TIINA RAUHAVIRTA

Characterizing and Modifying the Intestinal Barrier in Coeliac Disease

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ACADEMIC DISSERTATION
To be presented, with the permission of
the Board of the School of Medicine of the University of Tampere,
for public discussion in the Auditorium of Finn-Medi 5,
Biokatu 12, Tampere, on August 15th, 2014, at 12 o'clock.

UNIVERSITY OF TAMPERE

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Acta Universitatis Tamperensis 1952
Tampere University Press
Tampere 2014

UNIVERSITY
OF TAMPERE

ACADEMIC DISSERTATION
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Cover design by
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Distributor:
kirjamyynti@juvenes.fi
http://granum.uta.fi

Acta Universitatis Tamperensis 1952
ISBN 978-951-44-9504-5 (print)
ISSN-L 1455-1616
ISSN 1455-1616

Acta Electronica Universitatis Tamperensis 1437
ISBN 978-951-44-9505-2 (pdf)
ISSN 1456-954X
http://tampub.uta.fi

Suomen Yliopistopaino Oy – Juvenes Print
Tampere 2014

ABSTRACT

An impairment of the epithelial barrier has been shown to be a critical determinant in intestinal inflammation and several gastrointestinal malfunctions, including coeliac disease. Coeliac disease is an autoimmune-mediated disorder triggered by dietary gluten and related prolamines from wheat, rye and barley in genetically susceptible individuals. The condition develops from minor changes in the small-bowel mucosa to damage with villus atrophy, crypt hyperplasia and massive inflammation. An immune response to ingested gluten results in the formation of antibodies against both transglutaminase 2 (TG2) and deamidated forms of gluten-derived gliadin peptides. These antibodies are found in the circulation and anti-TG2 antibodies also as deposits in the small-bowel mucosa. In coeliac patients the ingestion of gluten triggers a wide spectrum of clinical manifestations, including intestinal and extraintestinal symptoms. At this moment the only effective treatment for coeliac disease is a strict and life-long gluten-free diet.

In this investigation of gluten-induced damage in coeliac patients, small-bowel mucosal biopsies were taken before the development of villus atrophy, during overt disease and one year after the initiation of a gluten-free diet. Immunohistochemical staining revealed the expression of intestinal epithelial junction proteins to be disrupted in the early stages of the disease, although the villus structure was still normal. When coeliac disease progressed to the stage of damaged mucosa, the expression of junction proteins remained low. Junction protein expression started to normalize during a gluten-free diet.

A human Caco-2 cell monolayer served as an *in vitro* model for intestinal epithelial permeability experiments. Pepsin-trypsin (PT)-digested gliadin caused a breakdown of this barrier and increased the permeability of the cell monolayer, observed as a lower transepithelial resistance (TER) and increased flux of fluorescently-labelled dextran molecules when compared to control samples. Interestingly, treatment of Caco-2 cells with IgA class antibodies derived from coeliac patients enhanced the translocation of labelled gluten-derived gliadin

peptides through the epithelial cell layer. According to measurements of TER, coeliac IgA had no effect on paracellular permeability, suggesting that translocation of gliadin peptides occurs transcellularly rather than by paracellular route. In addition, coeliac antibodies increased TG2 activity in Caco-2 cells, which effect could be prevented by treatment with a TG2 inhibitor, R281. This TG2 inhibitor also prevented coeliac antibody-induced translocation of gliadin peptides. In addition, TER measurements showed that TG2 inhibitors R281 and R283 prevented the PT-gliadin-induced increase in permeability. Fluorescence staining of Caco-2 cells showed PT-gliadin-induced disruptions of the cell cytoskeleton and junction proteins to be reduced by pre-treatment with TG2 inhibitors. In addition to the effects on gliadin-induced alterations in the epithelium, TG2 inhibitors prevented the PT-gliadin-induced increase in the number of CD25-positive lymphocytes, IL-15-positive cells, regulatory T cells and proliferative crypt enterocytes in coeliac patient-derived small-bowel mucosal biopsies.

This study showed that the integrity of the small-bowel mucosal epithelium is already impaired in early developing coeliac disease, as shown by the disruption of junctions between epithelial cells, although villus structure was still normal. Permeability experiments suggested that IgA-class antibodies from coeliac patients enhanced the translocation of harmful gliadin peptides through the intestinal epithelium. This effect could be prevented by inhibition of TG2. Furthermore, TG2-inhibitors reduced or removed many PT-gliadin-induced effects in an intestinal epithelial cell model and cultured biopsies. These findings reveal new aspects of the pathogenesis of coeliac disease in the intestinal mucosal epithelium and offer opportunities for developing new therapies for the disorder.

TIIVISTELMÄ

Suolen läpäisevyyttä säätelee epiteelin muodostama este, jonka häiriöillä on ratkaiseva merkitys suolen tulehdustilassa ja monissa ruoansulatuskanavan sairauksissa, kuten keliakiassa. Keliakia on perinnöllisesti alttiiden henkilöiden autoimmuunivälitteinen sairaus, jonka aiheuttavat ravinnosta saatava vehnän, rukiin ja ohran gluteeni sekä muut gluteeninkaltaiset prolamiinit. Keliakia kehittyy vähitellen alkaen pienistä muutoksista ohutsuolen limakalvolla päätyen pahimmassa tapauksessa nukkalisäkkeiden tuhoutumiseen, kryptien liikakasvuun ja laajaan tulehdustilaan. Gluteenin aiheuttama immuunivaste suolen limakalvolla johtaa vasta-aineiden muodostumiseen sekä elimistön omaa transglutaminaasi 2 (TG2)-entsyymiä vastaan että TG2:n muokkaamia gluteenista peräisin olevia peptidejä, esimerkiksi vehnän gliadiinipeptidejä, vastaan. Näitä vasta-aineita on verenkierrossa ja lisäksi TG2-vasta-aineita löytyy kertyminä ohutsuolen limakalvolta. Ruuan sisältämä gluteeni aiheuttaa keliaakikoilla paitsi monenlaisia suolioireita, myös suolen ulkopuolella esiintyviä oireita. Tällä hetkellä ainoa tehokas hoito keliakiaan on tiukka ja loppuelämän kestävä gluteeniton ruokavalio.

Tässä työssä gluteenin aiheuttamia vaurioita keliakiapotilaissa tutkittiin ottamalla koepaloja ohutsuolen limakalvolta ennen suolinukan häviämistä, aktiivisen taudin aikana ja vuosi gluteenittoman dieetin aloittamisen jälkeen. Immunohistokemialliset värjäykset paljastivat, että suolen epiteelisolujen välisten liitosproteiinien ilmentyminen oli alentunut jo keliakian varhaisessa vaiheessa, vaikka suolen limakalvon nukkarakenne oli vielä normaali. Kun keliakia eteni vaiheeseen, jossa suolinukka oli tuhoutunut, myös epiteelikerroksessa olevien liitosproteiinien määrät pysyivät alhaisina. Gluteeniton dieetti käynnisti liitosproteiinien normalisoitumisen.

Läpäisevyystutkimuksessa käytettiin ihmisperäisiä Caco-2-soluja, jotka muodostavat suolen epiteelin kaltaisen yhtenäisen solukerroksen. Pepsiinillä ja trypsiinillä pilkottu gliadiini (PT-gliadiini) lisäsi solukerroksen läpäisevyyttä, mikä havaittiin transepiteelisen resistanssin (TER) alentumisena ja fluoresenssi-leimalla merkittyjen dekstraani-molekyylien lisääntyneenä virtauksena solukerroksen läpi,

kun mittaustuloksia verrattiin kontrollinäytteisiin. Yllättävää kyllä, keliaakikkojen seerumeista eristetyt IgA-luokan vasta-aineet edistivät gluteenista peräisin olevien gliadiinipeptidien kulkeutumista epiteelisolukerroksen läpi, mikä todettiin käyttämällä leimattuja gliadiinipeptidejä. TER-mittausten perusteella pääteltiin, että keliaakikon IgA:lla ei ollut vaikutusta solujen välisiin liitoksiin, mikä viittaa siihen, että gliadiinipeptidien kulkeutumisen epiteelin läpi täytyi tapahtua transsytoosin avulla eikä solujen välisten liitosten kautta. Lisäksi keliaakikkojen vasta-aineet näyttivät lisäävän solujen TG2:n aktiivisuutta, mikä puolestaan voitiin poistaa TG2-estäjällä, R281:lla. Tällä TG2-estäjällä voitiin poistaa myös keliaakion vasta-aineiden vaikutus gliadiinipeptidien kulkeutumiseen epiteelisolukerroksen läpi. Lisäksi TER-mittauksissa nähtiin, että PT-gliadiini lisäsi epiteelisolukerroksen läpäisevyyttä mutta TG2-estäjät R281 ja R283 poistivat tämän vaikutuksen. Caco-2-solujen fluoresenssivärjäykset osoittivat, että solujen esikäsittely TG2-estäjillä vähensi PT-gliadiinin aiheuttamia vaurioita solun tukirangassa ja solujen välisissä liitoksissa. Epiteelisoluisssa havaittujen muutosten lisäksi TG2-estäjät vähensivät PT-gliadiinin vaikutuksesta lisääntyneiden CD25-positiivisten T-imusolujen, IL-15-positiivisten solujen, säätelijä-T-imusolujen ja jakautuvien kryptasolujen määriä keliaakikkojen suolen limakalvolta otetuissa koepaloissa.

Tässä väitöskirjatutkimuksessa havaittiin, että alkavassa keliakiassa suolen limakalvon eheydessä näkyy vaurioita epiteelisolujen välisissä liitoksissa, vaikka suolinukka on vielä normaali. Läpäisevyyskokeet osoittivat, että keliaakikkojen IgA-luokan vasta-aineet saattavat edistää haitallisten gliadiinipeptidien kulkeutumista suolen epiteelin läpi, mikä puolestaan voidaan estää TG2-estäjällä. Lisäksi TG2-estäjät voivat vähentää tai poistaa kokonaan useita gliadiinin aiheuttamia haittavaikutuksia suolen epiteelisolumallissa ja viljellyissä koepaloissa. Tutkimustulokset tuovat uutta tietoa keliakian kehittymisestä suolen limakalvolla ja tarjoavat mahdollisuuksia uusien hoitomuotojen kehittämiseen.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals **I – III**:

I Rauhavirta T, Lindfors K, Koskinen O, Laurila K, Kurppa K, Saavalainen P, Mäki M, Collin P, Kaukinen K (2014) Impaired epithelial integrity in the duodenal mucosa in early stages of celiac disease. Transl Med. In press. DOI:10.1016/j.trsl.2014.02.006

II Rauhavirta T, Qiao SW, Jiang Z, Myrsky E, Loponen J, Korponay-Szabó IR, Salovaara H, Garcia-Horsman JA, Venäläinen J, Männistö PT, Collighan R, Mongeot A, Griffin M, Mäki M, Kaukinen K, Lindfors K (2011) Epithelial transport and deamidation of gliadin peptides: a role for coeliac disease patient immunoglobulin A. Clin Exp Immunol 164: 127-36

III Rauhavirta T, Oittinen M, Kivistö R, Männistö PT, Garcia-Horsman JA, Wang Z, Griffin M, Mäki M, Kaukinen K, Lindfors K (2013) Are transglutaminase 2 inhibitors able to reduce gliadin-induced toxicity related to coeliac disease? A proof-of-concept study. J Clin Immunol 33: 134-42

