (Lesort *et al.* 1998) and in the mitochondria (Park *et al.* 2010). The stabilizing role of TG2 by cross-linking intracellular proteins in the later stages of apoptosis has been suggested to prevent the loss of intracellular components prior to clearance by phagocytosis (Piredda *et al.* 1997; Piacentini and Colizzi 1999). Studies on TG2 knock-out mice have revealed that despite the overt normal foetal phenotype, lack of TG2 affects clearance of dying cells by macrophages, and mice develop age-dependent autoimmunity (Szondy *et al.* 2003; Rose *et al.* 2006). A significant number of intra- and extracellular substrate proteins of TG2 have been identified by proteomic methods *in vitro* and *in vivo*, and the physiological

relevance of some of these post-translational modifications has been explored (Esposito and Caputo 2005). Transamidation of proteins by TG2 typically leads to the formation of cross-linked, polymerized protein scaffolds with altered functional properties (Griffin *et al.* 2002).

2.3.2. TG2 and cell adhesion

Although primarily considered as an intracellular protein, TG2 is also expressed on the cell surface and in the ECM (Zemskov *et al.* 2006; Belkin 2011; Wang and Griffin 2012). The mechanism of TG2 transport to the plasma membrane and secretion to the ECM is largely unknown, as TG2 has no leader peptide for externalization (Belkin 2011). Even though the Ca^{2+} concentration is higher in the extracellular space, the extracellular TG2 is catalytically inactive in normal physiological conditions, but can be rapidly activated after chemical or physical injury (Siegel *et al.* 2008). Cross-linking of extracellular proteins by TG2 is functionally related to the stabilization of ECM in normal tissue modelling as well as in the response to injury as it occurs in wound healing (Zemskov *et al.* 2006).

Independent of its transamidase activity, TG2 is also involved in the cell adhesion process through interactions with proteins of the cell membrane and ECM (Akimov *et al.* 2000; Verderio *et al.* 2003). The role of TG2 in cell adhesion is related to its high affinity binding to fibronectin (FN), a multi-functional ECM protein (Gaudry *et al.* 1999; Hang *et al.* 2005). FN is a high molecular weight modular glycoprotein, forming a homodimer of 440 kDa. FN promotes cell adhesion in its polymerized form and serves as a scaffold for the assembly of other ECM molecules (Zemskov *et al.* 2006). The FN binding region of TG2 is in the N-terminal domain (amino acids 88–106), and two TG2 molecules bind to the FN homodimer (Hang *et al.* 2005). Cell surface TG2 is involved in FN polymerization and is closely associated with the FN fibrillar matrix (Verderio *et al.* 1998; Akimov and Belkin 2001a). A complex formation between FN, TG2 and cell surface integrins promotes cell adhesion and spreading (Akimov *et al.* 2000; Belkin 2011). Integrins can directly bind to FN by recognizing the Arg-Gly-Asp (RGD) sequence containing sites within the type III domain of FN (Pierschbacher and Ruoslahti 1984) or by interacting via β1/β3/β5 subunits, with TG2 as a co-receptor for FN (Akimov *et al.* 2000). Both FN and TG2 can also interact with the heparan sulphate (HS) residues of the cell surface proteoglycans, and through this, support RGD-independent cell adhesion (Verderio *et al.* 2003; Telci *et al.* 2008). Syndecan-4 is thought to be the main TG2-binding heparan sulphate proteoglycan (HSPG) on the cell surface (Telci *et al.* 2008; Scarpellini *et al.* 2009). A model has been proposed in which the FN-TG2 matrix promotes cell adhesion via interactions with syndecan-4 and integrin α5β1, leading to the activation of protein kinase C-α and focal adhesion kinase (Telci *et al.* 2008; Verderio *et al.* 2009; Wang *et al.* 2010).

HSPGs are composed of a core protein and one or more linear polysaccharide chains, consisting of sulphated L-iduronic /D-glucuronic acid and glucosamine units. HSPGs are involved in processes of cellular communication by binding a variety of protein ligands like ECM proteins, growth factors, chemokines and cytokines (Bishop *et al.* 2007). Structurally HS is very close to heparin, which is widely used as a model to study protein-HS interactions. The mechanism of protein and heparin/HS binding is based on the electrostatic interactions between negatively charged residues of heparin/HS and positively charged basic amino acid residues of arginine and lysine in polypeptide chains. Most of the heparin/HS binding sites in proteins contain clusters of basic amino acids, and two common heparin binding consensus sequences have been proposed (XBBBXXBX and XBBXBX; B-basic, X-non basic amino acids) (Cardin and Weintraub 1989).

The binding of TG2 to heparin is known about from earlier studies and this feature was exploited for the affinity purification of TG2 on the heparin-Sepharose matrix (Signorini *et al.* 1988). Scarpellini *et al.* (2009), demonstrate high-affinity binding of guinea pig TG2 (gpTG2) to heparin and HS by the surface plasmon resonance (SPR) method.

2.3.3. TG2 in CD

TG2 is involved in a variety of pathologic conditions including celiac disease (Molberg *et al.* 2000), neurodegenerative disorders (Kim *et al.* 2002), fibrotic diseases (Wu and Zern 2004), and cancer (Mehta *et al.* 2010). While the expression and activity of TG2 are primarily regulated by stress signals, its involvement in chronic inflammatory diseases can be viewed as part of the stress/ inflammatory response (Iismaa *et al.* 2009). In the inflammatory environment, TG2 may protect cells from death, promote their motility and advance the clearance of apoptotic cells by macrophages (Mehta *et al.* 2010; Szondy *et al.* 2003). However, TG2 can also enhance the inflammatory process by activating NF-κB via non-canonical pathways (Lee *et al.* 2004; Luciani *et al.* 2009). High transamidase activity of TG2 is involved in forming pathogenic, cross-linked protein aggregates in Alzheimer's, Huntington's, and Parkinson's diseases (Kim *et al.* 2002). The changed antigenic properties of proteins modified by TG2 could induce autoimmunity in inflammatory conditions. This hypothesis is indirectly supported by the notion that TG2 substrate proteins often are known autoantigens in various autoimmune diseases (Kim *et al.* 2002). The relevance of TG2 for carcinogenesis depends on the type and stage of cancer as well on the localization and functional state of the TG2. Typically, TG2 is down-regulated in primary tumours, whereas TG2 expression is elevated in secondary, metastatic tumours where TG2 is implicated in promoting the invasive potential of the cells and conferring resistance to chemotherapeutic drugs (Kotsakis and Griffin 2007; Mehta *et al.* 2010). TG2 is considered to be a promising therapeutic target for treating drug-resistant and metastatic tumours (Mehta *et al.* 2010).

TG2 came to the attention of CD researchers after Dieterich and colleagues identified it as the main autoantigen for anti-endomysium IgA antibodies (EmA-IgA) (Dieterich *et al.* 1997). Increased transglutaminase activity in the jejunal biopsies of CD patients, with gliadin as the preferential substrate among dietary proteins, has been described earlier (Bruce *et al.* 1985). The higher expression of TG2 in the small intestinal mucosa of CD patients has since been revealed both at the mRNA and protein level (Ciccocioppo *et al.* 2003; Esposito *et al.* 2003; Biagi *et al.* 2006). TG2 is present in the lamina muscularis and to a lesser extent in the lamina propria of the normal small intestinal mucosa (Esposito *et al.* 2003; Sakly *et al.* 2005). The expression of TG2 is more pronounced in the subepithelial region of lamina propria in the atrophic small intestinal mucosa of CD patients (Esposito *et al.* 2003; Sakly *et al.* 2005; Gorgun *et al.* 2009), and in the enterocytes (Esposito *et al.* 2003; Biagi *et al.* 2006; Gorgun *et al.* 2009). However, some studies have found no difference in intestinal TG2 expression and activity between children with and without CD (Skovbjerg *et al.* 2004a), or between different stages of the disease (Villanacci *et al.* 2009). What is more an increase in TG2 expression does not seem to be disease specific, as similar upregulation of TG2 was detected in other inflammatory conditions of the small intestine (Esposito *et al.* 2003; Gorgun *et al.* 2009; Villanacci *et al.* 2009).

The crucial role of TG2 in CD immunopathogenesis was revealed by the finding that TG2 enhances the gliadin-specific CD4+ T-cell response by deamidating certain glutamine residues to glutamate in gliadin peptides (Molberg *et al.* 1998). TG2 could also mediate the formation of covalently linked gliadin-gliadin and gliadin-TG2 complexes *in vitro* (Dieterich *et al.* 1997; Uhlig *et al.* 1998; Fleckenstein *et al.* 2004; Dieterich *et al.* 2006). Sollid *et al.* (1997) suggested that TG2-specific B-cells, by processing and presenting TG2 cross-linked with gliadin, could get help from gliadin-specific T-cells to produce autoantibodies against TG2. Furthermore, via such a hapten-carrier model, autoantibodies could be induced towards other autoantigens complexed with gliadin (Sollid *et al.* 1997; Uhlig *et al.* 1998; Molberg *et al.* 2000; Skovbjerg *et al.* 2004b). *In vitro* studies have demonstrated cross-linking of gliadin peptides with collagens by TG2, and elevated serum IgA antibodies against these proteins were found in CD patients (Dieterich *et al.* 2006). Deamidation or transamidation of gliadin peptides and other substrate proteins is believed to be catalyzed by extracellular TG2, localized below the intestinal epithelial cell layer in a CD lesion (Fleckenstein *et al.* 2002; Ciccocioppo *et al.* 2003; Skovbjerg *et al.* 2004b; Siegel *et al.* 2008; Skovbjerg *et al.* 2008). The potential role of TG2 on the surface of intestinal dendritic cells in deamidation and presentation of gluten peptides to T-cells has been explored but no evidence was found to support this hypothesis (Ráki *et al.* 2007).

2.4. Antibodies in CD

2.4.1. Anti-gliadin antibodies

Berger (1958), first described antibodies against gluten proteins in the blood sera of CD patients, and this was followed by the development of various methods for the detection of anti-gliadin antibodies (AGA) from human sera (Bürgin-Wolff *et al.* 1976; O'Farrelly *et al.* 1983). IgA and IgG class AGA were found to be useful serologic markers for CD screening and monitoring the adherence to a gluten free diet, as antibody levels were dependent on gluten consumption in CD patients (Savilahti *et al.* 1983; Bürgin-Wolff *et al.* 1991; Mäki 1995). The diagnostic accuracy was higher for AGA-IgA, reaching over 90% of sensitivity and specificity, and lower for AGA-IgG, which was also detected in other diseases and in healthy subjects (Mäki 1995). AGA was found to be a more accurate CD diagnostic marker for children than for adults (Mäki 1995), and this was also supported by the finding that AGA-IgA positivity increases with age in the general population (Uibo *et al.* 1993). The new era of AGA as a biomarker for CD came after the identification of gliadin peptide epitopes, where certain glutamine residues were replaced with glutamate, providing an antigen for more accurate CD screening tests (Schwertz *et al.* 2004). Anti-deamidated gliadin antibody (AdGA) IgA and IgG assays showed equally high sensitivity and specificity for CD, and are suggested as valuable serologic markers for CD screening in combination with anti-TG2 antibodies (Sugai *et al.* 2006; Agardh 2007; Volta *et al.* 2008).

2.4.2. Anti-reticulin and anti-endomysium antibodies

The first study on CD-associated serum autoantibodies, which bind to autologous or homologous jejunal mucosa, was published by Malik and colleagues (Malik *et al.* 1964). This was followed by a description of specific IgG and IgA class autoantibody staining patterns on rodent tissue sections – called anti-reticulin antibodies (ARA) (Seah *et al.* 1971a; Seah *et al.* 1971b), and on primate oesophagus tissue sections – called anti-endomysium antibodies (EmA) (Chorzelski *et al.* 1983). Both autoantibodies were found in sera of CD patients when studied by the indirect immunofluorescence (IIF) technique. As an advance in assay optimization, human umbilical cord was used as a suitable antigenic substrate for detection of IgA class EmA (Ladinser *et al.* 1994), becoming a highly specific (97–100%) and sensitive (71–100%) serologic marker for CD (Lewis and Scott 2006). A somewhat lower prevalence of EmA-IgA was observed in children under the age of two (Grodzinsky *et al.* 1995) and in adults with CD (Sulkanen *et al.* 1998a).

EmA-IgA binds to the connective tissue surrounding smooth muscle and stromal cells and produces a staining pattern clearly distinctive from other known autoantibodies. ARA and EmA were thought to be close antibody

subtypes, detected on tissues of different types and from different species (Hällström 1989). Both antibodies appeared to be gluten-dependent – the antibody titers decreased or antibodies disappeared in response to a GFD lasting for several months and were induced again after introducing gluten to the diet (Hällström 1989; Bürgin-Wolff *et al.* 1991). Moreover, EmA was produced in small intestine biopsy samples of CD patients after *in vitro* gliadin challenge (Picarelli *et al.* 1996). The association of EmA-IgA with small intestinal lesion was less clear as, after starting a GFD, antibodies tended to disappear before complete mucosal recovery (Dickey *et al.* 2000).

Several attempts were made to identify EmA antigens (Marttinen and Mäki 1993; Börner *et al.* 1996), until Dieterich and colleagues identified TG2 as the main, if not the only EmA antigen (Dieterich *et al.* 1997). They used immunoprecipitation of the fibrosarcoma cell lysate with serum IgA antibodies from CD patients, followed by the identification of precipitated proteins – TG2 and associated FN, using proteomic methods. TG2 was conclusively confirmed to be a target antigen of both EmA and ARA in CD and dermatitis herpetiformis patients in further studies, and anti-TG2 autoantibodies were found to recognise extracellular TG2 in normal rodent and primate tissue (Korponay-Szabó *et al.* 2000; Korponay-Szabó *et al.* 2003b).

2.4.3. Anti-TG2 antibodies

The discovery of TG2 as the primary autoantigen for CD-associated autoantibodies led to the development of anti-TG2 antibody assays, both in the ELISA format (Dieterich *et al.* 1998; Sulkanen *et al.* 1998b), and by using immunoprecipitation of *in vitro* transcribed TG2 (Bazzigaluppi *et al.* 1999). Commercially available gpTG2 was used at first as the antigen in the anti-TG2 ELISA, and then replaced by human recombinant TG2 (hrTG2), resulting in the higher diagnostic accuracy of the assay for CD (Sárdy *et al.* 1999; Sblattero *et al.* 2000). The diagnostic sensitivity (93%) and specificity (> 98%) of those second generation anti-TG2 assays were in the same range as the EmA-IgA test (sensitivity 93%, specificity > 99%), based on a large meta-analysis, suggesting anti-TG2 IgA as the preferred serologic marker for CD screening (Lewis and Scott 2006).

Anti-TG2 antibodies of the IgG class are detected less frequently among CD patients, but have been found to be useful for recognizing CD patients with IgA-deficiency (Cataldo *et al.* 2000; Korponay-Szabó *et al.* 2003a). There are only a few studies where anti-TG2 IgM has been examined in CD, and the findings show low antibody prevalence (Agardh *et al.* 2003; Feighery *et al.* 2003). The combined assessment of anti-TG2 IgA and IgG is advised for serologic screening of CD because of the increased diagnostic accuracy (Picarelli *et al.* 2001; Dahlbom *et al.* 2008). The occurrence of anti-TG2 antibodies but not EmA, have been reported in a small proportion of patients with hepatic diseases (Bizzaro *et al.* 2003; Vecchi *et al.* 2003; Villalta *et al.* 2005), heart diseases

(Di Tola *et al.* 2008), and other autoimmune diseases (Sárdy *et al.* 2007), without having a CD diagnosis. These results are mostly regarded as analytical false-positives rather than true anti-TG2 antibodies, prompting the need for further assay optimization (Villalta *et al.* 2005; Sárdy *et al.* 2007).

A common feature of solid-phase anti-TG2 antibody assays is the inclusion of Ca^{2+} ions when coating the plate surface with the TG2 antigen, resulting in better diagnostic accuracy (Sulkanen *et al.* 1998b; Agardh *et al.* 2005). This is explained by the "open" conformational structure of TG2 in the presence of Ca^{2+}, which is preferentially recognized by CD anti-TG2 antibodies (Lindfors *et al.* 2011). In order to broaden the methodical range of anti-TG2 assessment, point-of-care anti-TG2 antibody rapid assays have been developed, comparable sensitivity and specificity of ELISA for CD screening (Sorell *et al.* 2002; Korponay-Szabó *et al.* 2005; Raivio *et al.* 2006; Baviera *et al.* 2007). Interestingly, one lateral flow immunochromatographic assay is based on the principle of autoantibody binding to self-TG2 from blood erythrocytes, and capturing the antibody-TG2 complex to immobilized FN (Korponay-Szabó *et al.* 2005).

The time-dynamics of the anti-TG2 IgA antibodies in the sera of CD patients, like EmA-IgA, follows gluten consumption (Bürgin-Wolff *et al.* 2002). The sensitivity of anti-TG2 IgA is considered lower in children under two years of age, and the assessment of AGA or AdGA is suggested as a complimentary test (Bürgin-Wolff *et al.* 2002; Agardh 2007; Lagerqvist *et al.* 2008). Epidemiological studies have described the first appearance of AGA, followed by anti-TG2 antibodies in children at genetic risk of CD (Liu *et al.* 2007; Simell *et al.* 2007). The phenomenon of the spontaneous disappearance of the anti-TG2 antibodies in about half of the gluten-consuming children, who had positive results earlier, has also been reported (Simell *et al.* 2007).

The production of anti-TG2 antibodies in the intestinal mucosa of CD children is demonstrated by antibody deposits colocalizing with TG2 (Korponay-Szabó *et al.* 2004), as well as by characterisation of the anti-TG2 antibody clones from the gut (Marzari *et al.* 2001). Detection of anti-TG2 IgA deposits in the intestinal mucosa of CD patients is suggested as an additional diagnostic tool in seronegative CD patients (Salmi *et al.* 2006a), and also as a predictive marker for forthcoming CD (Salmi *et al.* 2006b).

2.4.4. Anti-cytoskeletal antibodies

The presence of smooth muscle antibodies (SMA) of the IgA class have been observed in CD patients when studying serum antibody binding in IIF studies, although in lower titers and with less sensitivity and specificity than EmA-IgA (Uibo *et al.* 1995; Volta *et al.* 1995; Amara and Husebekk 1998). However, in some cases masking of the EmA-IgA pattern by SMA-IgA staining can occur, and by increasing the serum dilution, EmA-IgA detection can be improved (Lasagni *et al.* 1999).

SMA types are classified according to the staining pattern on different tissues (Bottazzo *et al.* 1976), and are mainly related to antibodies against different cytoskeletal proteins which are making up microfilaments, intermediate filaments and microtubules (Dighiero *et al.* 1990; Toh 1991). Both IgG class SMA and antibodies to individual cytoskeletal proteins, especially to actin, are associated with autoimmune hepatitis (Whittingham *et al.* 1966; Mackay *et al.* 2008), but low titer and transient SMA/anti-cytoskeletal antibodies have been found in various disease conditions (Bottazzo *et al.* 1976; Toh 1991), and also in the general population (Uibo *et al.* 1998). Autoantibodies reacting with microfilaments primarily recognize actin (Bottazzo *et al.* 1976; Mackay *et al.* 2008), whereas antibodies reacting with intermediate filaments are specific to vimentin and desmin proteins (Kurki and Virtanen 1985; Dighiero *et al.* 1990). Autoantibody binding depends on the conformation of the cytoskeletal proteins, as, for example, polymerised, filamentous actin is recognized more frequently than monomeric actin by autoantibodies of autoimmune hepatitis patients (Mackay *et al.* 2008). Therefore, considerable differences exist between anti-cytoskeletal antibody binding to tissues or cells by IIF histochemistry and to individual cytoskeletal proteins using the ELISA and western blot methods (Girard and Senécal 1995; Mackay *et al.* 2008).

Clemente and colleagues described the presence of gluten-dependent, mainly IgA class serum autoantibodies against filamentous actin in 71% of CD patients using IIF on HEp-2 cells (Clemente *et al.* 2000). The diagnostic value of anti-actin antibodies for CD, detected by IIF, ELISA, or both, was assessed in follow-up studies, and the association with the degree of mucosal lesion was revealed (Clemente *et al.* 2004; Granito *et al.* 2004; Carroccio *et al.* 2007). However, the lower diagnostic performance of anti-actin IgA compared to anti-TG2 IgA has given no support for the use of anti-actin antibodies in CD screening, but rather as an additional test for specific disease cases (Fabbro *et al.* 2008; Bazzigaluppi *et al.* 2010).

2.4.5. Other CD-associated autoantibodies

Autoantibodies against other self-proteins can be associated with CD or with extraintestinal manifestations of the disease. Transglutaminase 3 (TG3) or epidermal transglutaminase has been identified as the target antigen in DH, a skin disease closely associated with CD (Sárdy *et al.* 2002). In addition to the small intestinal lesions characteristic of CD, and circulating anti-TG2 IgA, patients with DH have anti-TG3 IgA antibodies in their blood and antigen-antibody deposits in the skin (Sárdy *et al.* 2002; Cannistraci *et al.* 2007; Hull *et al.* 2008). TG3 is expressed in keratinocytes, and is involved in cell envelope formation during keratinocyte differentiation (Griffin *et al.* 2002). Supported by experimental data, diffusion of TG3 from the epidermis and anti-TG3 IgA from the blood circulation leads to the formation of immune complexes in the papillary dermis in DH patients (Zone *et al.* 2011). Epitope spreading from TG2

to TG3 has been proposed as a mechanism for the development of anti-TG3 IgA antibodies in DH patients (Sárdy *et al.* 2002; Zone *et al.* 2011). A distinct population of antibodies against TG6, a recently described neuronal transglutaminase, is suggested to be associated with cerebellar ataxia in a subgroup of patients with the gluten-sensitive type of this neurological disease (Hadjivassiliou *et al.* 2008). In addition to antibodies against TG3 and TG6, cross-reactive anti-TG2 antibodies could recognize other members of the TG family, although this has not been experimentally studied (Alaedini and Green 2008).

Calreticulin, a multifunctional intracellular protein, was identified as an enterocyte antigen cross-reactive with the anti-gliadin antibodies of CD patients (Tučková *et al.* 1997). IgA and IgG class antibodies against calreticulin were found at significantly higher levels in the sera of CD patients compared with healthy controls, and the levels decreased in response to a GFD (Sánchez *et al.* 2000). However, antibodies to calreticulin also occur in various autoimmune and infectious diseases (Sánchez *et al.* 2003). Also neuronal protein synapsin I was shown to be recognised by anti-gliadin antibodies, and such antibodies were detected in several patients with CD (Alaedini *et al.* 2007). IgA antibodies against the tight junction regulatory protein zonulin, the expression of which is increased in the small intestinal mucosa of CD patients, have been detected in about a fifth of CD patients with the acute phase of the disease (Fasano *et al.* 2000). In smaller subsets of CD patients, autoantibodies were identified targeting osteoprotegerin in association with osteoporosis (Riches *et al.* 2009), or ATP synthase β chain and enolase α, the clinical relevance of which remained elusive (Stulík *et al.* 2003).

Several autoantibodies occurring in CD can be affiliated with an associated disease rather than with enteropathy itself. Antibodies against gangliosides, a type of glycolipid expressed on neuronal cells, were described in CD patients with neurological disorders (Alaedini *et al.* 2002; Volta *et al.* 2006). Similarly, autoantibodies associated with type 1 diabetes, autoimmune thyroiditis and some other autoimmune conditions can be observed as more prevalent in CD patients than in the general population (Shaoul and Lerner 2007).

2.4.6. Autoantibodies in the pathogenesis of CD

The question of whether and how autoantibodies contribute to CD pathogenesis has been addressed in many studies, the majority exploring the effect of anti-TG2 antibodies on the enzymatic activity of TG2 and on various cellular functions *in vitro* (Caputo *et al.* 2009; Caja *et al.* 2011). The epitope analysis of TG2, providing possible insights into the functional role of autoantibodies, has revealed that intact N-terminal, catalytic, and C-terminal domains are needed for antibody binding (Seissler *et al.* 2001; Sblattero *et al.* 2002; Nakachi *et al.* 2004; Byrne *et al.* 2007; Simon-Vecsei *et al.* 2012). A recent study identified a conformational epitope of TG2, involving amino acid residues Glu-153 and Glu-154 in the catalytic domain, and Arg-19 in the N-terminal domain, as the

main autoantibody binding site for anti-TG2 autoantibodies in CD patients (Simon-Vecsei *et al.* 2012).

A review of the studies shows that the effects of anti-TG2 antibodies of CD patients on the main biochemical activity of TG2 – transamidation, vary from the inhibition (Esposito *et al.* 2002; Dieterich *et al.* 2003; Byrne *et al.* 2010) to the enhancement of enzyme activity (Király *et al.* 2006; Myrsky *et al.* 2009). Although methodological differences may account for some of the variability in the results, autoantibody populations with opposite effects on TG2 transamidation activity may exist in the sera of patients with CD (Király *et al.* 2006). According to the recent report, the effect of autoantibodies on TG2 transamidation activity can be indirect, as the binding of anti-TG2 antibodies to the cell-surface activated the intracellular TG2 pool through the modulation of Ca^{2+} homeostasis (Caputo *et al.* 2012).

TG2 is involved in multiple cellular processes and the effects described for anti-TG2 antibodies from CD patients in various cell culture models are wide ranging: inhibiting differentiation (Halttunen and Mäki 1999) and increasing proliferation of intestinal epithelial cells (Barone *et al.* 2007a), disturbing angiogenesis (Halttunen and Mäki 1999; Myrsky *et al.* 2008; Caja *et al.* 2010), increasing intestinal (Zanoni *et al.* 2006) and endothelial cell permeability (Myrsky *et al.* 2009), modulating endocytosis of gliadin peptides (Caputo *et al.* 2010), and inducing apoptosis in trophoblastic cells (Di Simone *et al.* 2010). These findings are explained either by the modulation of the transamidation activity of TG2 by autoantibodies (Myrsky *et al.* 2009; Caja *et al.* 2010), disorganising the actin cytoskeleton of the cells (Barone *et al.* 2007a; Myrsky *et al.* 2008), or by interfering with the outside-in signalling of cell surface TG2-integrin complexes (Barone *et al.* 2007a; Caputo *et al.* 2010). The increase of ERK phosphorylation (Caputo *et al.* 2010), and activation of small GTPase RhoA (Myrsky *et al.* 2009) in cells have been observed in response to treatment with anti-TG2 autoantibodies. TG2 is required for the formation of active TGF-β from the latent TGF-β complex (Nunes *et al.* 1997), therefore the role of anti-TG2 autoantibodies hindering this process, and consequently cellular differentiation, has been proposed (Halttunen and Mäki 1999).

The possible pathogenic effects of anti-TG2 antibodies have been also studied in animal models, closely resembling situation at organism level. A systemic immune response was induced in mice when immunized with hrTG2, including the appearance of anti-TG2 IgG antibodies in the sera, and the development of focal lymphocytic infiltrates in the lacrimal glands, but not in the intestine or in the skin (Freitag *et al.* 2004). When expressing anti-TG2 single chain antibody fragments cloned from CD patients in a mouse model no apparent pathologies or antibody deposits were observed at the tissue level but mice developed strong anti-idiotypic antibody responses (Di Niro *et al.* 2008). Transient cerebellar ataxia, thought to occur through antibody-antigen interaction at the vascular level was induced in the animals by injecting monoclonal human anti-TG2 antibodies into the central nervous systems of the

mice (Boscolo *et al.* 2010). It has also been demonstrated that sera from DH patients transferred to human skin-grafted mice mimics DH immunopathology (Zone *et al.* 2011). These results would not support a central role for anti-TG2 antibodies in CD pathogenesis. However, no definitive conclusions can be made for human CD, based on animal studies only, while not modelling CD with chronic inflammation and increased vascular permeability (Di Niro *et al.* 2008). The presence of anti-TG2 antibody deposits in the small intestinal lesions of CD patients in the early phase of the disease (Korponay-Szabó *et al.* 2004), as well as in extraintestinal tissue (Sárdy *et al.* 2002; Hadjivassiliou *et al.* 2008), and the biological effects of anti-TG2 antibodies obtained *in vitro* and *in vivo*, would suggest an active role for anti-TG2 antibodies in inflammation and tissue remodelling in CD.

3. AIMS OF THE STUDY

1. To detect the presence of anti-cytoskeletal antibodies in the sera of CD patients and to identify the main cytoskeletal antigen(s); to compare the diagnostic value of anti-cytoskeletal antibodies and anti-TG2 antibodies, and to evaluate the impact of a gluten free diet on autoantibody levels (Study I).
2. To compare the diagnostic performance of an anti-TG2 ELISA, based on TG2 binding to fibronectin, with a conventional ELISA for serum anti-TG2 IgA, IgG and IgM assessment in children with CD (Study II).
3. To evaluate the effect of anti-TG2 antibodies from the sera of CD patients on TG2 interactions with heparin/heparan sulphate and on the TG2-dependent adhesion of intestinal epithelial cells (Study III).
4. To delineate the main heparin-binding regions of TG2 by biosensor analysis of the putative heparin-binding TG2 peptides; to characterize immuno-reactivity and the effect on cell adhesion of the heparin-binding TG2 peptides (Study IV).