ABBREVIATIONS

AGA	anti-gliadin antibodies
ANOVA	analysis of variance
APC	antigen presenting cell
ARA	anti-reticulin antibodies
BSA	bovine serum albumin
Caco-2	human colorectal adenocarcinoma cell line
Da	dalton
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DGP	deamidated gliadin peptides
DMEM	Dulbecco's modified Eagle's medium
EBV	Ebstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
EmA	endomysial antibodies
ERK1/2	extracellular-signal-regulated kinase 1/2
FBS	foetal bovine serum
FITC	fluorescein-isothiocyanate
FOXP3	forkhead box P3
GALT	gut-associated lymphoid tissue
GSRS	Gastrointestinal Symptom Rating Scale
HLA	human leukocyte antigen
HRP	horseradish peroxidase
hTPO	human thyroid peroxidise
IEL	intraepithelial lymphocyte
IFN-γ	interferon γ
IgA	immunoglobulin A
IgG	immunoglobulin G

IL	interleukin
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-light
MHC	major histocompatibility complex
NK	natural killer
PEP	prolyl-endopeptidase
pERK1/2	phosphorylated extracellular-signal-regulated kinase 1/2
PT	pepsin-trypsin-digested
PT-G	pepsin-trypsin-digested gliadin
PT-BSA	pepsin-trypsin-digested bovine serum albumin
RCD	refractory coeliac disease
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE-HPLC	size-exclusion high performance liquid chromatography
SIgA	secretory IgA
TCR	T cell receptor
TER	transepithelial resistance
TG2	transglutaminase 2
TNF-α	tumour necrosis factor α
Treg	regulatory T cell
Vh/CrD	villus height crypt depth ratio
ZO-1	zonula occludens-1

INTRODUCTION

The barrier function of the small-intestinal epithelium is essential in controlling transport of nutrients and pathogens. Junction proteins connect epithelial cells to each other and disruption of their expression or alteration of their intracellular localization can cause failures in intestinal barrier function. In coeliac disease, as in other disorders, the intestinal barrier plays a role in the pathogenesis. When intestinal epithelial permeability increases, components of gluten may translocate through the epithelium more easily, thus contributing to the immune reaction and inflammation in the gut.

The prevalence of coeliac disease is approximately 1 % worldwide, but for example in Finland it is already over 2 % and has increased during the last decade (Lohi et al. 2007). The development of this disorder requires a certain genotype. Genetically susceptible individuals express human leukocyte antigen (HLA) types DQ2 or DQ8. Some 30 – 40 % of the general population have this genotype, but the majority never develop coeliac disease (Koning 2005, Megiorni and Pizzuti 2012).

In addition of genotype, coeliac disease requires an external trigger, namely dietary gluten. This is a storage protein from wheat, rye and barley. It consists of alcohol-insoluble glutenins and soluble prolamines, called gliadin in wheat, secalin in rye and hordein in barley. In coeliac patients ingestion of gluten induces an immune reaction on the small-bowel mucosa. This reaction involves factors from both innate and adaptive immunity, resulting in a complex inflammation in the gut. The immune process leads to the production of antibodies against some components of gluten and autoantibodies against transglutaminase 2 (TG2), also known as tissue transglutaminase, a multifunctional enzyme expressed almost everywhere in the body (Dieterich et al. 1997).

Coeliac disease progresses gradually from lymphocytosis in normal villus architecture to massive inflammation and crypt hyperplasia with villus atrophy (Marsh 1992). Before visible damage to the small-bowel mucosal villus structure,

patients may evince various gastrointestinal symptoms. Interestingly, coeliac disease-specific antibodies are already present in early stages of the disease (Kaukinen et al. 2001, Kurppa et al. 2009, Salmi et al. 2006). However, microscopical alterations in epithelial integrity have not to date been studied in early-stage coeliac disease.

The role of coeliac autoantibodies in the pathogenesis is disputable. Do they have a protective effect or do they contribute to the progression of the disease? Nevertheless, *in vitro* experiments have shown that they may have effects on many biological functions related to coeliac disease. In contrast, the role of TG2, the target of autoantibodies, in the coeliac pathogenesis is indisputable. TG2 modifies gluten components by deamidation, turning them into antigens which are recognised by the immune system (Molberg et al. 1998, van de Wal et al. 1998). When these deamidated gluten-derived gliadin peptides permeate the intestinal epithelium, they are able to induce both innate and adaptive immune responses the in small-bowel mucosa (Hüe et al. 2004, Meresse et al. 2004, Shan et al. 2002).

A strict gluten-free diet is at present the only known effective treatment for coeliac disease. Patients who develop coeliac disease have this disorder for the rest of their lives, even if remission can be attained by excluding gluten from the diet. In fact, serological and intestinal markers of the disease return if a coeliac patient reverts to dietary gluten (Burgin-Wolff et al. 1991, Lähdeaho et al. 2011). Although the diet is effective in alleviating symptoms as well as intestinal and serological alterations, it can be a burden for patients. Therefore, alternative treatments would be desired. Approaches in the search for alternative therapies for coeliac patients have been varied, ranging from enzymatically and genetically modified gluten proteins to inhibition of factors involved in the pathogenesis. For example, the inhibition of TG2 might prevent the formation of immunogenic gluten peptides and probably the progression of the disease.

REVIEW OF THE LITERATURE

1. Intestinal barrier

The intestinal epithelium is a single layer of cells lining the gut lumen and fulfilling two important functions. First, it acts as a barrier to prevent the passage of harmful intraluminal enteric flora, foreign antigens, micro-organisms and their toxins. Second, the intestinal epithelium acts as a selective filter which allows the translocation of essential dietary nutrients, electrolytes and water from the intestinal lumen into the circulation via either transcellular or paracellular pathway (Groschwitz and Hogan 2009, Suzuki 2013). The transcellular transport pathways, including endo- and exocytosis, allow macromolecules access to the subepithelial compartment sampling them from the intestinal lumen into the enterocytes by vesicular transport (Menard et al. 2010).

In addition to intestinal epithelium, the barrier functions include also secreted components, such as immunoglobulins (Ig), mucous and other antimicrobial products (Vanheel et al. 2013). The transcellular transport of antigens can also be mediated by the formation of immunocomplexes with Ig, including isotypes IgA, IgM and IgG (Horton and Vidarsson 2013). IgA is the most common at the mucosal surface and the transport mechanism of these IgA polymers in the intestine comprises a receptor-mediated basal-to-apical secretion (Horton and Vidarsson 2013, Menard et al. 2010). Secretory IgA (SIgA) is an important factor in intestinal protective immunity since that retains potentially harmful antigens in the intestinal lumen and is able to block toxins and pathogens from adhering to the intestinal epithelium (Horton and Vidarsson 2013, Menard et al. 2010). The production of SIgA against specific intestinal mucosal antigens is dependent on antigen sampling by Peyer's patch M cells, on their processing by antigen presenting-cells, on T cell activation and on the B cell class switch recombination in gut-associated lymphoid tissues (GALT), mesenteric lymph nodes and possibly the *lamina propria* (Cerutti and Rescigno 2008, Mantis et al. 2011).

1.1 Junction proteins

Junctions connecting the epithelial cells form an essential part of the intestinal barrier function. These junctions offer a potential route for solute traffic not regulated by brush border membrane transporters and channels (Arrieta et al. 2006). This paracellular pathway is regulated by intercellular junctions, including tight junctions close to the luminal surface and adherens junctions underneath (Figure 1) (Vanheel et al. 2013). In addition to the intercellular connection of adjacent epithelial cells, both tight and adherens junctions are associated with the actin cytoskeleton inside the cells (Madara et al. 1987, Perez-Moreno and Fuchs 2006).

Tight junctions are composed of the proteins occludin (Furuse et al. 1993), and claudins (Kotler et al. 2013). The claudin family consists of 24 members which can be divided into sealing claudins reducing permeability and pore-forming claudins increasing permeability (Kotler et al. 2013). In addition, junction adhesion molecule A (Mandell et al. 2005) and tricellulin, which forms tricellular connections (Ikenouchi et al. 2005), are located in tight junctions. All these transmembrane junction proteins interact directly with membrane-associated zonula occludens (ZO)-proteins. ZOs assemble complexes at the cytoplasmic surface of intercellular junctions and also provide a link between the integral membrane proteins and the filamentous cytoskeleton (Bauer et al. 2010).

Adherens junctions initiate and stabilize cell-cell adhesion, regulate the actin cytoskeleton, mediate intracellular signalling and regulate transcription. Adherens junctions are formed mainly by interactions between transmembrane proteins of the cadherin superfamily such as E-cadherin, and the catenin family members, including β-catenin, α-catenin and p120-catenin (Hartsock and Nelson 2008). These E-cadherin-catenin complexes are critical for the proper functioning of the epithelium (Baum and Georgiou 2011). Firstly, the extracellular parts of E-cadherin molecules provide mechanically strong adhesive links between the cells (Baum and Georgiou 2011). Secondly, adherens junctions help to define an apical-basal axis of epithelial cells and act as a reference point for the coordination of cell polarity (Baum and Georgiou 2011). Thirdly, individual junctions connecting epithelial cells can form polarized cortical domains in the plane of the epithelium, contributing to planar cell polarity (Baum and Georgiou 2011).

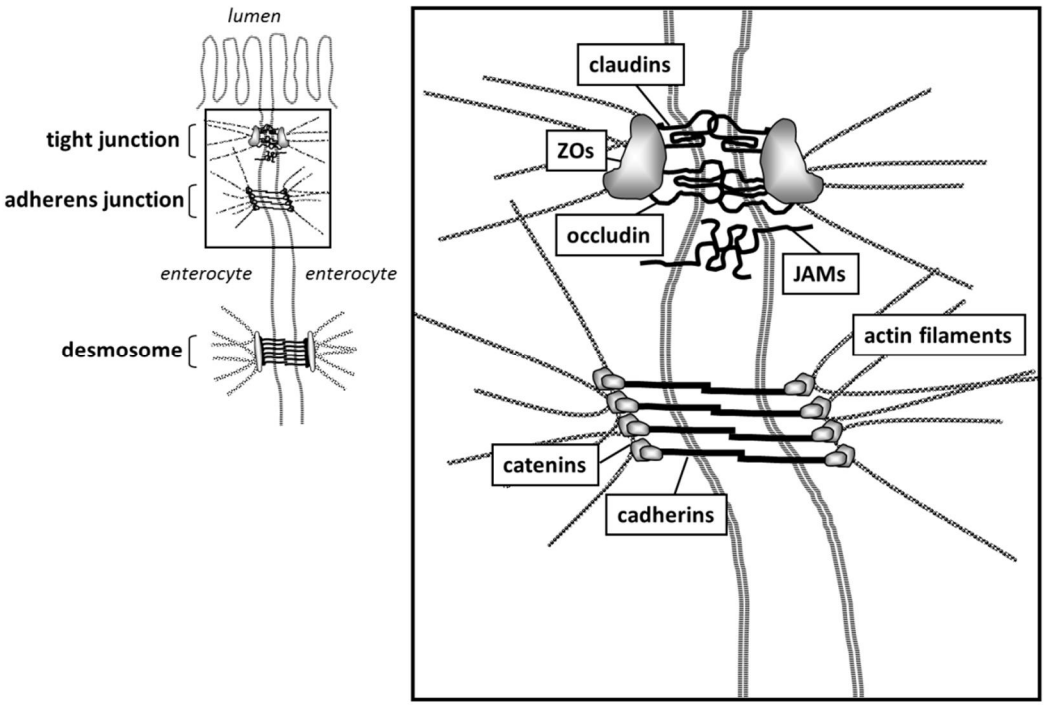


Figure 1. Schematic structure of an intercellular junction between intestinal epithelial cells and proteins involved in tight junctions and adherens junctions. The figure is based on Suzuki 2013. ZOs, zonula occludens proteins; JAMs, junction adhesion molecules.