4. MATERIAL AND METHODS

4.1. Study subjects

Blood sera from 436 children and adolescents were studied, including 216 patients with CD and 220 diseased or healthy control subjects. The general characteristics of the study groups are provided in Table 1. Patients were enrolled at two centres: the Children's Clinic, Tartu University Hospital in Estonia, from 1990–2009 (n = 221), and the Department of Pediatrics, Malmö University Hospital at Lund University in Sweden, from 2000–2007 (n = 215). The informed consent of the participants and/or their parents was obtained for the intended use of the serum samples. The studies were approved by the Ethics Review Committees on Human Research of the University of Tartu and Lund University. Serum samples were stored at –20°C or at –75°C for longer storage.

Celiac disease group

Celiac disease was diagnosed in 216 children (73 from Estonia, 143 from Sweden) in accordance with the European Society of Pediatric Gastro-enterology, Hepatology and Nutrition (ESPGHAN) criteria (Walker-Smith *et al.* 1990), based on biopsy findings of the small intestinal mucosa, characteristic of CD. According to the degree of mucosal lesion, intraepithelial lymphocytosis (Marsh grade I–II) was observed in 7 patients; partial villous atrophy (Marsh IIIa) was detected in 46 patients, subtotal villous atrophy (Marsh IIIb) in 95, and total villous atrophy (Marsh IIIc) in 56 patients; 9 patients had undifferentiated Marsh III grade lesions. Three patients refused intestinal biopsies, but were included in the study group because of their clinical symptoms of CD and high anti-TG2/EmA levels. The following associated disease conditions were diagnosed in CD patients: type 1 diabetes (n = 20), IgA-deficiency (n = 3), iron deficiency anaemia (n = 2), DH (n = 1), gastritis (n = 1), epilepsy (n = 1), Turner's syndrome (n = 1), Down's syndrome (n = 1). Sera were obtained at the same time as the small intestinal biopsy or up to 1 month before. Serum samples from 26 patients on a GFD for 6–18 months (mean 9.6 months) were also studied (Study I).

Control groups

The biopsy control group included 122 patients (50 from Estonia, 72 from Sweden), who had undergone upper endoscopies to exclude CD, and had normal intestinal mucosa on histological examination. Most of the patients (n = 82) had no established diagnosis with recurrent abdominal pains, temporary gastrointestinal symptoms, or growth problems as the main symptoms. Other disease conditions in this group were food allergies (n = 12), gastritis (n = 9), selective IgA-deficiency (n = 8), lipase deficiency (n = 3),

esophagitis (n = 3), duodenal ulcers (n = 2), iron deficiency anaemia (n = 1), Down's syndrome (n = 1) and chromosome 18p deletion syndrome (n = 1). The disease control group consisted of 50 children, diagnosed at the Children's Clinic, Tartu University Hospital with the following disease conditions: type 1 diabetes (n = 26), juvenile chronic arthritis (n = 14), reactive arthritis (n = 6), systemic connective tissue disorders (n = 3), and muscular dystrophy (n = 1). The healthy children (n = 48) were recruited during regular medical examinations at the Children's Clinic, Tartu University Hospital. No intestinal biopsies were performed among healthy children and disease controls.

Table 1. General characteristics of the study groups and involvement of subjects in studies (I–IV).

	CD	***Biopsy controls***	***Disease controls***	***Healthy controls***
Total, n	216	122	50	48
Origin, Estonia/Sweden	73/143	50/72	50/0	48/0
Female, n (%)	136 (63.0)	58 (47.5)	25 (50.0)	24 (50.0)
Median age, years	5.2	7.0	8.0	4.0
Age range, years	0.6–21.1	0.6–22.2	2.0–15.0	0.5–14
Study I, n	42	18	50	48
Study II, n	173*	97*	–	–
Study III, n	27	13	–	–
Study IV, n	10	11	–	–

* Including 143 patients with CD and 72 biopsy controls from Department of Pediatrics, Malmö University Hospital at Lund University, Sweden

Use of patient samples in different studies

In Study I, where the main focus was on defining the autoantigenic targets of CD and on the diagnostic significance of the corresponding autoantibodies, patients with CD (n = 42) and control subjects (biopsy controls, n = 18; disease controls, n = 50; healthy controls, n = 48) were recruited at the Children's Clinic, Tartu University Hospital in Estonia. Sera from Swedish patients (CD, n = 143; biopsy controls, n = 72) were used together with samples of Estonian origin (CD, n = 30; biopsy controls, n = 25) in Study II, in which the main objective was the comparison of two methods for anti-TG2 determination. Serum samples from CD patients and biopsy controls were also collected in Tartu for Study III (CD, n = 27; biopsy controls, n = 13) and Study IV (CD, n = 10; biopsy controls, n = 11), which focussed on the effect of anti-TG2 antibodies on TG2 binding to heparin/HS and the related cell adhesion function. Only sera from CD patients positive for anti-TG2 IgA or IgG were selected for these two studies.

4.2. Antibodies and peptides

Mouse monoclonal anti-TG2 antibodies (clone CUB7402), anti-tubulin antibodies (clones DM1A+DM1B), and rabbit polyclonal anti-TG2 antibodies were obtained from Thermo Fisher Scientific (Fremont, USA), mouse monoclonal anti-vimentin antibodies (clone V9) and rabbit anti-desmin antisera were from Sigma-Aldrich (St. Louis, USA), mouse monoclonal anti-actin (clone C4) was from Boehringer Mannheim (Mannheim, Germany), and rabbit polyclonal anti-human FN antibodies were from DakoCytomation (Glostrup, Denmark).

The amino acid sequences of peptides used in the studies are shown in Table 2. Integrin-binding RGD peptide (G1269) and a control peptide with no effect on integrin function (S3771) were from Sigma-Aldrich (St. Louis, USA). TG2 peptides and a control peptide from enterovirus VP1 protein, which is a common epitope for all known enteroviruses (Viskari *et al.* 2004), were synthesized by Storkbio Ltd (Tallinn, Estonia) using Fmoc chemistry and purified by reversed-phase HPLC to over 95% purity. Peptides were analyzed with MALDI-TOF mass spectrometry for correct molecular weight. TG2 peptides were selected based on the predicted heparin/HS binding sequences in the protein, as described earlier (Utt *et al.* 2001); peptide P3 represented the TG2 control peptide (Zanoni *et al.* 2006).

Table 2. Synthetic peptides used in the studies. The parent molecule, peptide location in protein sequence, as well as the theoretical p*I* and molecular weight of the peptides are shown. Lysin (K) and arginine (R) residues are underlined.

Peptide	***Parent molecule***	***Position***	***Sequence***	***Theoretical pI/Mw***
P1	Human TG2	202–215	KFLKNAGRDCSRRS	10.9/1637.9
P2	Human TG2	261–274	LRRWKNHGCQRVKY	11.0/1844.2
P3[1]	Human TG2	476–487	RIRVGQSMNMGS	12.0/1335.6
P4	Human TG2	590–603	KIRILGEPKQKRKL	11.2/1707.1
P5	Human TG2	671–681	DKLKAVKGFRN	10.3/1275.5
VP1[2]	Enterovirus VP1	ND	KEVPALTAVETGATC	4.5/1489.7
RGD	ND	ND	GRGDSPK	8.8/715.7
DGR	ND	ND	SDGRG	5.6/490.4

[1] Zanoni *et al.* 2006; [2]Viskari *et al.* 2004; ND – not defined

4.3. Preparation of antigens

Cytoskeletal proteins

In Study I, the human umbilical cord (HUC) was used as the antigenic source for extraction of cytoskeletal proteins for autoantibody screening. While the HUC was the antigenic substrate for detection of both EmA-IgA and SMA-IgA

by IIF, the same tissue was chosen for searching candidate cytoskeletal antigens. A complex extraction protocol was developed to achieve the enrichment of a 57 kDa candidate antigen, identified as the intermediate filament protein desmin. Using western blot analysis, the other major components of the desmin-enriched extract were identified as vimentin and actin. In order to get more purified desmin for an ELISA analysis, desmin was prepared from the smooth muscle of chicken (the gizzard) according to a published protocol (Geisler and Weber 1980). Buffers containing 6 M urea were used in both methods to solubilise cytoskeletal proteins.

TG2

Human recombinant TG2 was produced in order to develop an ELISA assay (Study II) to investigate the biological effects of anti-TG2 antibodies (Study III), and to study the binding characteristics of TG2 to heparin (Study IV). Our approach was designed to obtain sufficient recombinant TG2 while retaining the biological activity of the protein. Using human TG2 cDNA (Genbank accession no 55153) (Gentile *et al.* 1991) as the template, a plasmid vector was designed encoding human full length TG2 with a C-terminal polyhistidine tag (amino acids RSHHHHHH), and transformed into *E. coli*. TG2 expression was induced by a low concentration (10 μM) of isopropyl-β-D-thiogalactoside (IPTG) at 25°C to increase the yield of soluble TG2. A two-step purification of hrTG2 was performed with subsequent Ni^{2+}-chelate and anion-exchange chromatography to obtain recombinant protein purity of over 90%. The average amount of purified TG2 was 5 mg per litre of bacterial culture. The human recombinant TG2 possessed transamidase activity, comparable to that of commercial gpTG2 (Slaughter *et al.* 1992), as well as the ability to bind to its natural ligand FN (Study II).

4.4. SDS-PAGE, western blot, protein identification

SDS-PAGE was performed on discontinuous 10% polyacrylamide gels, and separated proteins were stained with Coomassie Brilliant Blue G-250 or transferred to nitrocellulose membranes for probing with human sera or monospecific antibodies. Western blot was used for detecting IgA reactivity against human desmin (Study I), monitoring the expression, correct molecular weight and purity of hrTG2 (Study II), and analysing the protein composition of affinity purified anti-TG2 antibody samples (Study III).

In Study I, the 57 kDa antigen of interest was identified as desmin, using peptide mass-mapping by MALDI-TOF mass spectrometry and direct sequence analysis of selected peptides by Edman degradation. The peptide masses were analysed using the ProFound program (http://prowl.rockefeller.edu/prowl-cgi/profound.exe). The ten highest scores identified desmin from different species and all four sequences obtained corresponded to muscle desmin.

4.5. Detection of autoantibodies

EmA and umbilical cord smooth muscle antibodies (uSMA)

An IIF assay on cryosections of HUC was used to detect IgA class EmA and SMA in the sera of CD patients and the controls (Study I). Serum was considered positive for EmA-IgA when a typical staining pattern around the smooth muscle cells of the blood vessels was observed at 1:10 serum dilution (Ladinser *et al.* 1994). The intracellular staining of the smooth muscle cells was designated as uSMA-IgA to discriminate it from the SMA determined on rodent tissues.

Anti-TG2 ELISA

Since TG2 was identified as an autoantigen of EmA-IgA, an ELISA for anti-TG2 IgA detection from the sera of CD patients was established using commercially available TG2 from guinea pig livers (Sigma-Aldrich) as the antigen (Dieterich *et al.* 1998; Sulkanen *et al.* 1998b). We used a similar ELISA in Study I to compare the diagnostic value of anti-desmin and anti-TG2 IgA antibodies for CD. Universal binding 96-well microtiter plates (Thermo Fisher Scientific Oy, Vantaa, Finland) were used. A consensus finding of advanced studies was the lower diagnostic accuracy of a gpTG2 based ELISA compared to those using human TG2 (Sblattero *et al.* 2000; Sárdy *et al.* 2002), so we developed ELISAs utilizing hrTG2 as the antigen in Study II. A novel ELISA using FN as the binding ligand for TG2 was compared with the traditional method for the detection of anti-TG2 antibodies of the IgA, IgG, and IgM classes in CD and the control groups. In the modified ELISA, instead of coating the plastic surface directly with hrTG, plate wells were coated first with purified human FN (F2006; Sigma-Aldrich) and then with hrTG2. Both assays were run in parallel with the same serum dilutions (1:100), and tested in duplicate wells on one plate. No human serum antibody reactivity with the wells only coated with FN was observed, whereas some conjugated secondary antibodies against human IgG and IgM had slightly higher background binding to FN, which was minimized by the selection of appropriate conjugate and dilution. Anti-TG2 ELISA results were expressed in arbitrary units (AU) as percentages of the reference serum optical density values. The cut-off values were chosen to achieve high diagnostic specificity without significant loss of sensitivity. The intra- and inter-assay variability of the assays remained below coefficient of variation (CV)% value of 15.

Anti-desmin ELISA

An ELISA procedure similar to that of anti-gpTG IgA determination was used for anti-desmin IgA detection (serum dilution 1:100). The antibody levels were expressed in AU, and the same reference serum was used in the ELISA and

western blot for detection of anti-desmin IgA. The positive cut-off value was defined as the mean AU + 3 standard deviations (SD) of healthy children serum reactivity.

Anti-peptide ELISA

The reactivity of the heparin-binding synthetic peptides of TG2 with serum IgA from CD patients was estimated using a peptide ELISA in Study IV. Enhanced binding 96-well microtiter plates (Thermo Fisher Scientific Oy, Vantaa, Finland) were coated with peptides and air-dried. Patient and control sera were tested at a dilution 1:50 and the results were expressed in optical density (OD) units.

4.6. Affinity purification of autoantibodies

The affinity purification of serum antibodies was carried out to characterize the antigen-specificity of anti-desmin antibodies (Study I) and the functional role of anti-TG2 antibodies (Study III). In Study I, chicken desmin (6 mg) was conjugated to divinylsulphone activated Sepharose CL-6B matrix (2 ml). Three serum pools (including one control, 0.5 ml each) were applied on separate 0.5 ml columns and bound antibodies were eluted with 1 M potassium thiocyanate. Antibody samples were dialyzed against phosphate buffered saline (PBS) and tested for the presence of autoantibodies (anti-desmin, anti-TG2, EmA, uSMA). In Study III, hrTG2 protein (2 mg) was coupled to 2 ml of N-hydroxysuccinimide activated Sepharose 4 Fast Flow beads according to the manufacturer's instructions (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The affinity purification was performed with 13 sera from CD patients and 6 control sera (0.5 ml each) on two hrTG2-Sepharose columns (1 ml). Antibodies were eluted with acidic glycine buffer (pH 2.5) and columns were regenerated with 6 M guanidinium hydrochloride after each purification cycle. Antibody samples were dialyzed against PBS, concentrated by centrifugal ultrafiltration, and the protein concentration was determined by the Bradford method. Anti-TG2 IgA and IgG levels in samples were assessed by a quantitative ELISA. The average recovery of specific anti-TG2 IgA from sera was 35 ± 17% and no anti-TG2 antibodies were found in the control samples.