1.2 Intestinal barrier in diseases

A wide variety of environmental agents in the intestinal lumen can initiate or perpetuate mucosal inflammation if they cross the epithelial barrier (Arrieta et al. 2006, Groschwitz and Hogan 2009). Impairment of the epithelial barrier and increased permeability have been shown to be critical determinants in intestinal inflammation and a number of gastrointestinal diseases such as Crohn's disease (Hollander et al. 1986, Wyatt et al. 1997), ulcerative colitis (Buning et al. 2012) and coeliac disease (Reims et al. 2002, Schulzke et al. 1998, van Elburg et al. 1993). The altered intestinal barrier function and increased permeability can be a consequence of a disease but also a primary causative factor predisposing to disease development (de Kort et al. 2011, Groschwitz and Hogan 2009).

Several factors may mediate intestinal barrier dysfunction, dysregulation of tight junction formation and increased permeability. For example, cytokine-mediated tight junction disruption includes downregulation of tight junction protein transcription (Mankertz et al. 2000, Youakim and Ahdieh 1999), decreased Na^+/K^+-

ATPase expression (Sugi et al. 2001), epithelial apoptosis (Gitter et al. 2000), and cytoskeletal contraction (Wang et al. 2005). Interferon γ (IFN-γ) and tumour necrosis factor α (TNF-α), central mediator cytokines in intestinal inflammatory diseases, promote reorganization and dysregulate the expression of tight junction proteins, thus hampering epithelial barrier function (Mankertz et al. 2000, Zolotarevsky et al. 2002). Animal experiments suggest that activation of anti-CD3-induced $CD4^+$ T cells promotes an increase in transcellular and paracellular intestinal permeability as well as release of proinflammatory cytokines IFN-γ and TNF-α (Clayburgh et al. 2005, Musch et al. 2002). T cell activation induces luminal fluid accumulation by increasing mucosal permeability and reducing epithelial Na^+/K^+-ATPase activity, which leads to diarrhoea due to decreased intestinal Na^+ and water absorption (Musch et al. 2002). In addition to IFN-γ and TNF-α, other pro-inflammatory cytokines such as IL-4 and IL-13, may also play a role in increased intestinal permeability (Berin et al. 1999, Ceponis et al. 2000). Intestinal immune cells can contribute to increased permeability in some gastrointestinal disorders. For example, intraepithelial lymphocytes (IELs), which comprise antigen-experienced T cells belonging to the T cell receptor (TCR)-$\alpha\beta^+$ and TCR$\gamma\delta^+$ lineages, are in direct contact with enterocytes and also in immediate proximity to antigens in the gut lumen (Cheroutre et al. 2011).

Dysfunction of the epithelial barrier is often associated with abnormal expression of junction proteins. In the small intestine of coeliac disease patients pore-forming claudin-2 is strongly expressed, while the expression of sealing-related proteins such as claudin-3, occludin and ZO-1 are decreased compared to the healthy gut (Schumann et al. 2012, Szakal et al. 2010). For example in Crohn's disease, upregulation of claudin-2 in addition to downregulation and redistribution of claudin-5 and -8 lead to altered tight junction structure and distinct barrier dysfunction already in the early stage of the disease (Zeissig et al. 2007). The discovery of *Vibrio cholera* zonula occludens toxin and its connection to increased tight junction permeability led to the identification of its eukaryotic counterpart, zonulin, later also known as a precursor of haptoglobin 2 (Tripathi et al. 2009). Bacterial exposure induces the release of zonulin, thus affecting intestinal barrier function, but gluten-derived gliadin in coeliac disease has the same effect when it binds to the chemokine receptor CXCR3, which is overexpressed in coeliac disease patients (Lammers et al. 2008). In addition to coeliac disease, previous studies

suggest that increased intestinal permeability in type 1 diabetes might also be zonulin-mediated and inhibition of zonulin might thus prevent loss of intestinal barrier function in these autoimmune diseases (Fasano 2012).

2. Coeliac disease

European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN) has defined coeliac disease as "an immune-mediated systemic disorder elicited by gluten and related prolamines in genetically susceptible individuals and characterized by the presence of a variable combination of gluten-dependent clinical manifestations, coeliac disease-specific antibodies, human leukocyte antigen (HLA)-DQ2 or HLA-DQ8 haplotypes, and enteropathy" (Husby et al. 2012). Thus besides genetic susceptibility, the development of coeliac disease also requires an external trigger, gluten (Husby et al. 2012). Gluten is a storage protein in cereals; it is composed of alcohol-insoluble glutenin and alcohol-soluble fractions called gliadins in wheat, hordeins in barely and secalins in rye (Shewry et al. 1995).

Ingestion of dietary gluten triggers a wide spectrum of clinical manifestations in genetically susceptible coeliac disease patients (Kaukinen et al. 2010). Abdominal and intestinal symptoms such as diarrhoea, malabsorption and weight loss are typical (Kaukinen et al. 2010). Also anaemias resulting from deficiency of iron, folate and vitamin B_{12} are relatively common in coeliac patients as a consequence of malabsorption in the intestine (Bergamaschi et al. 2008, Harper et al. 2007). One characteristic manifestation of coeliac disease is small-intestinal mucosal damage consisting mainly in the disappearance of the villus structure resulting from crypt hyperplasia, which is also a criterion for as coeliac disease diagnosis along with a positive coeliac disease-specific serology and response to a gluten-free diet (Husby et al. 2012).

Coeliac disease cannot be considered solely as a gastrointestinal disorder but in view of the presence of various extraintestinal manifestations in these patients rather as a systemic condition (Reilly et al. 2012). These manifestations include low bone-mineral density leading to osteopenia and osteoporosis (Pistorius et al. 1995, Valdimarsson et al. 1994), dental enamel defects (Aine et al. 1990, Wierink et al. 2007), liver disorders (Kaukinen et al. 2002) and infertility in both women and men

(Collin et al. 1996, Di Simone et al. 2010, Farthing et al. 1982). In addition, coeliac disease-associated neurological dysfunctions such as gluten ataxia and neuropathy are observed in some patients (Cicarelli et al. 2003, Hadjivassiliou et al. 1998). A specific cutaneous presentation of coeliac disease is *Dermatitis herpetiformis*, also known as skin coeliac disease (Bonciani et al. 2012). This extraintestinal manifestation is characterized by a blistering rash on the skin in genetically susceptible *dermatitis herpetiformis* patients whose diet includes gluten (Spurkland et al. 1997).

The development of coeliac disease requires one or both of the genes coding for the major histocompatibility complex (MHC) class II molecules HLA-DQ2 or HLA-DQ8, and more than 97 % of coeliac disease patients express at least one of these genes (Meresse et al. 2012, Mubarak et al. 2013). The majority of them carry the DQ2 gene, either an isoform DQ2.5 (*cis DQA1*05:01, DQB1*02:01* and *trans DQA1*05:05, DQB1*02:02*) or DQ2.2 (*DQA1*02:01, DQB1*02:02*), while fewer than 10 % carry the DQ8 gene (*DQA1*03:01, DQB1*03:02*) (Meresse et al. 2012, Mubarak et al. 2013). The genotype with HLA-DQ2 or -DQ8 is considered a necessary, albeit not sufficient, factor for the development of coeliac disease (Catassi and Fasano 2008). Although 30 – 40 % of the general population have this genotype, the prevalence of coeliac disease is about 1 % worldwide, varying between populations (Koning 2005, Megiorni and Pizzuti 2012). For example, in Finland the prevalence in adults is 2.4 % and in Germany 0.3 % (Mustalahti et al. 2010). In addition to coeliac disease, the expression of DQ2 and DQ8 molecules predisposes to other autoimmune disorders such as type 1 diabetes mellitus and autoimmune thyroid diseases, which can also prevail concomitant with coeliac disease (Collin et al. 2002). Recently, many non-MHC genes and risk alleles such as immunity-related CTLA4/ICOS and IL-2/IL-12, have also been found to be associated with coeliac disease and other autoimmune diseases, which suggests a shared genetic basis of immune-related disorders (Smyth et al. 2008, Trynka et al. 2010, Zhernakova et al. 2011).

Currently the diagnosis of coeliac disease is based on the finding of crypt hyperplasia and villus atrophy in small-bowel mucosal biopsies, but recently the requirements have been revised (Husby et al. 2012). The new criteria suggest that a duodenal biopsy is not necessary for the diagnosis of coeliac disease if the disease-specific antibody levels are high. The diagnosis may thus be based on a combination

of symptoms, HLA haplotypes and antibodies (Husby et al. 2012). Dietary gluten induces an immune response in the small intestine, leading to the formation of coeliac disease-specific autoantibodies against TG2 and antibodies against deamidated gliadin peptides (Husby et al. 2012). Especially anti-TG2 autoantibodies are remarkably sensitive and specific for coeliac disease, and in fact are currently used in serological analysis and diagnostics (Dieterich et al. 1997, Sulkanen et al. 1998, Troncone et al. 1999).

2.1 Early developing coeliac disease

Many signs of coeliac disease are already present in early phases of the disease when the villus morphology is still normal. Prior to small-bowel mucosal villus atrophy, coeliac disease patients may have, for example, anaemia or they may suffer from various gastrointestinal symptoms such as dyspepsia, diarrhoea, flatulence, indigestion or abdominal pain (Kaukinen et al. 2001, Kurppa et al. 2009, Salmi et al. 2006). On the other hand, some asymptomatic individuals may have normal intestinal histology but anti-TG2 antibodies in the serum, and later a subset of them can develop intestinal lesions with increased numbers of TCR$\gamma\delta^+$ IELs and TG2 antibody deposits (Koskinen et al. 2008, Salmi et al. 2006).

Small-intestinal mucosal damage in coeliac disease develops gradually from minor changes to severe damage to the gut. These changes from increased amounts of lymphocytes to damaged mucosa with villus atrophy and crypt hyperplasia in the gut can be rated using the Marsh classification (Marsh 1992) (Figure 2). In addition, the degree of injury of the small-intestinal mucosa can also be evaluated morphometrically by quantifying villus height crypt depth ratio (Vh/CrD) from biopsy specimens, this indicating the degree of gluten-induced damage (Taavela et al. 2013).

According to Marsh, the increase in IELs is one of the earliest signs of developing coeliac disease (Marsh 1992). The increased density of $CD3^+$ IELs in otherwise morphologically normal duodenal biopsies indicates inflammation in the gut and may predict forthcoming coeliac disease (Järvinen et al. 2004). However, an increased density of IELs is not specific for coeliac disease, since other intestinal

disorders, for example Helicobacter pylori infection, can induce similar duodenal intraepithelial lymphocytosis with normal villus architecture (Memeo et al. 2005). Thus the number of TCR$\gamma\delta^+$ lymphocytes, a subgroup of IELs, is a more accurate assay in predicting coeliac disease (Järvinen et al. 2003).

At early stage of coeliac disease, autoantibodies are already present in the serum while duodenal biopsies are morphologically normal (Kurppa et al. 2009, Salmi et al. 2006). In addition, TG2-specific antibody deposits can be found in the small-bowel mucosa before villus atrophy sets in (Koskinen et al. 2008, Salmi et al. 2006). Also antibodies against deamidated gliadin peptides (DGP-AGA) are present in coeliac patients in early-stage coeliac disease (Kurppa et al. 2011).