4.7. Biochemical analysis of TG2

Transamidation assay

A transamidation assay of FN bound TG2 was used (Uibo *et al.* 2011), based on the method by Király *et al.* (2006) to estimate the effect of anti-TG2 antibodies from CD patients on the enzymatic activity of TG2 in Study III. Human recombinant TG2 was immobilized to a FN coated surface and treated with human affinity purified anti-TG2 antibodies (10 μg/ml) or CUB 7402 monoclonal anti-TG2 antibodies (5μg/ml) with known transamidation-inhibiting properties. Thereafter, the incorporation of a 5-(biotinamido)-pentylamine substrate molecule was measured using alkaline phosphatase (AP)-conjugated streptavidin for detection (Pierce Biotechnology, Rockford, USA), and the results were expressed as percentages of OD values as compared to the control sample without antibodies.

Heparin/HS binding assay

In order to evaluate TG2 binding to heparin/HS and the effect of anti-TG2 antibodies on this interaction, a method of protein binding to non-covalently immobilized heparin (Mahoney *et al.* 2004) was used in Study III. Heparin, heparan sulphate, or hyaluronic acid (Sigma-Aldrich) were used to coat Heparin Binding Plates (BD Biosciences, Bedford, USA) followed by incubation with TG2 at 2.5 μg/ml. For the binding inhibition studies, TG2 was preincubated either with heparin (0.25–64 μg/ml), human sera (dilution 1:100) or affinity purified anti-TG2 antibodies (20 μg/ml) before applying it to the wells. The binding of TG2 was detected with mouse monoclonal anti-TG2 antibodies (CUB7402) and subsequently with AP-conjugated rabbit anti-mouse Ig antibodies (Dako, Glostrup, Denmark). The substrate colour absorbance was read at 405/492 nm and the binding of TG2 in inhibition experiments was expressed as percentages of the results obtained without antibodies.

Surface plasmon resonance

In order to characterize the affinity of TG2 and its putative heparin-binding peptides to heparin, surface plasmon resonance (SPR) analysis was performed with a Biacore 3000 instrument (Biacore AB, Uppsala, Sweden), enabling real-time monitoring of binding kinetics. Heparin was biotinylated via a reducing terminus (Osmond *et al.* 2002), and immobilized onto a streptavidin coated CM5 sensor chip. Binding of hrTG2 (5–100 nM) and synthetic peptides (5–67.5 μM) were measured at flow rates 10 μl/min and 20 μl/min. In binding competition experiments, TG2 was incubated with soluble heparin or hyaluronic acid for 15 min before injecting the mixture into heparinized surface. Surface regeneration to baseline was achieved with 1 M NaCl. A control sensorgram, obtained from a flow cell coated only with streptavidin, was always subtracted from the binding sensorgram, and the data was analyzed using

BIAevaluation software 3.1 (Biacore AB). The binding rate constants (k_a, k_d) and the dissociation equilibrium constant (K_d) were calculated using a Langmuir 1:1 binding model and a global fitting algorithm for concentration series.

4.8. Cell attachment studies

Cell lines

In Studies III and IV, the Caco-2 human colon adenocarcinoma cell line (American Type Culture Collection, Rockville, USA) was used in cell attachment experiments. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum and indicated supplements (PAA Laboratories GmbH, Pasching, Austria). Cells were maintained at 37°C in a 5% CO_2 environment and passaged or used in the experiment when reaching 80–90% confluence.

Cell attachment assay

Cell attachment was evaluated according to the method described by Balklava *et al.* (2002). Ninety six-well cell culture plates (BD Biosciences, Bedford, USA) coated with human FN or FN and TG2 (FN-TG2), were treated with dilutions of human sera (1:50) or affinity purified antibodies (20 μg/ml) for one hour or left untreated. Then trypsin-ethylenediaminetetraacetic acid (EDTA) detached Caco-2 cells were incubated for 15 min at room temperature with the adhesion inhibiting (RGD), adhesion control (DGR), or TG2 peptides P1–P3 (20 μg/ml, 100 μg/ml), and seeded on FN or FN-TG2 coated wells. Cells were allowed to attach for 90 min at 37°C, and after a gentle wash, were fixed and stained with crystal violet. After this, the stain was solubilised and the absorbance was read at 540 nm by a spectrophotometer. The results were expressed as the percentage normalized to the cell attachment in the presence of the DGR control peptide at 20 μg/ml (100%).

Fluorescence staining of cells

Fluorescence staining of cells was performed in Study III to visualize the Caco-2 cell attachment on the FN and FN-TG2 surface in various conditions. FN and FN-TG2 coated 8-well chamber glass slides (Nalge Nunc International, Rochester, USA) were treated with human serum dilutions, and the Caco-2 cells were allowed to attach for 2 hours, as described in the previous paragraph. Attached cells were fixed with 3.7% paraformaldehyde, permealized with 0.2% Triton X-100, and then the actin filaments were made visible with Alexa Fluor 594 phalloidin (Invitrogen Molecular Probes, Eugene, USA). Slides were examined under an Olympus IX70 fluorescence microscope (Olympus Corporation, Tokyo, Japan), and images were obtained with an integrated digital camera.

4.9. Statistics

Statistical analyses were performed using MedCalc software (Mariakerke, Belgium). The following parametric and nonparametric statistical tests were used for data analysis: one way analysis of variance (ANOVA) for comparison of the groups with an additional Student-Newman-Keuls test for pairwise comparisons (Study III); repeated measures ANOVA for comparing peptides' immunoreactivity; Student's t-test for independent samples for comparing other datasets (Study IV); the Mann-Whitney U-test for comparison of the antibody values between the groups (Study I); Fisher's exact test (Study I) and McNemar's paired proportion test (Study II) for comparing antibody prevalences; Pearson's test (Studies III, IV) and Spearman's rank test (Studies I, II) for correlation analysis; regression analysis and the Kruskal-Wallis test for associations of assay values with age and degree of mucosal lesion, respectively (Study II). The areas under the receiver operating characteristic (ROC) curves of two assays in Study II were compared according to the method of Hanley and McNeil (1983). $p < 0.05$ was considered significant in all analyses. Average value ± standard deviations (SD) of parameters are presented if not indicated otherwise.

5. RESULTS

5.1. Anti-desmin IgA antibodies (Study I)

Identification and prevalence of anti-desmin IgA in CD

The starting point of this study was the observation of a SMA type IgA antibody staining pattern in some of the CD patients when tested for EmA-IgA on tissue sections (Uibo *et al.* 1995; Volta *et al.* 1995; Amara and Husebekk 1998). SMA-IgA detection by IIF might be underestimated in CD because of strong EmA-IgA staining, therefore, the western blot method was chosen to detect IgA antibodies against cytoskeletal antigens, the main protein targets of SMA.

Serum IgA of many CD patients recognized a 57 kDa antigen in a crude cytoskeletal extract of HUC. Enrichment of the 57 kDa protein was obtained after further extraction steps with other dominant proteins at 45 kDa, 59 kDa, and over 200 kDa (Figure 3). The 57 kDa protein band was excised from the gel and, after protease (trypsin or endoproteinase LysC) treatment, subjected to identification by peptide mass-mapping using MALDI-TOF mass spectrometry and by amino acid sequencing of selected peptides. Both methods clearly identified the 57 kDa antigen as the muscle specific intermediate filament protein, desmin. The result was also confirmed by rabbit anti-desmin polyclonal antibodies reacting with the 57 kDa antigen on western blots. The proteins of 45 kDa and 59 kDa were stained by mouse monoclonal anti-actin and anti-vimentin antibodies, respectively. Mouse monoclonal anti-TG2 antibodies also recognized a narrow band at 85 kDa (Figure 3).

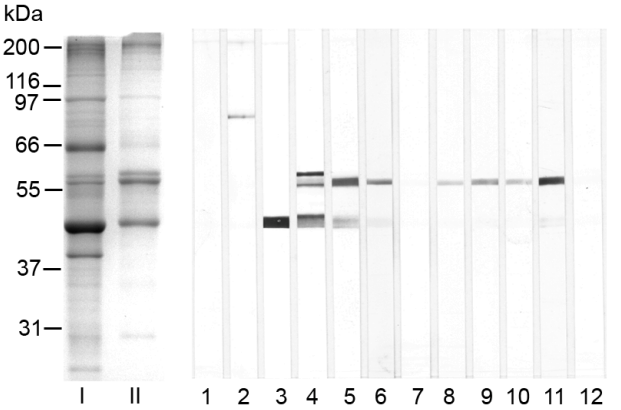

Figure 3. SDS-PAGE of cytoskeletal extracts from HUC and western blot with control antibodies and human serum IgA. Crude cytoskeletal extract (lane I) and desmin-enriched extract (lane II), separated on 10% polyacrylamide gel and stained with Coomassie Brilliant Blue G-250. Western blot of desmin-enriched extract with: mouse monoclonal antibodies to tubulin (lane 1), to TG2 (lane 2), to actin (lane 3), to vimentin (lane 4), rabbit polyclonal antibodies to desmin (lane 5), anti-desmin positive control serum (lane 6), negative control serum (7), and sera from patients with CD (8–12).

Table 3. Prevalence of IgA autoantibodies in sera of CD patients and controls.

Group	*n =*	*EmA-IgA*	*gpTG2-IgA*	*uSMA-IgA*	*Desmin-IgA (WB)*	*Desmin-IgA (ELISA)*
CD	42	30 71.4%	30 71.4%	7 16.7%	22 52.4%	21 50.0%
CD-GFD	26	9 34.6%*	12 46.2%*	2 7.7%	4 15.4%*	5 19.2%*
Controls[1]	68	1 1.5%*	2 2.9%*	9 13.2%	11 16.2%*	11 16.2%*
Healthy	48	0 *	2 4.2%*	4 8.3%	1 2.1%*	1 2.1%*
All controls	116	1 0.9%*	4 3.4%*	13 11.2%	12 10.3%*	12 10.3%*

[1] Including 18 biopsy controls and 50 patients with other diseases
* $p < 0.05$ compared with untreated CD (Fisher's exact test)
CD, celiac disease; WB, western blot;

Positive IgA reactions against human desmin on blots were detected in 22 of the 42 (52.4%) patients with CD and in 12 of the 116 (10.3%) control subjects (Figure 3; Table 3). The protein bands corresponding to actin and vimentin were only recognized by the IgA of four CD patients in each case. Two sera from CD patients reacted with all three cytoskeletal proteins but no sera recognized the 85 kDa band corresponding to TG2.

When the same sera were tested for anti-desmin IgA by an ELISA, using purified chicken desmin as the antigen, the antibody results correlated significantly with those obtained in the WB assay in patients with untreated CD ($r = 0.65$, $p < 0.001$). A similar proportion of CD sera was positive in anti-desmin ELISA (50.0%), although many sera were positive in one assay only. The discrepancy in the results were mainly due to the different immunoreactivity of human and chicken desmin (sharing 84% amino acid sequence identity), but were also due to the differences in the methods used. The uSMA-IgA was detected less frequently in the CD sera (16.7%) than anti-desmin IgA antibodies, but the frequency in the control sera (11.2%) was similar to that of anti-desmin IgA (10.3%). However, the majority of the uSMA-IgA positive sera (63.6%) were positive for at least one of the anti-desmin IgA tests.

Comparison of anti-desmin and anti-TG2 IgA

In order to estimate the diagnostic value of anti-desmin IgA for CD, anti-desmin antibody results were compared with most accurate antibody markers for CD, EmA-IgA and anti-TG2 IgA. Both assays were positive in 71.4% of patients with untreated CD, representing diagnostic sensitivity. Although higher than the

sensitivity of anti-desmin IgA, this number was considerably lower than the sensitivity of EmA-IgA and anti-TG2 IgA for CD reported previously (Dieterich *et al.* 1998; Sulkanen *et al.* 1998b). We explained the low anti-TG2 seropositivity with the high proportion of very young patients in our CD group (60% of CD patients were under 2 years of age). Eight of the 10 EmA-IgA and anti-TG2 negative CD patients where under the age of 2, and 6 of those were positive for AGA, as detected by ELISA (Uibo and Maaroos 1993).

Anti-desmin IgA and anti-TG2 IgA levels, determined by ELISA, were significantly correlated among CD patients' sera ($r = 0.53$, $p < 0.001$). Twenty six of the 30 CD patients with elevated anti-TG2 IgA had positive results in at least one anti-desmin IgA antibody test. Conversely, anti-TG2 IgA was detected in 19 of 21 sera positive in ELISA for anti-desmin IgA and in all 22 sera positive in WB. Moreover, the levels of anti-desmin IgA and anti-TG2 IgA changed in parallel in CD patients after a period of a GFD (Table 2 in Study I).

In order to examine the possible serum IgA cross-reactivity between desmin and TG2, serum preadsorption experiments, as well as affinity purification of anti-desmin antibodies from CD patients' sera, were performed. The results confirmed the specificity of anti-desmin IgA antibodies, as only soluble desmin, but not TG2, reduced or abolished IgA binding to the human desmin on the WB (Table 3 in Study I). More directly, affinity-purified anti-desmin IgA antibodies from CD patients' sera recognized both human and chicken desmin on blots (Figure 4 in Study I), but had no reactivity against TG2 in the ELISA. Moreover, affinity purified anti-desmin IgA stained smooth muscle cells on HUC tissue in a similar pattern to uSMA-IgA, while the EmA-IgA reactivity detected in the original serum could not be found (Figure 4).

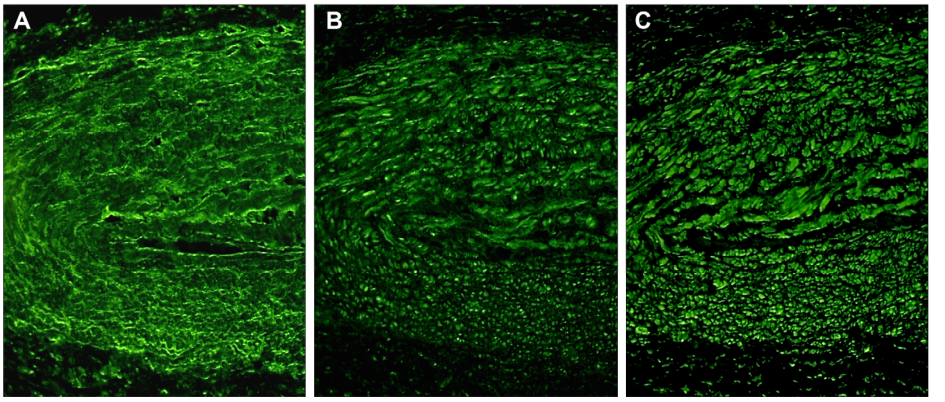

Figure 4. Serum antibody staining on HUC tissue sections by IIF microscopy (original magnification 150×). (A) EmA-IgA binding pattern (surrounding smooth muscle cells of umbilical blood vessel) by CD patient's sera. (B) Staining of smooth muscle cells by affinity purified anti-desmin IgA antibodies from EmA-IgA positive CD patient's sera. (C) uSMA-IgA staining pattern by sera from a CD patient.