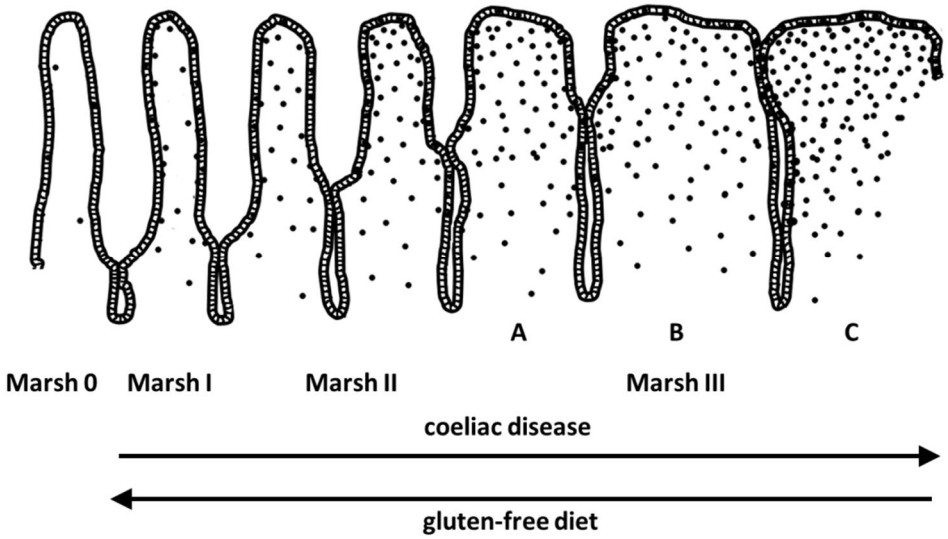


Figure 2. Progress of small-intestinal mucosal damage in coeliac disease. Ingested gluten induces lymphocytosis and lengthening of crypts, leading to villus in the small intestine. The level of damage can be rated by Marsh classification: Marsh 0 (normal mucosa), Marsh I (lymphocytotic enteritis), Marsh II (lymphocytotic enteritis with crypt hyperplasia) and Marsh III (A: partial villus atrophy, B: subtotal villus atrophy, C: total villus atrophy). Elimination of gluten from the diet can normalize the intestinal mucosa. The figure is based on Green et al. 2005 and Marsh 1992.

2.2 Pathogenesis of coeliac disease

Both genetic and environmental factors, as well as innate and adaptive immunity, contribute to the pathogenesis of coeliac disease (Figure 3) (Sollid and Jabri 2013). The primary trigger in the disease is ingested gluten, contained in wheat and other cereals. Its incompletely digested peptides can induce an immunological cascade in the small intestine of the patient (Sollid and Jabri 2013). Specific effects of gluten have also been widely studied in different *in vitro* models, including organ cultures of coeliac patient-derived small-intestinal mucosal biopsies, patient-derived lymphocytes and intestinal epithelial cell cultures such as human colon adenocarcinoma cell lines Caco-2, T84, and LoVo (Tables 1 and 2).

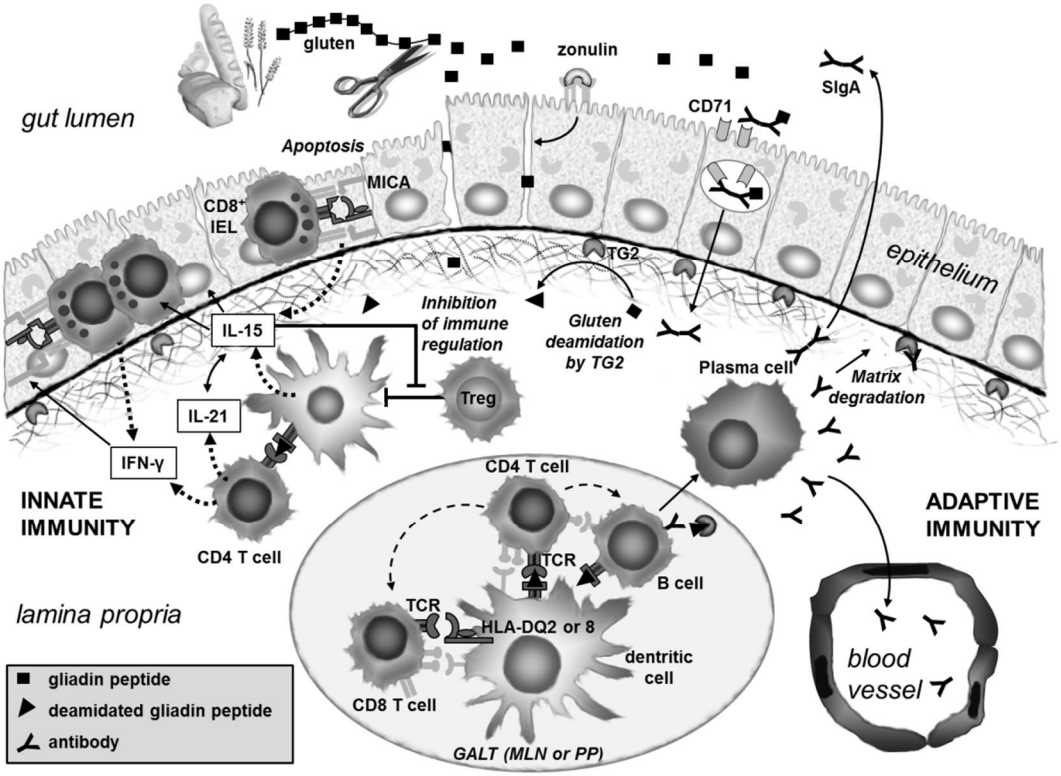


Figure 3. Schematic summary of mechanisms in coeliac disease pathogenesis. CD71, transferrin receptor; SIgA, secretory immunoglobulin A; IEL, intraepithelial lymphocyte; MICA, major histocompatibility class 1 polypeptide-related sequence A; TG2, transglutaminase 2; IL, immunoglobulin; Treg regulatory T cell; IFN-γ, interferon γ; TCR, T cell receptor; GALT, gut-associated lymphoid tissue; MLN; mesenteric lymph node; PP, Peyer's patch. The figure is based on Meresse et al. 2012 and Sollid and Jabri 2013.

*Table 1. In vitro e*ffects of gluten and pepsin-trypsin-digested wheat gliadin in coeliac patient-derived small-intestinal biopsies, isolated lymphocytes and dendritic cells

Model	Effect	Reference
Organ culture	Secretion of autoantibodies	Picarelli et al. 1996, Picarelli et al. 1999, Stenman et al. 2008
	Activation and migration of T cells	Halstensen et al. 1993, Maiuri et al. 1996, Stenman et al. 2008
	Increased proliferation of crypt epithelial cells	Barone et al. 2007, Maiuri et al. 2000, Maiuri et al. 2001
	Increased apoptosis in intestinal epithelial cells	Barone et al. 2007, Maiuri et al. 2001
	Zonulin release and binding to its receptor	Drago et al. 2006
	Altered expression of junction proteins	Dolfini et al. 2005, Drago et al. 2006, Elli et al. 2011, Sander et al. 2005, Szakal et al. 2010
	Decreased enterocyte cell height	Fluge and Aksnes 1981, Stenman et al. 2008
	Increased secretion and overexpression of IL-15	Maiuri et al. 2000
Isolated intraepithelial and peripheral lymphocytes and intestinal epithelial cells	Increased secretion and overexpression of IL-15	Borrelli et al. 2013, Maiuri et al. 2000, Mention et al. 2003, Meresse et al. 2004
Isolated peripheral dendritic cells	Maturation of dendritic cells	Palova-Jelinkova et al. 2005
	Migration	Chladkova et al. 2011
	Remodelling of cytoskeleton	Chladkova et al. 2011

IL, interleukin

Gluten is composed of approximately 15 % proline and 35 % glutamine residues (Stern et al. 2001). The high proline content together with improper digestion by prolyl endopeptidase in the human gastrointestinal tract results in relatively long gluten-derived peptides (Shan et al. 2002). Gluten-derived gliadin fractions can be classified according to their different primary structures into α-, β-, γ- and ω-types, of which α-gliadins have been found to be the most harmful in coeliac disease (Stoven et al. 2012). Subsets of these incompletely cleaved peptides are identified based on their damaging features in coeliac disease. The so-called toxic α-gliadin peptides, for example p31-49 and its shorter form p31-43, cause an innate immunity response (Barone et al. 2011, Maiuri et al. 2003). Another identified coeliac-related subset of gliadin peptides consists of the so-called immunogenic gliadin peptides,

for example 33-mer and its shorter form p57-68, which are able to induce an adaptive immune response in coeliac disease (Fraser et al. 2003, Qiao et al. 2004). Effects of these gliadin-derived peptides are collected in Table 3.

*Table 2. In vitro e*ffects of gluten and pepsin-trypsin-digested wheat gliadin in different cell lines

Cell line	Effect	Reference
Caco-2	Increased intestinal epithelial permeability	Drago et al. 2006, Sander et al. 2005
Caco-2 IEC6	Zonulin release and binding to its receptor	Drago et al. 2006
Caco-2 IEC6 Three-dimensional LoVo cultures	Reorganization of actin filaments	Barone et al. 2007, Dolfini et al. 2005, Drago et al. 2006, Sander et al. 2005, Stenman et al. 2009
Caco-2	Phosphorylation of EGFR and ERK	Barone et al. 2007
Caco-2 Three-dimensional LoVo cultures	Altered expression of junction proteins	Dolfini et al. 2005, Drago et al. 2006, Elli et al. 2011, Sander et al. 2005, Szakal et al. 2010
Co-culture of T84 and patient-derived lymphocytes LoVo INT-407	Increased secretion and overexpression of IL-15	Borrelli et al. 2013, Maiuri et al. 2000, Mention et al. 2003, Meresse et al. 2004
Caco-2	Apoptosis	Giovannini et al. 2000, Maiuri et al. 2001, Sakly et al. 2006
Caco-2 NIH3T3	EGF-like effects on cell cycle and proliferation	Barone et al. 2007, Barone et al. 2011

Caco-2, human colorectal adenocarcinoma cell line; IEC6, rat small intestine epithelial cell line; LoVo, human colon adenocarcinoma cell line; EGFR, epidermal growth factor receptor; ERK, extracellular-signal-regulated kinase; T84, human colonic adenocarcinoma cell line; INT-407, human embryonic intestine cell line; IL, interleukin; NIH3T3 mouse embryonic fibroblast cell line

Table 3. In vitro effects of cytotoxic gliadin peptides p31-49 and p31-43, and immunogenic gliadin peptides p56-88 (33-mer) and p57-68