5.2. Comparison of anti-TG2 ELISAs (Study II)

The new generation of antibody assays for anti-TG2 IgA detection, using human TG2 as the antigen, have reached a diagnostic accuracy for CD screening as high as 93% sensitivity and 98% specificity (Lewis and Scott 2006). However, in a number of studies lower diagnostic specificities of anti-TG2 IgA assays compared to the EmA-IgA test have been reported (Villalta *et al.* 2005; Bizzaro *et al.* 2006; Carroccio *et al.* 2007; Sárdy *et al.* 2007). Keeping this in mind, we modified an ELISA for anti-TG2 detection by binding hrTG2 to its high affinity ligand FN. The purities of the protein components used in the assay are presented in Figure 5, showing that over 90% purity of hrTG2 was achieved after two-step chromatographic purification.

The diagnostic performance of the FN-TG2 ELISA was compared with the traditional ELISA for the detection of anti-TG2 antibodies in the sera of CD patients and biopsy controls. ROC analysis was used to compare the tests estimating the diagnostic sensitivity and specificity at every possible cut-off level, which can be expressed as the area under the curve (AUC) value (Zweig and Campbell 1993). ROC curves for each Ig class assay are represented in Figure 6, showing that the detection of IgA class anti-TG2 antibodies was most effective for distinguishing between the CD and control subjects. The area under the ROC curve was high for both ELISAs detecting IgA antibodies (0.977 for FN-TG2-IgA and 0.970 for TG2-IgA). Compared to the IgA antibodies, AUC values were lower for assays detecting anti-TG2 IgG or IgM antibodies, but here the FN-TG2 ELISA (AUC = 0.930 for IgG, AUC = 0.850 for IgM) performed significantly better than the corresponding TG2 ELISA (AUC = 0.809 for IgG, $p < 0.001$; AUC = 0.811 for IgM, $p = 0.019$).

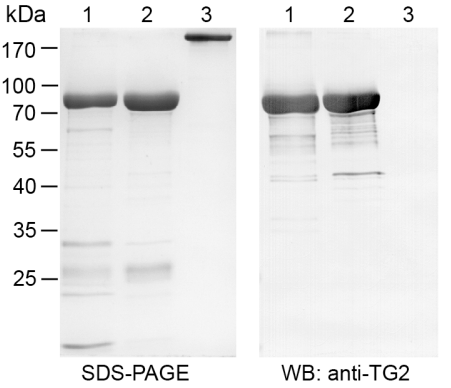

Figure 5. SDS-PAGE and western blot analysis of human purified recombinant TG2. Protein samples were separated on 10% polyacrylamide gels and stained either with Coomassie Brilliant Blue G-250 or western blotted with monoclonal anti-TG2 antibody: hrTG2 purified by Ni^{2+}-chelate chromatography (lane 1), hrTG2 after additional ion-exchange chromatography (lane 2), purified FN from human plasma (lane 3).

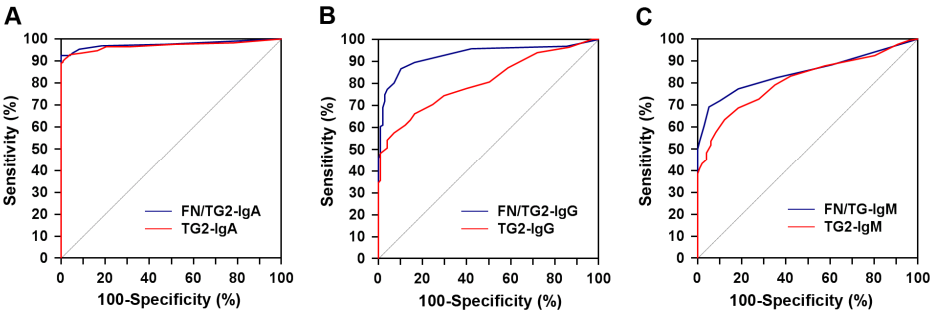

Figure 6. ROC curves for FN-TG2 and TG2 ELISAs for anti-TG2 IgA, IgG and IgM antibodies. Areas under the curve, representing overall diagnostic accuracy, were: (A) 0.977 for FN-TG-IgA and 0.970 or TG2-IgA ($p = 0.356$); (B) 0.930 for FN-TG2-IgG and 0.809 for TG2-IgG ($p < 0.001$); (C) 0.850 for FN-TG2-IgM and 0.811 for TG2-IgM ($p = 0.019$).

The anti-TG2 antibodies are the preferred serologic marker for screening CD and instrumental for intestinal biopsy (Lewis and Scott 2006), so a high diagnostic specificity (99–100%) was required for selecting the cut-off values in our study (Table 4). At these settings, the diagnostic sensitivity of all three FN-TG2 ELISAs (92.5% for IgA, 60.7% for IgG, 50.3% for IgM) was significantly higher than that of the corresponding TG2 ELISAs (89.0% for IgA, 48.6% for IgG, 38.7% for IgM).

Table 4. Median antibody levels and diagnostic accuracy of FN-TG2 ELISAs and TG2 ELISAs for anti-TG2 IgA, IgG, and IgM antibodies among CD patients and biopsy controls.

ELISA	*Cut-off AU*	*CD (n = 173)*			*Controls (n = 97)*		
		Median AU (range)	*Pos n=*	*Sensitivity %*	*Median AU (range)*	*Pos n=*	*Specificity %*
FN-TG2-IgA	≥ 8	72 (0–147)	160	92.5*	0 (0–7)	0	100.0
TG2-IgA	≥ 12	73 (0–167)	154	89.0	2 (0–11)	0	100.0
FN-TG2-IgG	≥ 12	17 (0–131)	105	60.7*	1 (0–19)	1	99.0
TG2-IgG	≥ 16	15 (2–113)	84	48.6	6 (1–24)	1	99.0
FN-TG2-IgM	≥ 8	8 (0–208)	87	50.3*	1 (0–7)	0	100.0
TG2-IgM	≥ 18	13 (1–179)	67	38.7	5 (1–17)	0	100.0

*$p<0.05$ McNemar paired proportion test, compared to sensitivity of corresponding TG2 ELISA.
AU, arbitrary units; CD, celiac disease.

The better differentiation between the CD and control patients using the FN-TG2 ELISAs was mainly due to the lower reactivity of the control sera in the FN-TG2 assay compared to the TG2 ELISAs, while in the CD patients the median antibody levels remained similar in both assays. This enabled lower cut-off values in the FN-TG2 ELISAs, at the same assay specificity, to detect new positive CD cases with moderate anti-TG2 antibody levels and thus increase the diagnostic sensitivity of FN-TG2 assays for CD.

The distribution of positive results among CD patients in all three FN-TG2 assays (IgA, IgG, IgM) revealed that 117 (67.6%) of the patients were positive in at least two antibody assays, 43 (24.9%) were positive in only the FN-TG2-IgA ELISA, and 5 (2.9%) patients were positive in the FN-TG2-IgG ELISA. The remaining 8 (4.6%) CD patients were negative in all of the the FN-TG2 tests; four of them were younger than two years old. All CD patients positive in the FN-TG2-IgM ELISA also had positive values in the FN-TG2-IgA ELISA. The combined use of the FN-TG2 ELISAs for IgA and IgG antibodies resulted in 95.4% sensitivity and 99.0% specificity for childhood CD. Interestingly, the levels of anti-TG2 IgG were inversely associated with the age of the CD patients ($p = 0.004$, linear regression analysis), while no such association was observed for the anti-TG2 IgA and IgM antibodies. Unsuprisingly, the antibody levels of all the anti-TG2 antibody classes were positively associated with the degree of mucosal damage in the small intestine of CD patients ($p < 0.01$, Kruskal-Wallis test).

5.3. Anti-TG2 autoantibodies: inhibition of TG2-heparin/HS interaction and the adhesion function of TG2 (Study III)

Other than being an accurate serologic marker for CD, the role of anti-TG2 antibodies in the disease pathogenesis has been less clearly defined, although various and even contradictory effects of anti-TG2 antibodies on the biochemical activity and cellular functions of TG2 have been described (Caputo *et al.* 2009; Lindfors *et al.* 2009). Relying on the recent findings on the role of TG2 in cell adhesion involving TG2 interactions with HS proteoglycans, we aimed to study whether anti-TG2 antibodies from CD patients' sera could have an impact on the TG2-related cell adhesion function, by interfering with TG2 binding to heparin/HS residues. The effects of sera and affinity purified anti-TG2 antibodies on TG2 binding to immobilized heparin/HS and on Caco-2 intestinal cell attachment to the FN-TG2 matrix were studied.

Anti-TG2 autoantibodies inhibit TG2 binding to heparin/heparan sulphate

The specificity of TG2 binding to immobilized heparin on a heparin binding plate was confirmed by applying different concentrations of TG2 to immobilized

heparin or to nonspecific glycosaminoglycan hyaluronic acid, and by competing TG2 binding with soluble heparin. Control experiments also demonstrated that the binding of monoclonal anti-TG2 (CUB 7402), used as the detection antibody for TG2, was not affected by anti-TG2 autoantibodies from CD patients (Figure 2 in Study III).

When testing the serum effect on TG2 binding to immobilized heparin, sera from CD patients were divided into two groups based on anti-TG2 IgA levels: the CD1 group (anti-TG2 IgA ≥ 100 AU), and the CD2 group (anti-TG2 IgA < 100 AU). The CD1 sera decreased TG2 binding to heparin (23 ± 13% of TG2 binding without sera) compared to the CD2 and the control sera (46 ± 17% and 51 ± 18%, respectively, $p < 0.001$; Figure 7A). Anti-TG2 IgA levels in sera of CD patients were negatively correlated with TG2 binding to heparin

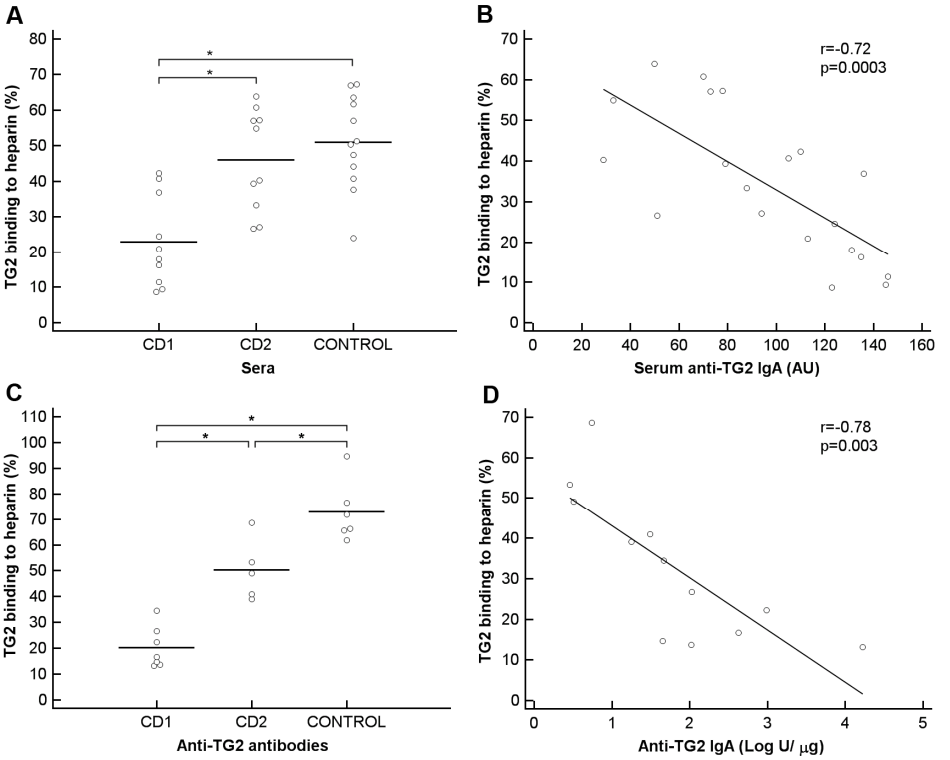

Figure 7. The effect of human sera and affinity purified anti-TG2 antibodies on TG2 binding to immobilized heparin. (A) Sera from CD patients with high anti-TG2 IgA levels (CD1) decreased TG2 binding to heparin compared to those with low antibody levels (CD2) and controls. (B) Negative correlation between anti-TG2 IgA values and inhibition of TG2 binding to heparin among CD sera. (C) Affinity purified anti-TG2 antibodies from CD patients significantly inhibited TG2 binding to heparin as compared with controls, and (D) inhibition was inversely correlated with anti-TG2 IgA levels. * $p < 0.05$

(r = –0.72, p = 0.0003; Figure 7B). Similar results were obtained using HS instead of heparin in TG2 binding inhibition experiments. In line with the effect produced by the CD1 sera, affinity purified anti-TG2 antibodies inhibited TG2 binding to heparin when comparing TG2 binding values after treatment with the CD1 samples (20 ± 8%), the CD2 samples (50 ± 18%), and the controls (73 ± 25%, $p < 0.001$; Figure 7C). There was also a high correlation between anti-TG2 IgA levels and the ability of samples to inhibit TG2 binding to heparin (r = –0.78, p = 0.003; Figure 7D). The concentration-dependent effect of human affinity purified anti-TG2 antibodies, but not rabbit anti-TG2 antibodies, on TG2 interactions with heparin and HS, was revealed when studying selected samples at different concentrations (Figure 4 in Study III).

Anti-TG2 autoantibodies reduce TG2-dependent Caco-2 cell adhesion

The finding that cell attachment to a FN coated surface can be reduced by RGD-containing peptides, the potent inhibitors of integrin-mediated adhesion (Pierschbacher and Ruoslahti 1984), was also true in our experiments using the Caco-2 intestinal epithelial cell line. Similar to previous studies but using Caco-2 cells, we found that the RGD peptide inhibited cell adhesion to the FN-TG2 matrix less than to FN. The TG2-related cell adhesion pathway has been shown to complement the RGD-dependent adhesion process (Verderio *et al.* 2003; Telci *et al.* 2008), so we performed our antibody inhibition experiments in the presence of the RGD peptide and normalized the results to the level obtained with the DGR control peptide with no effect on cell adhesion. Initially we found that treatment of the FN-TG2 coated surface with mouse monoclonal or rabbit polyclonal anti-TG2 antibodies had no significant effect on cell attachment compared with the normal IgG controls, but when the FN-TG2 matrix was treated with CD patients' sera, it resulted in lower Caco-2 cell attachment compared to the control sera (66 ± 18% versus 97 ± 14%, $p < 0.05$), and a similar difference persisted in the presence of the DGR control peptide (Figure 8A, 8B). The cell adhesion reducing effect was also obtained by using affinity purified antibodies: the incubation of the FN-TG2 surface with the CD1 antibody samples decreased the Caco-2 attachment compared with the control samples (65 ± 14% versus 86 ± 22%, respectively, $p < 0.05$; Figure 8C). When affinity purified anti-TG2 antibodies were directly added to the cell suspension, no difference in the attachment assay was found between samples from CD patients and controls (49 ± 6% versus 53 ± 14%, respectively; p = 0.31).