Gliadin peptide	Model	Effects	Reference
p31–49	Organ culture	Reduction in enterocyte cell height	Shidrawi et al. 1995
		Expression of MIC-A on epithelial cell surface and	Hüe et al. 2004, Meresse et al. 2004
p31–43	Organ culture	Epithelial expression of HLA-DR molecules	Maiuri et al. 1996, Maiuri et al. 2003
		Increased IL-15 expression	Maiuri et al. 2003
		MAP kinase activation	Maiuri et al. 2003
		Appearance of $CD3^+CD25^+$ cells	Maiuri et al. 1996, Maiuri et al. 2005
		Anti-endomysial antibody production	Picarelli et al. 1999
		Apoptosis	Maiuri et al. 2003, Maiuri et al. 2005
		Increased expression and activation of TG2	Maiuri et al. 2005
		Increased proliferation of coeliac crypt enterocytes	Barone et al. 2007, Barone et al. 2011
	Caco-2 cells	Increased proliferation	Nanayakkara et al. 2013
		Reorganization of actin filaments	Barone et al. 2007
		Phosphorylation of EGF receptor and IL-15 receptor	Barone et al. 2007, Nanayakkara et al. 2013
		Phosphorylation of ERK1/2	Barone et al. 2007b, Nanayakkara et al. 2013
		Promoting of dendritic cell migration and activity	Chladkova et al. 2011
		Mobilization of intracellular Ca^{2+} from endoplasmic reticulum and mitochondria	Caputo et al. 2012
		Increased expression and activation of TG2	Maiuri et al. 2005, Caputo et al. 2012
	T84 cells	Apoptosis	Maiuri et al. 2005
		Increased IL-15 expression and activation	Luciani et al. 2010
		MAP kinase activation	Luciani et al. 2010
		Induction of TG2-mediated PPARγ cross-linking and proteasome degradation	Luciani et al. 2010
	NIH3T3 cells	EGF-like effects on cell cycle and proliferation	Barone et al. 2007
	Isolated dendritic cells	Induced migration	Chladkova et al. 2011

p56–88, 33-mer	Patient-derived T cells	Binding to HLA-DQ2 and proliferation of T cells	Meresse et al. 2004, Qiao et al. 2004
	Isolated dendritic cells	Inhibition of p31-43-induced migration	Chladkova et al. 2011
p57–68	Organ culture	Activation of TG2	Maiuri et al. 2005
		Appearance of $CD3^{+}CD25^{+}$ cells	Maiuri et al. 2005
	Caco-2 cells	Mobilization of intracellular Ca^{2+} from mitochondria	Caputo et al. 2012
		Activation of intracellular TG2	Caputo et al. 2012

MIC-A, major histocompatibility complex class I polypeptide-related sequence A; HLA, human leukocyte antigen; IL, interleukin; MAP, mitogen activated protein; Caco-2, human colorectal adenocarcinoma cell line; EGF, epidermal growth factor; EGF, epidermal growth factor; ERK1/2, extracellular-signal-regulated kinase 1/2; TG2, transglutaminase 2; T84, human colonic adenocarcinoma cell line; NIH3T3 mouse embryonic fibroblast cell line

There are several theories as to how gluten-derived peptides pass through the intestinal epithelium and reach the *lamina propria* (Figure 3). One suggested mechanism is a tight junction disassembly by gluten-induced release of zonulin, which increases paracellular leakage of the epithelium (Clemente et al. 2003). Another translocation process is enhanced transport of gluten peptides into the *lamina propria* via epithelial transcytosis (Schumann et al. 2008). Matysiak-Budnik and colleagues have suggested that transcytosis of gluten peptides is facilitated by abnormal retrotransport of SIgA via its binding to transferrin receptor CD71, which is up-regulated and apically expressed in active coeliac disease (Matysiak-Budnik et al. 2008). Several groups have demonstrated that gliadin peptides enter the enterocytes by endocytosis and that p31-43 and p57-68 have different trafficking routes in the cells. Moreover, it has been shown that p31-43 can interfere with vesicular trafficking in enterocytes and in coeliac patient-derived biopsies (Barone et al. 2010, Barone et al. 2011, Caputo et al. 2010, Luciani et al. 2010, Reinke et al. 2011).

2.2.1 Innate immune response

Toxic gliadin peptides are able to induce an innate immunity response, which is probably mediated by interleukin 15 (IL-15) secreted by epithelial cells, macrophages and dendritic cells, this leading to intestinal epithelial damage (Hüe et al. 2004, Maiuri et al. 2003), (Figure 3). In active coeliac disease the number and activity of cytotoxic $CD8^+$ IELs expressing $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ are substantially increased (Han et al. 2013, Järvinen et al. 2003, Savilahti et al. 1990). Further, IL-15 is responsible for the extracellular-signal-regulated kinase (ERK)-pathway-mediated upregulation of the natural killer (NK) cell receptors on $CD8^+$ IELs and their upregulated ligand MHC class I -related chains (MICs) on intestinal epithelial cells, which leads to apoptosis these cells (Hüe et al. 2004, Meresse et al. 2004). Consequently, T cells activated by IL-15 secrete pro-inflammatory cytokines, such as interferon-γ (IFN-γ) (Di Sabatino et al. 2006, Nilsen et al. 1998), which induce the release of tissue-damaging proteins, including matrix metalloproteases (Mohamed et al. 2006). $CD4^+$ T helper cells may participate in IEL activation via production of IL-21, which synergizes with IL-15 to activate cytotoxic $CD8^+$ T cells

(Sarra et al. 2013). In addition, due to IL-15, T cells appear to become resistant to suppression of the regulatory T cells (Tregs) which usually control immune responses (Hmida et al. 2012, Zanzi et al. 2011), (Figure 3).

2.2.2 Adaptive immune response

When immunogenic gliadin peptides enter the *lamina propria*, they can initiate an adaptive immune response in the small intestine of coeliac disease patients (Fraser et al. 2003) (Figure 3). TG2, the target of coeliac disease autoantibodies, is involved in this process by deamidating immunogenic gliadin peptides (Molberg et al. 1998, van de Wal et al. 1998). This deamidation increases the affinity of these peptides to predisposing HLA-DQ2 or -DQ8 molecules on the surface of antigen-presenting cells (APC), such as dendritic cells (DC) (Molberg et al. 1998, Sollid and Jabri 2013, van de Wal et al. 1998). Mature gluten-presenting APCs migrate to Peyer's patches or mesenteric lymph nodes, which belong to GALT, where they induce the activation of gluten-specific $CD4^+$ T helper cells (Meresse et al. 2012). Further, this cascade results in the activation of T cells in the *lamina propria* and secretion of proinflammatory Th1 type cytokines such as IFN-γ, eventually leading to damage to the small-bowel mucosa (Jabri and Sollid 2009).

In addition, T helper cells produce Th2 type cytokines to activate B cells primed in GALT (Jabri and Sollid 2009). B cells migrate to the *lamina propria* and differentiate into plasma cells which produce coeliac disease-specific antibodies against TG2 and gluten-derived peptides (Figure 3) (Di Niro et al. 2012, Meresse et al. 2012). Dimeric IgA antibodies are released into the intestinal lumen as SIgA and also into the circulation (Meresse et al. 2012). Furthermore, these TG2-targeted autoantibodies can be found deposited on the small-bowel mucosa of untreated coeliac disease patients (Korponay-Szabó et al. 2004).

2.3 Coeliac disease antibodies

In genetically predisposed coeliac patients the ingestion of gluten induces secretion of coeliac disease-specific IgA and IgG antibodies targeted against TG2 and gluten-derived deamidated gliadin peptides (Kaukinen et al. 2007, Sulkanen et al. 1998). These specific antibodies are found in the serum of untreated coeliac patients (Kaukinen et al. 2007, Sulkanen et al. 1998). The anti-TG2 autoantibodies are produced locally in the small-bowel mucosa and are also found in intestinal secretions (Mawhinney and Love 1975, Wahnschaffe et al. 2001) and as small-bowel mucosal deposits (Korponay-Szabó et al. 2004). The antibodies deposited in the small bowel are found to be targeted against extracellular TG2 (Korponay-Szabó et al. 2004). Characterization of anti-TG2 autoantibodies has revealed that certain core domain regions of TG2 can be recognised by these Igs and recognition depends on the conformation of the enzyme (Sblattero et al. 2002). It is noteworthy, that anti-TG2 autoantibody deposits may already be found below the small-intestinal epithelial basement membrane in untreated coeliac disease patients before the development of villus atrophy and crypt hyperplasia, and even before the appearance of serum autoantibodies (Kaukinen et al. 2005, Korponay-Szabó et al. 2004, Salmi et al. 2006).

2.3.1 Antibodies in the diagnostics of coeliac disease

Serum antibodies, especially autoantibodies against TG2, are used in the diagnostics of coeliac disease. Tests based on IgA- and IgG-class anti-gliadin antibodies (AGA) have also been used, but their sensitivity and specificity are not optimal (Kaukinen et al. 2007, Mäki et al. 1991). In addition, AGA levels also may be elevated in non-coeliacs, for example in patients suffering from chronic inflammatory bowel disease (Kull et al. 1999), and in the elderly population (Ruuskanen et al. 2010, Ruuskanen et al. 2013). However, detection of antibodies against deamidated gliadin peptides (DGP-AGA) has shown better accuracy than conventional AGA tests (Kaukinen et al. 2007, Sugai et al. 2006). In addition, Kurppa and colleagues have suggested that DGP-AGA would offer a promising method for case-finding among early-stage coeliac disease patients having normal villus morphology (Kurppa et al. 2011).

The discovery of coeliac autoantibodies and their detection by indirect immunofluorescence method have proved more specific than AGA tests (Burgin-Wolff et al. 1991, Sacchetti et al. 1996). First these autoantibodies were characterized as anti-reticulin antibodies (ARA) binding reticulin fibres of rodent tissues, and later as endomysial antibodies (EmA) binding endomysium from the smooth muscle of the monkey oesophagus (Chorzelski et al. 1983). The autoantigen target of EmA was later identified as TG2 (Dieterich et al. 1997). Nowadays monkey oesophagus can be replaced with human umbilical cord, which is used as a substrate for coeliac disease-specific autoantibodies in a more ethical, sensitive and specific manner (Kolho and Savilahti 1997, Ladinser et al. 1994). Nevertheless, since EmA detection is a semiquantitative method in the diagnosis of coeliac disease, interpretation may vary among different laboratories (Rostom et al. 2005).

Since TG2 was identified as the autoantigen of coeliac disease, measurement of anti-TG2 antibodies, usually by quantitative enzyme-linked immunosorbent assay (ELISA), has been a sensitive and specific option in the diagnosis of coeliac disease (Fabiani et al. 2004, Sulkanen et al. 1998, Troncone et al. 1999, Van Meensel et al. 2004). Further, detection of anti-TG2 antibodies has been utilized in the development of coeliac disease rapid tests for point-of-care detection from whole blood samples (Korponay-Szabó et al. 2005, Raivio et al. 2006).

2.3.2 Antibodies in the pathogenesis of coeliac disease

The role of coeliac-specific antibodies in the pathogenesis of coeliac disease is controversial, although based on *in vitro* studies patient-derived antibodies may have several biological effects. Anti-TG2 antibodies can inhibit differentiation of intestinal epithelial cells (Halttunen and Mäki 1999) and induce proliferation of intestinal epithelial cells (Barone et al. 2007), activate monocytes (Zanoni et al. 2006), induce apoptosis in neuronal cells (Cervio et al. 2007) and placental trophoblasts (Di Simone et al. 2010), inhibit angiogenesis (Kalliokoski et al. 2013, Myrsky et al. 2008), increase blood vessel permeability (Myrsky et al. 2009) and inhibit endothelial and intestinal epithelial cell attachment to the matrix (Nadalutti et al. 2014, Teesalu et al. 2012). Recent findings have suggested that secretory coeliac IgA could also mediate transcytosis of gliadin peptides through the intestinal

epithelium by binding to a transferrin receptor CD71, which is overexpressed on the apical side of intestinal epithelial cells in coeliac disease patients (Matysiak-Budnik et al. 2008) (Figure 3). Also, TG2 localized on the apical side of the epithelium may participate in this transcytosis process (Lebreton et al. 2012).

Coeliac disease-specific anti-TG2 autoantibodies can influence the enzymatic activity of TG2 and its cellular functions, but thus far the results from different studies have been contradictory. Coeliac patient-derived autoantibodies have been shown to both enhance (Kiraly et al. 2006, Myrsky et al. 2009) and inhibit (Byrne et al. 2010, Esposito et al. 2002) TG2 activity. However, the relevance of the inhibitory effect of anti-TG2 autoantibodies *in vivo* has been questioned in *in vivo* studies (Dieterich et al. 2003).