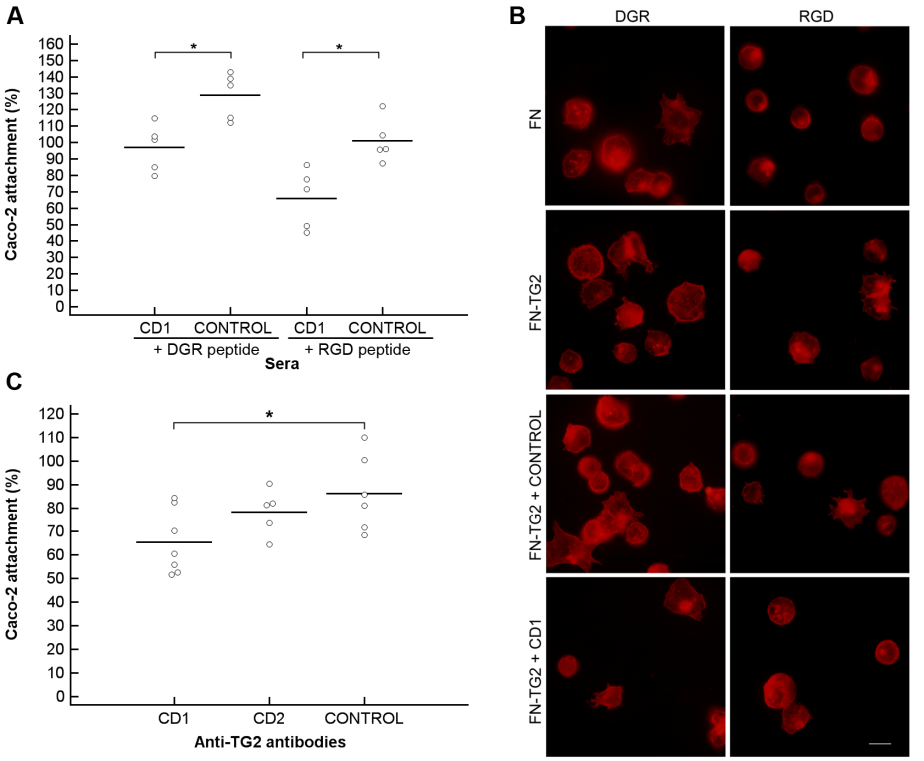

Figure 8. Caco-2 cell attachment to the FN-TG2 coated surface treated with sera or anti-TG2 antibodies from CD patients. Treatment of FN-TG2 wells with CD patients' sera containing high anti-TG2 (CD1) reduced Caco-2 cell attachment compared with the control sera both in the presence of DGR or RGD peptide, represented as (A) results of the attachment assay, (B) fluorescence images with stained actin filaments. (C) Attachment of Caco-2 cells to FN-TG2 was lower when treated with purified anti-TG2 from CD1 sera as compared with the control samples. Bar, 20 μm * $p < 0.05$

Anti-TG2 autoantibodies have no effect on transamidase activity

In order to investigate whether the cell attachment reducing effect of celiac patients' anti-TG2 antibodies could be related to the change in the transamidation activity of TG2 bound to FN, the impact of affinity purified antibodies on the enzymatic activity of TG2 was studied using *in vitro* assay. Used as a positive control, mouse monoclonal CUB 7402 antibody inhibited the TG2 activity down to the level of 13 ± 2%. In the contrary, the affinity purified anti-TG2 antibodies showed no effect on the transamidation activity of TG2 (activity 93 ± 5% for CD1 and 94 ± 5% for controls), with no correlation between TG2 transamidation and anti-TG2 IgA levels in the CD samples ($r = 0.04$, $p = 0.87$).

5.4. Heparin-binding peptides of TG2 (Study IV)

Binding of TG2 and synthetic peptides to heparin by SPR

SPR analysis was used to characterize the affinity of TG2 and its possible heparin-binding peptides to heparin, enabling the real-time monitoring of binding kinetics. High-affinity binding of TG2 to biotinylated heparin was detected by injecting different concentrations of TG2 over a sensor flow cell (Figure 9A). Using the Langmuir 1:1 binding model to fit sensorgram data, association and dissociation rate constants were obtained, resulting in an average dissociation constant of K_d = 34.7 nM for TG2. Of the four candidate heparin-binding TG2 peptides, P1 (202–215) and P2 (261–274) bound to heparin while the other two and a control peptide showed no detectable binding (Figure 9B). Kinetic analysis of P1 and P2 binding sensorgrams in different peptide concentrations revealed an average K_d = 12.7 μM for P1and K_d = 3.7 μM

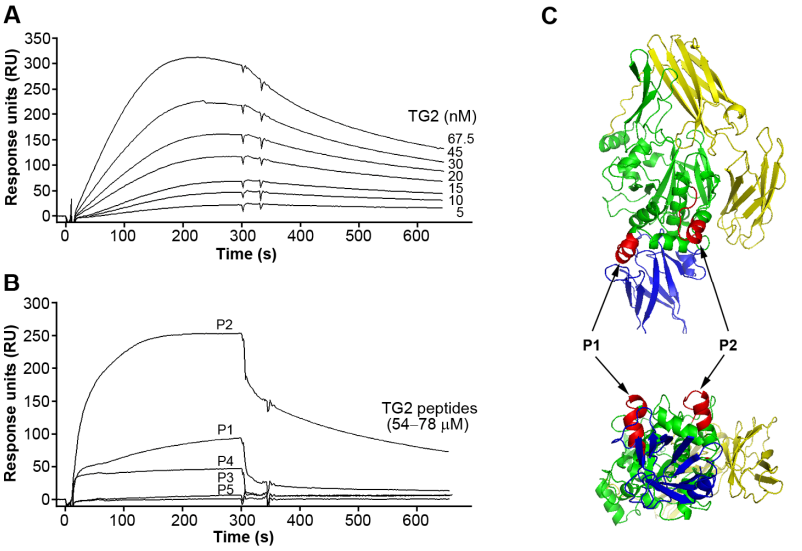

Figure 9. Binding of human recombinant TG2 and its peptides to immobilized heparin by SPR analysis. (A) Heparin binding sensorgrams of TG2 in the concentration range of 5–67.5 nM, at a flow rate 20 μl/min. (B) Binding of TG2 peptides P1–P5 to heparin at concentration 100 μg/ml (54–78 nM) at flow rate 10 μl/min. (C) Sequences corresponding to the heparin-binding peptides P1 (amino acids 202–215) and P2 (261–274) in the structural model of TG2 (PDB code: 1KV3; PyMol software). Sequences corresponding to peptides are in red and marked with arrows. The N-terminal domain is shown in blue, the catalytic domain in green and the C-terminal β-barrel domains in yellow. Side view (upper panel) and view from direction of N-terminal domain (lower panel).

for P2. The binding of TG2 and its peptides to immobilized heparin was completely abolished by an excess of soluble heparin, but not by hyaluronic acid. The sequences corresponding to the heparin-binding TG2 peptides P1 and P2 were localized within the structural model of TG2 as part of similarly oriented, alpha-helical structures, residing close to each other on the surface of the catalytic domain (Figure 9C).

Immunoreactivity of heparin-binding TG2 peptides with CD patients' IgA

By testing CD patients' sera IgA reactivity with TG2 peptides in ELISA, the peptides P1 and P2 were most immunoreactive compared to the other three TG2 peptides (Figure 3 in Study IV; $p < 0.001$). A similar trend was observed for the control sera, but the CD sera had higher reactivity with all TG2 peptides, respectively ($p < 0.01$). There was no difference between CD and control patient serum IgA reactivity towards the enterovirus VP1 control peptide ($p = 0.57$). Serum IgA binding levels with different TG2 peptides were significantly correlated with each other ($r = 0.66–0.93$; $p < 0.001$) while no correlation existed between serum reactivity with any of the TG2 peptides and enterovirus VP1 peptide ($r = 0.02–0.07$; $p > 0.7$).

Effect of TG2 peptides on Caco-2 cell attachment

The effects of the TG2 peptides P1–P3, the enterovirus VP1 peptide, as well as the adhesion inhibiting RGD and non-inhibiting DGR control peptides, on Caco-2 cell attachment to FN and FN-TG2 coated surfaces were studied (Figure 4 in Study IV). The RGD peptide inhibited cell attachment onto FN and FN-TG2 surfaces compared to that of the DGR peptide, whereas inhibition was less effective on the FN-TG2 coated surface. In order to reduce the role of integrin-mediated cell adhesion, the RGD peptide at 20 μg/ml was included when studying the effect of the TG2 peptides on cell attachment. In these conditions, the TG2 peptide P2 reduced Caco-2 attachment to both the FN and FN-TG2 coated surfaces compared with the enterovirus VP1 peptide or with only the RGD peptide. Surprisingly, the RGD-independent cell adhesion reducing effect of the TG2 peptide P2 was even more evident on the FN coated surface than on the FN-TG2 coated surface (Figure 4 in Study IV).

6. DISCUSSION

6.1. The role of anti-desmin IgA antibodies in CD

In addition to the specific markers for CD – EmA-IgA and anti-TG2 IgA, a number of CD-associated autoantibodies have been described, although they are less sensitive and specific for the disease (Shaoul and Lerner 2007; Alaedini and Green 2008). The reported higher prevalence of SMA-IgA antibodies among CD patients led us to identify the main cytoskeletal targets of IgA antibodies in CD. It is known that cytoskeletal proteins, which constitute microfilaments and intermediate filaments, are the main antigens of SMA (Dighiero *et al.* 1990; Toh 1991). We identified desmin, by proteomic methods, as the cytoskeletal antigen recognised by serum IgA in about half of the CD patients. Similar results were obtained by using purified desmin in ELISA experiments, and an even higher prevalence of anti-desmin IgA (86.7%) was found in anti-TG2 IgA positive sera.

One year before publishing our results, Clemente and colleagues reported on antibodies against filamentous actin, detected on HEp-2 cells, in the majority of CD patients (Clemente *et al.* 2000). Even though anti-actin reactivity was rare among our CD patients when tested by a western blot of HUC cytoskeletal proteins, we could not exclude the higher prevalence of anti-actin antibodies when tested by a different method. There are inherent differences between the methods such as IIF, WB and ELISA for autoantibody detection, concerning the complexity and the conformation of the antigen. Polymerized and filamentous, or unpolymerized and monomeric cytoskeletal proteins differ in their antigenic properties, which can explain, for example, the discrepancies between the IIF and ELISA results for anti-actin antibodies (Granito *et al.* 2004; Mackay *et al.* 2008) and our finding of the higher anti-desmin IgA prevalence than uSMA-IgA in CD sera. Masking of the weaker uSMA-IgA staining by the prominent EmA-IgA reactivity when testing sera from CD patients, could also account for the lower uSMA-IgA prevalence. This is also supported by our results demonstrating that the affinity purified anti-desmin IgA produced a staining pattern resembling uSMA-IgA, whereas in the original serum only EmA-IgA was detectable.

In the small intestinal mucosa of CD patients, desmin is coexpressed with α-smooth muscle actin in the lamina muscularis mucosa and in the smooth muscle strands traversing the lamina propria, and a similar distribution is observed in normal mucosa (Korhonen *et al.* 2000). Our finding, that anti-desmin IgA antibodies were correlated with an anti-TG2 response, and that both antibodies decreased in response to a GFD in patients with CD, suggests that anti-desmin IgA is related to the disease process in the intestinal mucosa. The majority of CD patients in this study were graded as having subtotal villous atrophy of the small intestinal mucosa (Marsh IIIb), so we could not properly evaluate the association of anti-desmin IgA with the degree of tissue

destruction. While desmin is an intracellular protein, not accessible to antibodies in viable cells, we suggest that anti-desmin antibodies can be induced in response to tissue injury occurring in the inflammatory intestinal mucosa after a gliadin exposure. Indeed, molecular signatures on the dying cells have been demonstrated to become a target for autoantibodies (Gensler et al. 2001; Hansen et al. 2001), and rearranged cytoskeleton is also partially exposed on the surface of the apoptotic cells (Moisan and Girard 2006; Ndozangue-Touriguine *et al.* 2008). Bearing this in mind, the high reactivity of CD patients' IgA antibodies with monomeric desmin in our study might be explained by antibody development against normally "hidden" epitopes when filamentous structures are disrupted. Interestingly, it has been shown that TG2 codistributes with stress-fibres in human vascular smooth muscle cells (Chowdhury *et al.* 1997), and with intermediate filaments in mouse dermal fibroblasts (Trejo-Skalli *et al.* 1995). Furthermore, many cytoskeletal proteins, including desmin and actin, could act as transamidation substrates for TG2 as shown *in vitro* (Gard and Lazarides 1979; Huang *et al.* 1992), and *in vivo* in cells undergoing apoptosis (Nemes *et al.* 1997). Therefore, it is tempting to hypothesize that TG2 could modify the antigenic properties of desmin and other cytoskeletal proteins in the inflamed small intestinal mucosa of CD patients and thus contribute to the production autoantibodies. For example, in a different autoimmune disease, rheumatoid arthritis, the citrullination of arginine residues of another intermediate protein, vimentin, induces a specific autoantibody response (Vossenaar *et al.* 2004).

The role of IgA antibodies against desmin and other cytoskeletal proteins in the pathogenesis of CD has not been experimentally studied. Still, considering the increase in IgA anti-cytoskeletal antibodies in various gut inflammatory conditions (Mayet *et al.* 1990; Fabbro *et al.* 2008), anti-cytoskeletal antibody response is obviously not a finding specific for CD. Autoantibodies against cytoskeletal components, especially against intermediate filaments, have also been found among natural antibodies – the germline-encoded, preimmune and polyreactive type of antibodies (Lacroix-Desmazes *et al.* 1998; Thorpe *et al.* 1998). The involvement of natural antibodies has been suggested in homeostatic functions like first line defence against pathogens, elimination of cell debris and immunoregulation (Lacroix-Desmazes *et al.* 1998; Lutz *et al.* 2009). Although differently regulated than the antigen-driven T-cell dependent antibody response, the transition of natural antibody specificities towards pathogenic autoantibodies can take place (Menge *et al.* 2002).