2.4 Transglutaminase 2

In the late 1990s, the target of coeliac disease autoantibodies was identified as transglutaminase 2 (TG2), also known as tissue transglutaminase (Dieterich et al. 1997, Dieterich et al. 1998, Sulkanen et al. 1998). TG2 is a multifunctional enzyme ubiquitously expressed in mammalian cells in many organs, including the liver, the heart, the intestine and blood (Klöck et al. 2012). TG2 is found in extracellular locations as well as inside the cell, where it is expressed especially in the cytosol but can also be found in mitochondria and the nucleus (Klöck et al. 2012). Intracellular TG2 acts mainly as a G-protein and at the cell surface it functions as a co-receptor involved in adhesion (Jones et al. 1997). In the extracellular space TG2 promotes crosslinking of peptide-bound glutamine residues to lysine residues, leading to proteolytically resistant ε-(γ-glutamyl)lysine isopeptide bonds (Griffin et al. 2002). The enzymatic activity of TG2 is regulated by several factors such as Ca^{2+} concentration, redox circumstances and guanine nucleotide binding (Figure 4) (Liu et al. 2002, Stamnaes et al. 2010).

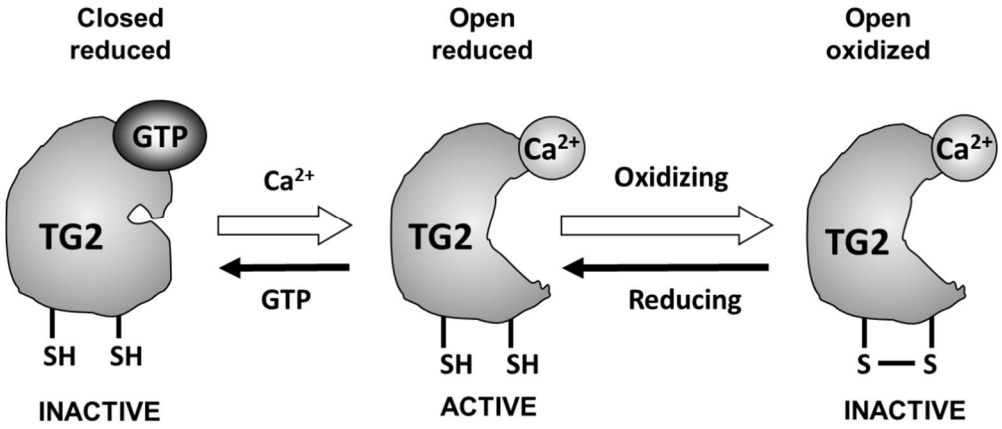


Figure 4. Stages of transglutaminase 2 (TG2) activity under different physiological conditions. In the presence of guanosine triphosphate (GTP) and in the absence of Ca^{2+}, TG2 is reduced, has a closed conformation and is inactive. TG2 is in an open conformation and active in reducing conditions with low GTP and high Ca^{2+} concentrations. Oxidizing conditions inactivate TG2 in its open conformation by the formation of disulphide bond. The figure is based on Kupfer and Jabri 2012.

TG2 is involved in many biological functions and only some of these are dependent on its cross-linking activity (Klöck et al. 2012). Depending on the cellular context and biological indications, TG2 can have either a pro-apoptotic or an anti-apoptotic role (Fesus and Szondy 2005). Extracellular TG2 is involved in matrix assembly (Collighan and Griffin 2009) and cell adhesion (Gaudry et al. 1999, Nadalutti et al. 2011). In addition, TG2 plays a role in wound healing (Haroon et al. 1999), receptor signalling (Nunes et al. 1997), and cell proliferation, invasion, motility and survival (Balklava et al. 2002, Mehta et al. 2006, Nadalutti et al. 2011, Zemskov et al. 2006). Regardless of these various biological effects, TG2-knockout mice have developed and reproduced normally (De Laurenzi and Melino 2001, Nanda et al. 2001).

In the pathogenesis of coeliac disease TG2 has an undisputed role, since the enzyme deamidates specific glutamine residues of gliadin peptides, thus creating epitopes with increased affinity to bind to DQ2 (Figure 5) (Molberg et al. 1998, van de Wal et al. 1998). This leads to enhanced T cell activation paralleled by B cell activation and secretion of TG2-targeted autoantibodies (Di Niro et al. 2012, Sollid

et al. 1997). Recently, Simon-Vecsei and colleagues identified an epitope of TG2 which is recognized by coeliac patient-derived antibodies (Simon-Vecsei et al. 2012). This epitope also has residues similar to those of the most typical immunogenic gluten-derived gliadin peptide bound to HLA-DQ2 (Simon-Vecsei et al. 2012). Iversen and colleagues have since identified other highly autoantibody-specific epitopes clustered in the N-terminal part of TG2 (Iversen et al. 2013). According to Hodrea and colleagues, TG2 is also expressed and catalytically active on the surface of coeliac patient-derived dendritic cells and monocytes, which could enable the direct deamidation of gliadin peptides, contributing thus to inflammatory processes (Hodrea et al. 2010).

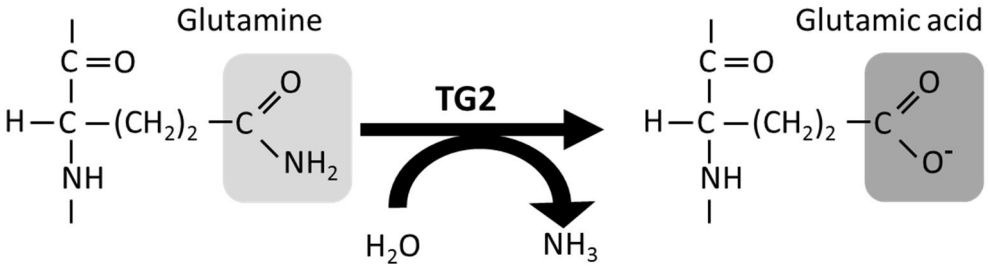


Figure 5. Deamidation of glutamine residue to glutamic acid residue by transglutaminase 2 (TG2). The figure is based on Sollid and Jabri 2011.

2.5 Treatment for coeliac disease

2.5.1 Gluten-free diet

The current treatment for coeliac disease is a strict and lifelong gluten-free diet which aims at relieving the symptoms and healing the intestine as well as revoking the consequences of malabsorption (See and Murray 2006). Avoiding dietary wheat, rye, barley and related grains can lead to clinical, serological and histological remission usually during 1 – 2 years (Kemppainen et al. 1998, Tursi et al. 2006, Wahab et al. 2002). On the other hand, recovery on a gluten-free diet may also take longer, especially in adult patients (Bardella et al. 2007, Wahab et al. 2002).

Moreover, the duodenal lesions do not always normalize completely in adult coeliac patients despite the disappearance of symptoms and negative coeliac disease-related serology (Lanzini et al. 2009). However, even if the villus morphology recovers to normal, IELs may still persist for years after the initiation of a gluten-free diet (Ilus et al. 2012). On a gluten-free diet intestinal permeability may recovered rapidly, even in a few weeks, and this precedes recovery of the small-intestinal morphology (Cummins et al. 1991, Cummins et al. 2001). Likewise gastrointestinal symptoms such as diarrhoea and pain begin to abate immediately after removal of gluten from the diet (Murray et al. 2004). In addition, a gluten-free diet improves nutritional parameters. For example, the body mass index (BMI) increases in underweight coeliac patients and, in contrast, decreases in overweight patients (Murray et al. 2004, Smecuol et al. 1997, Ukkola et al. 2012). Furthermore, a gluten-free diet raises bone mineral density in coeliac patients having osteopenia (Bai et al. 1997, Smecuol et al. 1997), and increases levels of vitamin B and D, folic acid, iron and magnesium (Rubio-Tapia et al. 2013, Vilppula et al. 2011). Among coeliac children with a growth delay, starting a gluten-free diet generally enables catch-up growth (Damen et al. 1994, De Luca et al. 1988). One important aspect considering dietary treatment is that in long-term studies a strict gluten-free diet has been shown to prevent potential later complications, such as cancers (Holmes et al. 1989).

Regardless of the many beneficial results of the gluten-free diet, studies have also revealed disadvantageous influences. A gluten-free diet may be nutrionally unbalanced with unfavourable energy intake from saturated fat and lower intake of dietary fibre (Bardella et al. 2000, Hopman et al. 2006, Mariani et al. 1998, Ohlund et al. 2010). In addition, essential micronutrients such as iron, calcium, magnesium, zinc and B vitamins may remain at a low level (Mariani et al. 1998, Martin et al. 2013, Wild et al. 2010). A poor vitamin status is not necessarily improved, even in 10 years, on a strict gluten-free diet (Hallert et al. 2002).

A small subset, 0.3 % - 10 % of coeliac patients, have refractory coeliac disease (RCD) which does not respond even to a strict gluten-free diet and intestinal villus atrophy with symptoms of malnutrition can persist for more than 6 – 12 months despite the diet (Ilus et al. 2014, Malamut et al. 2012). Based on the phenotype of IELs, this condition can be classified into non-clonal RCD I and clonal RCD II, which is regarded as an intraepithelial T lymphoma with a poor prognosis (Ilus et al. 2014, Malamut et al. 2012).

Although a gluten-free diet is mostly effective and can improve the quality of life (Kurppa et al. 2010, Ukkola et al. 2011), it can also be difficult to maintain for many coeliac patients due to its restrictive aspects as well as the poor availability and expense of gluten-free products (Hallert et al. 2002, Lee et al. 2007). In fact, recent studies have reported that many coeliac patients at the time of diagnosis and on a gluten-free diet desire a medicine and wish for more research on novel alternative therapies for coeliac disease (Aziz et al. 2011, Ukkola et al. 2012).

2.5.1 Alternative non-dietary therapy targets

The complexity of the coeliac pathogenesis offers several possible targets for drug development and there are at this moment therapy studies in different preclinical and clinical trial phases.

Gluten, the main trigger of coeliac disease, can be modified so as to be less toxic. Prolyl-endopeptidase (PEP) expressed in the human intestine is not capable of cleaving gluten-derived peptides, but PEPs expressed in micro-organisms such as bacteria and fungi can cleave and thus also inactivate immunodominant gluten peptides (Ehren et al. 2008, Fuhrmann and Leroux 2011, Shan et al. 2004, Stepniak et al. 2006). Lactobacilli or other probiotics used in sourdough fermentation are also able to proteolytically process proline- and glutamine-rich gluten peptides, thus detoxifying gluten and making it tolerable for coeliac patients (Di Cagno et al. 2004, Greco et al. 2011, Lindfors et al. 2008). On the other hand, identification of immunodominant coeliac disease-specific T cell epitopes in gliadins could offer a possibility of creating “non-toxic” wheat by the removal or modification of the antigenic sequence (Anderson et al. 2000, Tye-Din et al. 2010).

Secondly, ingested gluten can be rendered less toxic by using intraluminal therapies. Oral polymeric resin poly(hydroxyethyl methacrylate-co-styrene sulfonate) has been suggested to block access of the immunotoxic gluten peptides to the mucosal immune cell compartment (Liang et al. 2010, Pinier et al. 2009). Another intraluminal therapy option is gluten toleration and immune modulation. The “hygiene hypothesis” proposes that in developed countries the reduction of infectious diseases further enhances the increasing prevalence of allergic and autoimmune diseases (Bach 2002). In this light, it has been suggested that the

immunity mechanisms in coeliac patients could be modulated by the administration of parasites, for example the hookworm *Necator americanus*, to reduce an immune response to gluten (Daveson et al. 2011, McSorley et al. 2011).