The exact nature of anti-desmin IgA antibodies appearing in CD has not been explored in our study, but the specificity and gluten-dependence of these antibodies allows them to be considered as part of the complex immune response taking place in the small intestinal mucosa of CD patients. Based on our results, anti-desmin IgA detection is of no additional value for CD screening but its potential role as a supplemental marker for the integrity of the small intestinal mucosa needs further study.

6.2. Improved performance of a modified anti-TG2 ELISA for CD

TG2 was identified as the main autoantigen in CD in 1997 (Dieterich *et al.* 1997), and since then serum anti-TG2 antibodies have been utilized as useful diagnostic markers for CD. The second generation of anti-TG2 IgA immunoassays, using human antigen, have a diagnostic accuracy for CD comparable with the conventional EmA-IgA test (Sárdy *et al.* 1999; Sblattero *et al.* 2000; Lewis and Scott 2006). Anti-TG2 IgA antibodies have been shown to be highly sensitive and specific markers for CD while IgG and IgM class anti-TG2 antibodies are less common in CD (Sblattero *et al.* 2000; Agardh *et al.* 2003; Feighery *et al.* 2003). However, the lower specificity of anti-TG2 IgA immunoassays compared with EmA-IgA has been observed, suggesting the need for further assay optimization. The major limitations of anti-TG2 antibody assays relate to the purity and proper conformation of the TG2 antigen, resulting in a fraction of false-positive or false-negative results (Carroccio *et al.* 2002; Sárdy *et al.* 2007). In order to overcome these analytical problems, we developed an ELISA using FN as a linker molecule to bind TG2 for detection of anti-TG2 autoantibodies. The diagnostic accuracy of the modified ELISA for CD outperformed that achieved by traditional ELISAs for anti-TG2 of all three major immunoglobulin classes (IgA, IgG, and IgM).

Several methodological features could account for the better diagnostic performance of the FN-TG2 ELISA. First, the additional purification of TG2 by binding to its high affinity ligand FN could eliminate minor contaminant proteins with the ability to react with non-specific antibodies. Indeed, in our study, even after a two-step chromatographic purification of recombinant TG2 minor protein impurities could still be detected. The role of additional FN "affinity" purification of TG2 is also supported by the striking difference between the FN-TG2-IgG and TG2-IgG assay results, as IgG is the main serum antibody fraction that is most sensitive to any protein impurities. The reduction of nonspecific reactivity enabled us to lower the cut-off values for the FN-TG2 ELISAs without loss of specificity, and consequently to differentiate CD patients with "borderline" anti-TG2 antibody levels from the control group. The lower impact of the FN-TG2 ELISA on the anti-TG2 IgA assessment could be due to the high levels of anti-TG2 IgA antibodies in CD.

A second explanation for the improved performance of the FN-TG2 ELISA comes from the finding that anti-TG2 antibodies from CD patients recognized TG2 bound to FN with asimilar extent than TG2 alone. This suggests that in the FN-TG2 complex the major TG2 epitopes remain accessible for antibody binding. Even more importantly, the FN bound TG2 resembles the extracellular appearance of TG2 in tissues, both on sections used for EmA detection, and in the jejunum of CD patients, where colocalisation of TG2 with the FN network has been demonstrated (Korponay-Szabó *et al.* 2000; Korponay-Szabó *et al.* 2004). Therefore, it can be assumed that the FN-TG2 complex, mimicking the

in vivo state, and enabling the natural conformation and orientation of TG2 molecules, serves as an appropriate antigen for anti-TG2 detection.

We confirmed the high diagnostic sensitivity and specificity of anti-TG2 IgA as a marker for untreated CD in a large cohort of children with CD and control subjects with normal intestinal histology. Anti-TG2 IgG antibodies which are less sensitive for CD, have been previously detected in about half of CD patients (Sblattero *et al.* 2000; Agardh *et al.* 2003), but they have been found to be a valuable marker for detecting CD patients with IgA-deficiency (Cataldo *et al.* 2000), and in a small subgroup of anti-TG2 IgA negative patients with normal IgA levels (Picarelli *et al.* 2001). Both aspects were consistent with our results, supporting the accepted view that combining the anti-TG2 IgA and IgG assays gives maximum diagnostic accuracy for CD screening. The finding that higher levels of anti-TG2 antibodies of the IgG class were detected in younger CD patients, but not of the IgA or IgM classes, could reflect the dynamics of antibody class switching during CD immunopathogenesis. In a previous prospective study anti-gliadin IgG antibodies have shown to emerge earlier than IgA antibodies during follow-up of children with a genetic risk for CD (Simell *et al.* 2007).

Using the modified ELISA, we detected anti-TG2 IgM antibodies in half of the CD patients, which is a considerably higher prevalence than reported previously (Agardh *et al.* 2003; Feighery *et al.* 2003). The better diagnostic performance of the FN-TG2-IgM ELISA could be the main reason for the difference, as the positivity rate obtained with the traditional ELISA in our study was similar to that in a previous report (Feighery *et al.* 2003). Our results show that the assessment of anti-TG2 IgM had no additional diagnostic value for the serological screening of childhood CD. Still, possible associations with disease duration or other clinical parameters cannot be ruled out for anti-TG2 IgM and this should be addressed in further studies. An established ELISA, based on recombinant TG2 bound to FN, is suggested for the assessment of anti-TG2 IgA and IgG antibodies in childhood CD screening as the format is relatively simple, reliable, and, most importantly, has better diagnostic performance than using the same antigen without FN. The novel ELISA could be useful for the screening of disease groups with a reported higher prevalence of anti-TG2 antibodies, and where the presence of false positive results could come into question.

6.3. The functional effects of anti-TG2 autoantibodies

A number of studies have described the effects of anti-TG2 autoantibodies on the transamidase activity of TG2, and on various cellular functions *in vitro*, but despite this there is still no consensus on the role of anti-TG2 antibodies in the immunopathogenesis of CD. Also, not much is known about the mechanisms how anti-TG2 autoantibodies exert their effects on cell behaviour. Our

hypothesis-driven approach revealed that anti-TG2 antibodies from CD patients could interfere with the cell adhesion function of TG2. This was substantiated by the inhibiting effect of CD patients' autoantibodies on TG2 binding to heparin/HS, as well as on the RGD-independent attachment of Caco-2 intestinal epithelial cells onto the FN-TG2 matrix. Moreover, heparin-binding peptides of TG2 displayed increased immunoreactivity with the serum IgA of CD patients.

It is most likely that the inhibitory effect of anti-TG2 autoantibodies on TG2 binding to heparin/HS can be explained by antibody blocking of the TG2 regions responsible for interactions with heparin/HS. We began to define the heparin-binding sites of TG2 by using synthetic candidate peptides in heparin binding studies, revealing two heparin-binding candidate regions, corresponding to amino acids 202–215 and 261–274 in the core domain of TG2. The sequence 261–274 contains the typical heparin-binding consensus sequence XBBXBX (B-basic, X-non-basic amino acid) and both regions are part of alpha-helical structures, residing close to each other on the surface of the molecule. The high content of positively charged amino acid residues in these structures in a generally acidic TG2 molecule could allow the binding of negatively charged HS residues. Similar to our findings, Wang and colleagues recently demonstrated that TG2 amino acids 202 – 222 form the major heparin-binding site of TG2 by using molecular modelling, site-directed mutagenesis and corresponding peptide analysis (Wang *et al.* 2012). Previous studies show no significant effect of heparin binding on the transamidase activity of TG2 (Gambetti *et al.* 2005; Scarpellini *et al.* 2009), suggesting that heparin binding does not affect substrate recognition in the catalytic site. It has also been demonstrated earlier, that antibodies could react with protein heparin-binding sites (Oppermann *et al.* 2006; Inagaki *et al.* 2011), for example, the peptides of laminin 111 with heparin and syndecan-4 binding activity were predominantly recognized by serum antibodies from patients with endometriosis (Inagaki *et al.* 2011). The recently identified main conformational epitope of CD anti-TG2 antibodies also contains arginine residue which is critical for antibody binding (Simon-Vecsei *et al.* 2012). Therefore, the surface exposition and higher content of charged amino acids could form the structural basis for the higher immunogenicity of the protein heparin-binding regions.

Syndecan-4 has been demonstrated as the candidate HSPG for binding TG2 (Scarpellini *et al.* 2009), and has been suggested as being involved in the RGD-independent adhesion process (Verderio *et al.* 2003; Telci *et al.* 2008). We have not addressed interaction between TG2 and syndecan-4 in our studies. Still, reduced cell attachment on the anti-TG2 autoantibody treated FN-TG2 matrix provides indirect evidence that the adhesion function of TG2, related to the binding of cell surface proteoglycans, could be compromised by autoantibodies. The finding that the TG2 peptide 261–274 decreased cell attachment on the surfaces of both FN and FN-TG2, could also be explained by the inhibition of interactions between cell surface HSPGs and heparin-binding sites in FN and TG2. Our results suggest that the effect of anti-TG2 autoantibodies on the

intestinal epithelial cell attachment is related to targeting extracellular TG2 bound to the FN matrix, rather than to TG2 expressed on the cell surface. The improved performance of our FN-TG2 ELISA also supports the preferred binding of autoantibodies to the FN bound TG2, and most probably, extracellular TG2 is the primary target of CD patients' autoantibodies in the small intestinal mucosa (Korponay-Szabó *et al.* 2004).

Questions may arise as to whether anti-TG2 autoantibodies could interfere with FN binding to TG2 as there are mouse monoclonal antibodies available that demonstrate this (Akimov and Belkin 2001b). Also, in a recent work serum anti-TG2 autoantibody reactivity decreased when TG2 was linked to FN (Sóñora *et al.* 2011). In opposite to this, in our ELISA studies we have not seen the difference between autoantibody binding to TG2 directly coated on plastic and when bound to FN. In another study it has been suggested that, although anti-TG2 autoantibodies can form complexes with TG2 and FN in blood circulation, they do not seem to disrupt the FN-TG2 interaction (Lorand and Graham 2003).

Anti-TG2 autoantibodies had no effect on the transamidase activity of FN bound TG2, and therefore this mechanism is unlikely to be responsible for the reduced cell attachment in our studies. Although the protein cross-linking function of TG2 plays a role in cell-ECM interactions (Zemskov *et al.* 2006), the adhesion promoting effect of TG2 has been shown to be independent of TG2 transamidase activity (Akimov *et al.* 2000; Verderio *et al.* 2003). The variable effects of anti-TG2 autoantibodies on the transamidase activity of TG2 obtained so far could be due to differences in the methods and antibody samples used for testing TG2 activity.

Previous studies show that the IgA fraction or anti-TG2 antibodies from CD patients inhibits intestinal epithelial cell differentiation (Halttunen and Mäki 1999) and endocytosis (Caputo *et al.* 2010), promotes cell proliferation (Barone *et al.* 2007a), disturbs angiogenesis (Myrsky *et al.* 2008), and increases intestinal and endothelial cell permeability (Zanoni *et al.* 2006; Myrsky *et al.* 2009). These findings are explained either by the anti-TG2 antibody effects on transamidase activity (Myrsky *et al.* 2009) or the nonenzymatic functions of TG2 (Barone *et al.* 2007a). The mechanisms by which anti-TG2 autoantibodies could cause these phenomena are of interest, as is the possible contribution of the mechanism proposed by us – antibodies interfering with TG2 binding to cell surface HSPGs and the related cell adhesion function. The involvement of anti-TG2 antibodies in modifying cellular adhesion was also revealed by the recent finding, that IgG purified from CD patients' sera impaired the invasiveness of trophoblastic cells (Di Simone *et al.* 2010).

The question remains as to whether the proposed inhibitory mechanism of CD autoantibodies on the TG2 and heparin/HS interaction and on cell adhesion could have implications for the immunopathogenesis and tissue remodelling of small intestinal lesions in CD. TG2-related cell adhesion may play a particular role in cell survival in the inflamed tissues where the RGD-dependent adhesion

is impaired (Verderio *et al.* 2003). Based on this, we suggest that binding of anti-TG2 antibodies to extracellular TG2 and hindering its interactions with cell HSPGs could lead to the loosening of adhesion contacts between epithelial cells and the basement membrane in the small intestinal mucosa of CD patients (Figure 10). Indeed, blistering of the epithelial cells in the small intestinal lesions of CD patients has been previously reported (Kainulainen *et al.* 2002).

Further studies are needed, using human biopsy cultures or suitable disease models, in order to explore the possible role of TG2-mediated cell adhesion pathways and anti-TG2 antibodies in the pathogenesis of CD. Selection and cloning of antibodies from the target tissue, the tool successfully used for dissecting the anti-TG2 antibody response (Marzari *et al.* 2001; Di Niro *et al.* 2012), could lead to more detailed characterization of the nature and role of anti-TG2 autoantibodies in CD.

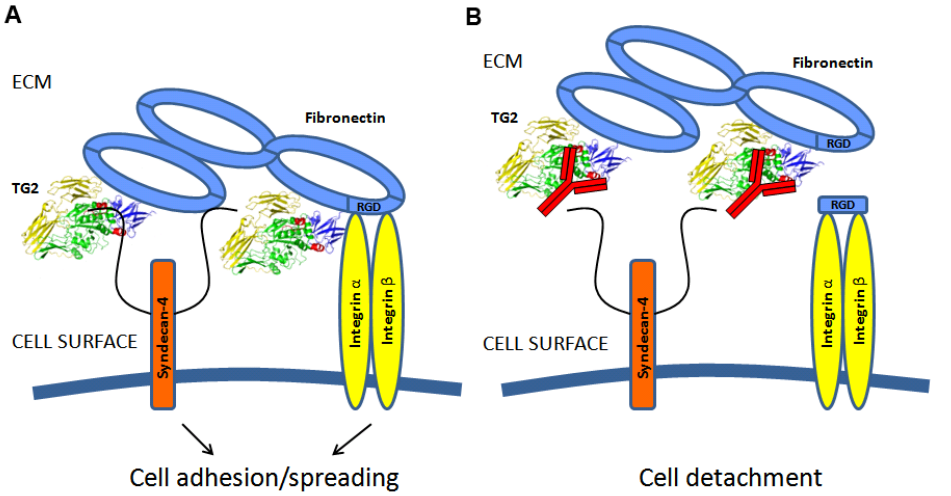

Figure 10. Schematic model of the inhibitory mechanism of anti-TG2 autoantibodies on cell adhesion in CD. (A) In a normal situation, cell adhesion is mediated by interactions of cell surface integrins and HSPGs (syndecan-4) with FN-TG2 matrix outside the cell. (B) In an inflamed tissue like in CD, the RGD-dependent adhesion is suppressed by short RGD peptides, and TG2 binding to HS chains of syndecan-4 is inhibited by anti-TG2 autoantibodies, resulting in decreased intestinal epithelial cell adhesion.

7. CONCLUSIONS

1. Desmin was identified as the most frequently recognized cytoskeletal antigen by serum IgA of children with CD, detected in more than half of patients. Anti-desmin IgA antibodies were not cross-reactive with TG2, and both anti-desmin and anti-TG2 IgA levels decreased in response to a gluten free diet. Anti-desmin antibodies could be induced in response to ongoing intestinal tissue destruction as part of the complex immune activation in CD.
2. The diagnostic performance of the modified ELISA, using FN bound TG2, was superior to the conventional ELISA for anti-TG2 IgA, IgG and IgM detection in children with CD. The combined use of an anti-TG2 IgA and IgG ELISA resulted in 95.4% sensitivity and 99.0% specificity for childhood CD, whereas anti-TG2 IgM detection had no additional value for CD screening. The purification effect of binding TG2 to its ligand FN, as well as the oriented conformation mimicking the situation *in vivo*, could be the main reasons for the superior accuracy of the modified ELISA for anti-TG2 detection. The novel ELISA could be useful for screening for childhood CD with high diagnostic accuracy.
3. Anti-TG2 antibodies from the sera of CD patients inhibited TG2 binding to heparin/HS in a concentration-dependent manner. The treatment of the FN-TG2 surface with anti-TG2 antibodies from CD sera reduced RGD-independent intestinal epithelial cell attachment. Anti-TG2 autoantibodies had no effect on the transamidase activity of TG2 *in vitro*. Our results suggest that anti-TG2 autoantibodies from CD patients affect the cell adhesion function of TG2. We hypothesize that the binding of anti-TG2 antibodies to extracellular TG2 and hindering its interactions with cell HSPGs can lead to the loosening of adhesion contacts between epithelial cells and the basement membrane in the small intestinal mucosa of CD patients.
4. Two heparin-binding TG2 sequences, consisting of amino acid residues 202–215 and 261–274, were identified by analysing corresponding peptides in SPR biosensor studies. Heparin-binding TG2 peptides were immuno-reactive with serum IgA of CD patients, and the TG2 peptide P2 (261–274) reduced intestinal epithelial cell adhesion on surfaces coated either with FN or FN-TG2. Identified heparin-binding sequences reside close to each other on the surface of the TG2 core domain and these regions could be involved in TG2 interaction with cell surface HSPGs.

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SUMMARY IN ESTONIAN

Autoantikehad desmiini ja transglutaminaas 2 vastu tsöliaakia korral: diagnostiline ja funktsionaalne tähendus

Tsöliaakia on krooniline peensoolehaigus, mille vallandavad nisus leiduvad gluteeni valgud, ning sarnased valgud rukkis ja odras. Tsöliaakia korral on häirunud normaalne immuuntolerantsus vastavate teraviljavalkude suhtes, mis viib immuunvastuse ja põletiku tekkeni peensoole limaskestas ning soolehattude kahjustumiseni. Peamised tsöliaakia kliinilised sümptomid, mida kirjeldati juba 19. saj. lõpus, on toitainete maladsorptsioonist tingitud kõhulahtisus, kõhupuhitus ja kasvu peetumine lastel. Pikka aega ongi tsöliaakiat peetud suhteliselt harvaesinevaks väikelaste haiguseks, mis tekib kui lapsed hakkavad sööma teraviljajahust toite ja avaldub kirjeldatud „klassikaliste" sümptomitega. Viimasel paaril aastakümnel on tsöliaakia muutunud sagedaseks haiguseks, millel on lai kliiniline spekter – alates „klassikalisest" kuni atüüpiliste ja subkliiniliste avaldumisvormideni. Kõrge levimuse, milleks on kuni 1% arenenud maade elanikkonnast, üheks põhjuseks on efektiivsete sõeluuringumeetodite kasutuselevõtt, teisalt on tegemist elukeskkonna muutustest tingitud levimuse tõusuga, mis on omane paljudele autoimmuunhaigustele.

Tsöliaakia patogeneesis on kesksel kohal loomulik ja adaptiivne immuunvastus gluteeni peptiidide vastu. Haigusel on ka oluline pärilik komponent ning tugevaim seos on HLA II klassi heterodimeere DQ2 ja DQ8 kodeerivate alleelidega, esinedes pea kõigil patsientidel. Tsöliaakiahaigete peensoole limaskestas esitletakse gluteeni peptiide DQ2/DQ8 vahendusel CD4+ T-rakkudele, mis produtseerivad põletikutsütokiine ja aktiveerivad omakorda teisi rakke. Teise järjestusega gluteeni peptiidid tekitavad stressireaktsiooni soole epiteelrakkudes, mis viib intraepiteliaalsete CD8+ T-rakkude aktivatsioonile ja epiteelrakkude hävitamisele. Immuunkahjustuse mehhanismide tulemusena kujuneb välja soolehattude atroofia, krüptide hüperplaasia ja soole läbilaskvuse suurenemine. Kõik need muutused taanduvad täieliku gluteenivaba dieedi pidamisel, mis ongi tsöliaakia ainsaks efektiivseks raviks.

Lisaks gluteenivastastele immuunreaktsioonidele tekivad tsöliaakiahaigetel autoantikehad transglutaminaas 2 (TG2) ja teiste autoantigeenide vastu. TG2 on multifunktsionaalne valk, mis omab transamideerivat aktiivsust ning osaleb protsessides nagu rakkude proliferatsioon, apoptoos, adhesioon, endotsütoos ja rakuvälise maatriksi kujunemine. Tsöliaakiahaigete peensooles on TG2 ekspressioon tõusnud ning TG2 poolt modifitseeritud gluteeni peptiidid võimendavad täiendavalt T-rakkude vastust. Samuti võib TG2 siduda gluteeni peptiide kovalentselt iseenda külge ja selliste komplekside teket peetakse oluliseks anti-TG2 antikehade indutseerimisel. Seerumi IgA tüüpi anti-TG2 antikehad on kujunenud tsöliaakia tundlikuks ja spetsiifiliseks haigusmarkeriks, mida määratakse haiguskahtluse või -riski korral. Vaatamata anti-TG2 IgA kõrgele haigusspetsiifilisusele, leitakse teatud haigusgruppides vähesel arvul valepositiivseid

tulemusi. Kuna peensoole biopsia teostamine on nõutav tsöliaakia diagnoosimiseks, siis on oluline võimalikult efektiivne patsientide eelnev selektsioon biomarkerite alusel. Seega, jätkuv anti-TG2 antikehade testide arendamine on vajalik parema diagnostilise võimekuse saavutamiseks. Samuti on vajadus täiendavate haigusseoseliste biomarkerite järele seerumis, mis võimaldaksid hinnata koekahjustuse ulatust peensoole limaskestas. Lisaks kasutusele biomarkerina, on võimalik ka anti-TG2 antikehade osalemine tsöliaakia immunopatogeneesis. Anti-TG2 IgA antikehi produtseeritakse tsöliaakiahaigete peensoole limaskestas ja nad kolokaliseeruvad rakuvälise TG2-ga basaalmembraani all. *In vitro* katsetes on näidatud, et tsöliaakiahaigete anti-TG2 antikehad mõjutavad TG2 transamideerivat aktiivsust ja mitmesuguseid TG2 rakulisi funktsioone, kuid täpsemad antikehade toimemehhanismid pole teada.

Käesolevas väitekirjas seatud eesmärkideks oli:

- iseloomustada tsütoskeleti valkude vastaste antikehade esinemist tsöliaakiahaigete laste vereseerumis ja identifitseerida peamised autoantigeenid;
- võrrelda uudse ensüümseoselise immunosorbentmeetodi (ELISA) diagnostilist võimekust senise ELISA testiga anti-TG2 antikehade määramiseks tsöliaakiahaigete laste seerumis;
- hinnata tsöliaakiahaigete anti-TG2 antikehade mõju TG2 seondumisele hepariini/heparaansulfaadiga ja rakkude TG2-sõltuvale adhesioonile;
- määratleda peamised hepariiniga seonduvad TG2 piirkonnad, kasutades kandidaatpeptiide seondumis- ja funktsionaalsetes katsetes.

Leidsime, et tsütoskeleti valkude hulgast reageeris tsöliaakiahaigete seerumi IgA kõige sagedamini desmiiniga, mis on lihasspetsiifiline vahepealsete filamentide hulka kuuluv valk. Desmiinivastaseid IgA antikehi leiti enam kui pooltel tsöliaakiahaigetest lastest nii immunoblotanalüüsil kui ELISA meetodiga. Desmiinivastane IgA ei ristreageerinud TG2-ga, kuid mõlemate antikehade tase langes gluteenivaba dieedi toimel. Antikehade teke desmiini vastu võib olla vastuseks juba kujunenud koekahjustusele tsöliaakiahaigete peensooles. Desmiinivastaste IgA antikehade kasutus koekahjustuse markerina tsöliaakia korral vajab täiendavat uurimist.

Meie poolt välja töötatud uudne ELISA test, mis põhineb TG2 seondumisel tema ligandi fibronektiini (FN) külge (FN-TG2 ELISA), oli parema diagnostilise võimekusega võrreldes senise testiga nii IgA, IgG kui ka IgM tüüpi anti-TG2 antikehade määramiseks tsöliaakiahaigete laste seerumis. Kombineeritud IgA ja IgG tüüpi anti-TG2 antikehade määramisel saavutati diagnostiline tundlikkus 95.4% ja spetsiifilisus 99.0%. Autorite arvates on modifitseeritud ELISA parem diagnostiline võimekus seotud nii TG2 täiendava puhastamisega seondumisel FN-ga kui ka seondunud TG2 konformatsiooniga, mis jäljendab olukorda *in vivo*.

Kõrge anti-TG2 antikehade tasemega tsöliaakiahaigete seerumid ja afiinsuspuhastatud anti-TG2 antikehad inhibeerisid TG2 seondumist hepariini ja

heparaansulfaadiga (HS) kontsentratsioonist sõltuvalt. Kuna TG2 seondumine HS jääkidega on seotud TG2 adhesioonifunktsiooniga, uurisime anti-TG2 autoantikehade mõju soole epiteelrakkude adhesioonile. FN-TG2 kompleksiga kaetud pinna mõjutamisel anti-TG2 autoantikehadega vähenes soole epiteelrakkude kinnitumine, mis oli sõltumatu integriinide ja RGD motiivi vahendatud adhesioonist. Uurides võimalikele hepariiniga seonduvatele TG2 järjestustele vastavaid sünteetilisi peptiide, leidsime et aminohapetele 202–215 ja 261–274 vastavad peptiidid seonduvad hepariiniga biosensoranalüüsil. Samad peptiidid olid immunoreaktiivsed tsöliaakiahaigete seerumi IgA-ga ning peptiid 261–274 vähendas sooleepiteelrakkude kinnitumist FN-TG2 kaetud pinnal. Leitud hepariinseonduvad järjestused paiknevad lähestikku TG2 pinnal ja need võivad olla olulised TG2 seondumisel rakupinna HS-proteoglükaanidega.

Lähtudes saadud tulemustest püstitasime uudse hüpoteesi, et tsöliaakiahaigete anti-TG2 antikehad, seondudes rakuvälise TG2-ga peensoole limaskestas ja takistades TG2 seondumist rakupinna HS-proteoglükaanidega, nõrgendavad soole epiteelrakkude adhesioonikontakte rakuvälise maatriksiga ning võivad seeläbi osaleda tsöliaakia koekahjustuse tekkel.

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PUBLICATIONS

CURRICULUM VITAE

Kaupo Teesalu

Date and place of birth:	April 16, 1971 in Karksi, Estonia
Citizenship:	Estonian
Address:	Immunology Group, Department of General and Molecular Pathology, University of Tartu, Ravila 19, Tartu 50411, Estonia
Phone:	+372 566 80701
E-mail:	kaupo.teesalu@ut.ee

Education

1987–1989	Tallinn Secondary School No 55
1992–1997	BSc studies in biotechnology and biomedicine, Faculty of Biology and Geography, University of Tartu
1997–1999	MSc studies in biomedicine, Faculty of Medicine, University of Tartu
2001	MSc degree in biomedicine, Faculty of Medicine, University of Tartu
2006–2011	Doctoral studies in medicine (immunology), Faculty of Medicine, University of Tartu

Professional employment

2000–2002	Technician, Immunology Group, Department of General and Molecular Pathology, University of Tartu
2002–2005	Researcher, Immunology Group, Department of General and Molecular Pathology, University of Tartu
2005–2006	Specialist, Department of Research and Institutional Development; University of Tartu
2006–2012	Researcher, Immunology Group, Department of General and Molecular Pathology, University of Tartu

Research activity

- Main research field is characterization of the autoantibody response in celiac disease and in the endocrine autoimmune diseases
- Co-author of 12 research articles in the international peer-reviewed journals
- Member of the Estonian Society for Immunology and Allergology

ELULOOKIRJELDUS

Kaupo Teesalu

Sünniaeg ja koht:	16. aprill 1971, Karksi, Eesti
Kodakondsus:	Eesti
Aadress:	immunoloogia õppetool, üld-ja molekulaarpatoloogia instituut, Tartu Ülikool, Ravila 19, Tartu 50411, Eesti
Telefon:	+372 566 80701
E-post:	kaupo.teesalu@ut.ee

Haridus

1987–1989	Tallinna 55. Keskkool
1992–1997	biotehnoloogia ja biomeditsiini bakalaureuseõpe, bioloogia-geograafiateaduskond, Tartu Ülikool
1997–1999	biomeditsiini magistriõpe, arstiteaduskond, Tartu Ülikool
2001	teadusmagistri kraad, arstiteaduskond, Tartu Ülikool
2006–2011	arstiteaduse doktoriõpe (immunoloogia), arstiteaduskond, Tartu Ülikool

Teenistuskäik

2000–2002	laborant, immunoloogia õppetool, üld- ja molekulaarpatoloogia instituut, Tartu Ülikool
2002–2005	teadur, immunoloogia õppetool, üld- ja molekulaarpatoloogia instituut, Tartu Ülikool
2005–2006	teadustalituse peaspetsialist, teadus- ja arendusosakond, Tartu Ülikool
2006–2012	teadur, immunoloogia õppetool, üld- ja molekulaarpatoloogia instituut, Tartu Ülikool

Teadustöö

- Peamine uurimisvaldkond on autoantikehade iseloomustamine tsöliaakia ja endokriinsete autoimmuunhaiguste korral
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