Since both innate and adaptive immune responses are involved in the coeliac pathogenesis, several players in the immune system have been suggested as therapeutic targets (Mukherjee et al. 2012). Blockage of HLA-DQ2 by gliadin peptide analogues could inhibit DQ2-mediated antigen presentation and T cell recognition (Kapoerchan et al. 2010, Xia et al. 2007). Also, blockage of the function of IL-15, IFN-γ, TNF-α and other cytokines or chemokines, and inhibition of their binding to receptors could offer targets for coeliac disease drug development (Mukherjee et al. 2012).

One option for a novel coeliac disease therapy is modulation of intestinal permeability. The structure of zonulin antagonist AT-1001 (larazodite acetate) is derived from zonula occludens toxin secreted by *Vibrio cholera*, and it functions as an inhibitor of paracellular permeability (Paterson et al. 2007). In one pilot experiment AT-1001 reduced intestinal barrier dysfunction, proinflammatory cytokine production and gastrointestinal symptoms in coeliac patients (Paterson et al. 2007). However, later studies showed that although larazodite acetate prevented gastrointestinal symptoms and reduced coeliac antibody titres in patients, there was no significant improvement in their intestinal permeability (Kelly et al. 2013, Leffler et al. 2012).

2.5.2 TG2 inhibitors as a non-dietary therapy target

Downregulation of the adaptive immune response provides a possible target for non-dietary coeliac disease therapy. Inhibition of the deamidation of gliadin peptides could reduce their binding to HLA-DQ2 and HLA-DQ8, thus preventing T cell activation (Siegel and Khosla 2007). Based on this, TG2 inhibitors have been suggested as a possible therapeutic means in coeliac disease (Siegel and Khosla 2007). The location of the enzymatic activity of TG2 in the *lamina propria* provides an opportunity to generate TG2 inhibitors for a localized function (Klöck et al. 2012, Sollid and Khosla 2011).

TG2 inhibitors can be classified into competitive amine inhibitors, irreversible inhibitors and reversible inhibitors based on their mechanism of action (Siegel and Khosla 2007). Irreversible inhibitors bind covalently to TG2, thus preventing deamidation of gliadin peptides, while reversible inhibitors block the access of substrate to the active site of TG2 without modifying the enzyme covalently (Siegel and Khosla 2007). Different TG2 inhibitors have been used to prevent harmful coeliac disease-related effects *in vitro*. Molberg and colleagues have shown that T cell proliferation induced by deamidated PT-gliadin can be reduced by blocking the activity of endogenous TG2 with cystamine, a competitive amine inhibitor (Molberg et al. 2001). Maiuri and colleagues have also described reduced T cell activation in studies with an irreversible TG2 inhibitor R283, which prevents crosslinking of gliadin peptides to endogenous proteins and deamidation of the peptides (Maiuri et al. 2005).

AIMS OF THE STUDY

The main goal of the present study was to investigate the impact of gluten and its derivative gliadin, and coeliac disease -specific autoantibodies in the intestinal epithelial mucosa, and further, means of eliminating their harmful effects.

Specific aims were

1. to study gluten-induced microscopic alterations in the small-bowel epithelium in early developing coeliac disease **(I)**;
2. to study gliadin-induced harmful effects in the small-bowel mucosa of coeliac disease patients **(I)**, an intestinal epithelial cell model **(II, III)** and organ culture of coeliac patient-derived small-bowel mucosal biopsies **(III)**;
3. to study the impact of coeliac disease-specific antibodies on the gliadin permeability in an intestinal epithelial cell culture **(II)** and
4. to study means of removing the effects of gliadin with a gluten-free diet in coeliac disease patients **(I)** and with TG 2 inhibitors in an intestinal epithelial cell culture and in an organ culture of coeliac-patient-derived small-bowel mucosal biopsies **(III)**.

3. MATERIALS AND METHODS

3.1 Patient samples, controls and study designs (I – III)

Coeliac disease patient samples for experiments were taken from EmA-positive adult patients. The patients underwent upper gastrointestinal endoscopy at Tampere University Hospital due to a clinical suspicion of coeliac disease. The study protocol was approved by the Ethical Committee of Tampere University Hospital and all patients gave their written informed assent.

The study cohort in original publication **I** comprised twenty adult EmA-positive patients with a suspicion of coeliac disease (Table 4). Ten patients had normal villus structure (Marsh I) compatible with early developing coeliac disease (Kurppa et al. 2009). Seven of them developed villus atrophy (Marsh IIII) during the follow-up when they continued on a gluten-containing diet, and later, after one year on a gluten-free diet, the mucosal damage was abolished. The remaining three patients with early developing coeliac disease (Marsh I) and ten with overt small-bowel villus atrophy (Marsh III) started a gluten-free diet directly after the first biopsy, and after one year clinical, serological and histological recovery was evident in all. The non-coeliac control group consisted of twenty EmA-negative subjects who had been investigated due to dyspepsia and had no relatives with coeliac disease. All had normal small-bowel mucosal morphology while consuming a standard gluten-containing diet. At every stage of the disease, six small-intestinal mucosal biopsies were taken upon upper gastrointestinal endoscopy from coeliac disease patients and controls. At each visit a clinical examination was also carried out and serum and whole blood samples were drawn. Further, gastrointestinal symptoms were evaluated by structured and validated Gastrointestinal Symptom Rating Scale (GSRS) questionnaires (Svedlund et al. 1988).

The study cohort in original article **II** comprised twelve coeliac disease patients on a gluten-containing diet and three non-coeliac healthy controls who provided

serum samples (Table 4). All sera from coeliac patients were positive for anti-TG2 and EmA, whereas those from non-coeliac control subjects were negative.

The study cohort in original article **III** comprised eight untreated coeliac disease patients (Table 4). All had subtotal villus atrophy with crypt hyperplasia and positive serum TG2 autoantibodies. Three biopsies from each participant were taken for organ culture.

Table 4. Numbers of coeliac and non-coeliac subjects, sample forms and methods in each original publication

Original article	Sample	Method	Early stage CD (n)	Overt CD (n)	Treated CD (GFD) (n)	Non-coeliac control (n)
I	Serum	TG2-ab (ELISA)	10	17	20	20
		EmA (IF)	10	17	20	20
	Biopsies (paraffin sections)	Morphology (IHC, Vh/CrD)	10	17	20	20
		Occludin (IHC)	10	14	15	17
		Claudin-3 (IHC)	10	16	15	17
		ZO-1 (IHC)	10	16	17	19
		E-cadherin (IHC)	9	15	12	17
	Biopsies (frozen sections)	$CD3^+$, $\alpha\beta^+$, $\gamma\delta^+$ IELs (IHC)	10	17	20	20
		Occludin (WB)	4	7	5	6
		Claudin-3 (WB)	4	7	5	6
		E-cadherin (WB)	4	7	5	6
II	Purified IgA from serum	Permeability of Caco-2 cells (TER, FITC-dextran, lissamine-labelled gliadin peptides)		12		3
		TG2 activity (live-cell ELISA)		12		3
III	Biopsies (frozen sections from organ culture)	$CD25^+$, $FOXP3^+$, $IL\text{-}15^+$ cells (IF)		8		3
		$Ki\text{-}67^+$ crypt cells (IHC)		8		3
		pERK1/2 / ERK1/2 (WB)		8		3

CD, coeliac disease; GFD, gluten-free diet; ELISA, enzyme-linked immunosorbent assay; IF, immunofluorescence; IHC, immunohistochemistry; Vh/CrD, villus height crypt depth ratio; IEL, intraepithelial lymphocyte; WB, Western blot; IgA, immunoglobulin A; TER, transepithelial resistance; FITC, fluorescein-isothiocyanate; FOXP3, forkhead box P3; IL, interleukin; (p)ERK1/2, (phosphorylated) extracellular-signal-regulated kinase 1/2

3.1.1 Small-bowel mucosal morphology (I)

For morphology studies small-bowel mucosal biopsy samples were fixed in formalin for haematoxylin-eosin staining. Small-intestinal mucosal morphology was

observed under light-microscopy and the villus structure further evaluated by measuring the villus height crypt depth ratio (Vh/CrD). A ratio less than 2.0 was considered compatible with villus atrophy (Marsh III) and overt coeliac disease (Taavela et al. 2013).

3.1.2 Coeliac serology and HLA genotype (I)

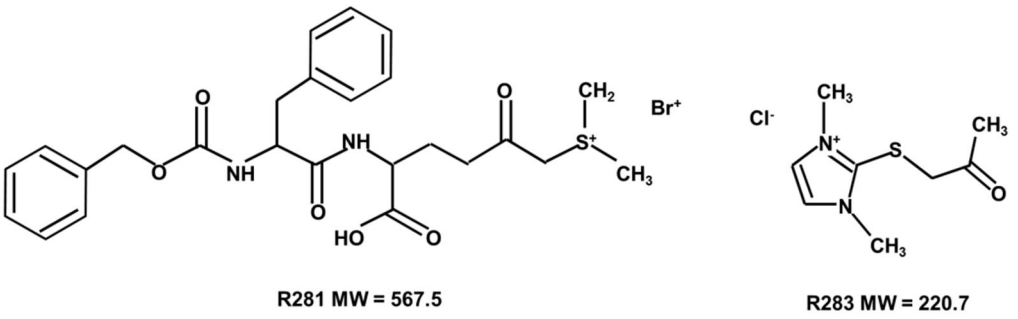
Serum IgA-class EmA was defined by an in-house indirect immunofluorescence method with human umbilical cord as substrate. A serum dilution of 1:≥5 was considered positive. Positive sera were further diluted 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000, and 1:4000 and serum IgA-class anti-TG2 antibodies were detected by ELISA (Celikey®, Phadia, Freiburg, Germany) according to the manufacturer's instructions. Here values >5.0 U were considered to be positive.

The coeliac-type HLA was determined using either the DELFIA Coeliac Disease Hybridization Assay (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland) or the SSP DQB1 low resolution kit (Olerup SSP AB, Saltsjöbaden, Sweden) according to the manufacturer's instructions.

Early developing and overt coeliac disease patients were HLA-DQ2/8-positive with various abdominal symptoms and positive serum TG2 autoantibodies. Non-coeliac patients with dyspepsia and negative coeliac antibodies were chosen as disease controls.

3.2 TG2 inhibitors (II, III)

Cell-impermeable TG2 active site inhibitor R281 and cell-permeable R283 (Figure 6) (Griffin et al. 2008), (Baumgartner et al. 2004) were dissolved in H_2O, the final concentration of the stock solution being 0.1 M. Further, the inhibitors were diluted in Hank's Balanced Salt Solution (Gibco, Invitrogen, the Netherlands) and supplemented in experiments to reach a final concentration of 200 µM. Inhibitors were added one hour prior to gliadin administration to Caco-2 cell cultures or small-intestinal mucosal biopsies in an organ culture system.



*Figur*e 7. Molecular structures of transglutaminase 2 inhibitors R281 and R283

3.3 Organ culture (III)

Three biopsies from each patient were subjected to organ culture (Figure 8): One of the three samples was supplemented with PT-gliadin, one with PT-gliadin and TG2 inhibitor R281, and one with medium only (Table 5). Organ cultures were incubated for 24 h in RPMI-1640 medium (Invitrogen-Gibco, Paisley, Scotland, UK) containing 15 % foetal bovine serum (FBS) (Invitrogen-Gibco), 100 µg/ml streptomycin (Invitrogen Gibco), 100 U/ml penicillin (Invitrogen-Gibco), 4 mM L-glutamine (Invitrogen-Gibco), 50 µg/ml insulin (Sigma-Aldrich Co, St. Louis, Missouri, USA) and 10 mM HEPES buffer (Invitrogen-Gibco). After incubation the culture media were collected and the biopsies snap-frozen with OCT. Both samples were stored at -20 °C until analysed. The levels of TG2-antibodies and IFNα were measured from the organ culture supernatants by ELISA (Celikey®, Phadia, Freiburg, Germany, and BioVendor, Brno, Czech Republic, respectively).

Table 5. Concentrations of study compounds used in *in vitro* experiments

Compound	Concentration	Original article
IgA (CD or non-CD)	1.0 µg/ml	II
p31-43 (unlabelled or lissamine-labelled)	0.5 mg/ml	II
p57-68 (unlabelled or lissamine-labelled)	0.5 mg/ml	II
Human thyroid peroxidase (hTPO) peptide (lissamine-labelled)	0.5 mg/ml	II
PT-BSA	1.0 mg/ml	III
PT-gliadin	1.0 mg/ml	II, III

IgA, immunoglobulin A; CD, coeliac disease; non-CD, non-coeliac; PT, pepsin-trypsin-didested; BSA, bovine serum albumin

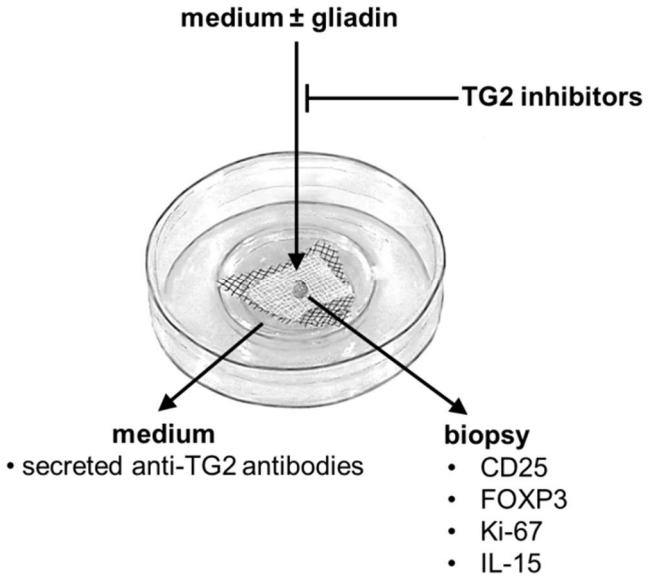


Figure 8. Organ culture system. Coeliac patient-derived intestinal mucosal biopsies were cultured with or without pepsin-trypsin-digested gliadin for 24 hours. A subset of samples were pre-incubated with transglutaminase 2 (TG2) inhibitors for one hour prior to gliadin treatment. Secreted autoantibodies were measured from media and the densities of $CD25^+$ T cells, forkhead box P3 $(FOXP3)^+$ regulatory T cells and proliferating $Ki\text{-}67^+$ enterocytes and interleukin $(IL)\text{-}15^+$ cells were quantified from frozen biopsies.

3.4 Immunostaining of biopsies (I, III)

Immunohistochemical and immunofluorescence staining were performed with sections from either paraffin-embedded or snap-frozen optimal cutting temperature compound (OCT)-embedded (Tissue-Tek, Sakura Finetek Europe, Holland) small-intestinal biopsies. The sections from paraffin blocks were deparaffinised and rehydrated before staining.

Immunohistochemical staining to detect $CD3^+$, $\alpha\beta^+$ and $\gamma\delta^+$ IELs was performed on the frozen sections of small-intestinal mucosal biopsies and positive IELs counted as previously described (Järvinen et al. Am J Gastro 2003). The reference values were 37 cells/mm for $CD3^+$ IELs, 25 cells/mm for $\alpha\beta^+$ IELs and for 4.3 cell/mm for $\gamma\delta^+$ IELs (Järvinen et al. Am J Gastro 2003).

Intestinal mucosal TG2-targeted autoantibody deposits were detected from frozen biopsy sections using direct immunofluorescence as previously described (Korponay-Szabó et al. 2004), (Salmi et al. 2006). The deposits were further graded from negative (0) to strong positive (3) based on their intensity in the villus-crypt area, as described elsewhere (Salmi et al. 2006).

In junction protein staining antigen retrieval was done in boiling 0.01 M citrate buffer or by protease from *Streptomyces griseus* (Sigma Aldrich). Non-specific binding was blocked with goat normal serum (1:20, Vector Laboratories inc., Burlingame, USA) in phosphate-buffered saline (PBS) supplemented with 5 % milk and 5 % bovine serum albumin. The sections were incubated with primary polyclonal rabbit antibodies either at room temperature for one hour or at +4 °C overnight depending on the antibody (Table 6). The sections were incubated with biotinylated anti-rabbit antibodies and endogenous peroxidase was blocked with 0.3 % H_2O_2. The avidin-biotin complex method was used for immunodetection and the sections were counterstained with Harris haematoxylin. The junction protein expression was analysed from microscope images (Olympus BX60F5, Olympus Optical co. LTD, Japan) using ImageJ software (National Institutes of Health, USA). The epithelium was demarcated and the threshold set to visualize epithelial junctions and results were given as a percentage of the measured epithelium area.

Table 6. Antibodies used in immunofluorescence and immunohistochemical staining

Antigen	Description	Producer	Dilution in IF (host)	Dilution in IHC (host)	Original article
β-catenin	Adherens junction protein	BD Transduction Laboratories	1:400 (mouse)		III
CD25	Interleukin-2 receptor	Imgenex, San Diego, CA, USA	1:25 (mouse)		III
CD3	Marker of T cell population	BD Biosciences, San Jose, CA, USA		1:15 (mouse)	I
Claudin-3	Tight junction protein	Zymed Laboratories, Invitrogen, Camarillo, CA, USA	1:100 (rabbit)	1:25 (rabbit)	I, III
E-cadherin	Adherens junction protein	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:100 (rabbit)	1:50 (rabbit)	I, III
FOXP3	Forkhead box P3, a marker of regulatory T cells	Abcam, Cambridge, UK	1:400 (rabbit)		III
IL-15	Interleukin 15, a cytokine of the innate immune system	R&D Systems, Minneapolis, USA	1:50 (mouse)		III
Ki-67	Marker of cellular proliferation	Novus Biologicals, Littleton, CO, USA	1:100 (rabbit)		III
Occludin	Tight junction protein	Zymed Laboratories, Invitrogen, Camarillo, CA, USA	1:100 (mouse)	1:25 (rabbit)	I, III
TCR αβ	T cell receptor	Thermo Scientific, Rockford, IL, USA		1:60 (mouse)	I
TCR γδ	T cell receptor	Thermo Scientific, Rockford, IL, USA		1:140 (mouse)	I
ZO-1	Zonula occludens 1, a tight-junction-associated protein	Zymed Laboratories, Invitrogen, Camarillo, CA, USA	1:300 (mouse)	1:25 (rabbit)	I, III

$CD25^+$, forkhead box P3 $(FOXP3)^+$, Ki-67^+ and IL-15^+ cells were stained in 5-µm-thick cryostat biopsy sections fixed in ice-cold acetone and incubated with goat normal serum (Vector Laboratories Inc., Burlingame, USA). Thereafter the samples were incubated in a primary antibody (Table 6) at 37 °C for 30 min. Bound anti-CD25, and anti-IL-15 primary antibodies were detected by Alexa-conjugated anti-mouse antibody (1:2000; Molecular Probes, Leiden, the Netherlands), anti-FOXP3 by Alexa-conjugated anti-rabbit antibody (1:2000; Invitrogen) and Ki-67 by

biotinylated anti-rabbit antibody (1:100; Vector Laboratories, Inc. Burlingame, CA, USA) at 37 °C for 30 min. Incubation with the biotinylated antibody was followed by treatment with peroxidase-conjugated streptavidin (BioGenex, San Ramon, CA). The densities of $CD25^+$ T cells in the *lamina propria* and $IL\text{-}15^+$ cells on the epithelial level were counted as the number of cells in a total area of one mm^2. The numbers of $FOXP3^+$ lymphocytes and $Ki\text{-}67^+$ proliferating crypt enterocytes were counted and presented as percentages of the total number of *lamina propria* cells and crypt cells, respectively.

3.5 Cell culture (II, III)

Caco-2 (human colorectal adenocarcinoma) epithelial cells (passage 25-81, American Type Culture Collection, HTB-37, Rockville, MD, USA) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco Invitrogen, Paisley, UK) supplemented with 20% FBS 1 % non-essential amino acids, sodium bicarbonate, penicillin (all from Gibco Invitrogen) and sodium pyruvate (Sigma Aldrich). The culture was maintained at 37 °C in a 5 % CO_2 atmosphere and passaged twice a week. Before experiments, confluent monolayers were starved overnight in DMEM supplemented with 1 % or 0.1 % FBS.

3.6 Epithelial permeability measurements (II, III)

Caco-2 cells were cultivated on semipermeable Millicell®-PCF Culture Inserts (pore size 0.4 μm, diameter 12 mm, Millipore, Carrigtwohill, Co. Cork, Ireland). The cells were treated with each study compound (Table 4) for up to 5 h and transepithelial resistance (TER) was measured once an hour using a Millicell® volt-ohm meter (Millipore). The TER values were normalized to control results.

Translocation of 4 kDa fluorescein isothiocyanate (FITC) dextran (Sigma Aldrich) through the Caco-2 cell monolayer was studied by measuring the amount of fluorescence in samples taken from the basal chamber. Fluorescence was detected with Wallac Victor2™ (Perkin Elmer) using wavelengths 485 nm and 535 nm for excitation and emission, respectively.

For the gliadin-peptide translocation assay, total IgA fractions from coeliac and non-coeliac individuals were purified using affinity chromatography and used in the experiments at a concentration of 1 µg/ml as described in earlier studies (Myrsky et al. 2008). Caco-2 cells cultivated on semipermeable culture inserts were treated with purified IgA together with a lissamine-labelled gliadin peptide p31-43 or p57-68, or a human thyroid peroxidase control peptide (Table 5). IgA and the peptides were supplemented apically to the cultures and basal medium samples were taken and measured at timepoints 0, 1, 3 and 5 h. Fluorescence was detected with Wallac Victor2™ using wavelengths 485 nm and 590 nm for excitation and emission, respectively.

All barrier function experiments were performed in triplicate and repeated at least three times.

3.6.1 Analyses of translocated gliadin peptides (II)

Transcytosed lissamine-labelled gliadin peptides were analysed by size-exclusion high performance liquid chromatography (SE-HPLC). The samples were diluted in running buffer (0.1 % sodium dodecyl sulphate (SDS), 20 % acetonitrile, 50 mM sodium phosphate, pH 6.9) and centrifuged (10 min, 11,000 g). The supernatants were analysed using a Superdex Peptide 10/300 GL column (GE Healthcare Biosciences AB, Uppsala, Sweden) with the HP 1050 series HPLC system (Hewlett Packard, Waldbronn, Germany) equipped with an HP 1046A fluorescence detector (Hewlett Packard). The fluorescence was detected using wavelengths 485 nm and 590 nm for excitation and emission, respectively. The results were analysed with ChemStation software (ChemStation for LC 3D systems, Agilent Technologies, Waldbronn, Germany).

An *in vitro* T cell proliferation assay was performed to test the ability of both apical and basal culture media to stimulate T cells. The $CD4^+$ T cell clone TCC387.9, which recognizes DQ2-α-I gliadin epitope in a strict deamidation-dependent manner, was derived from a coeliac intestinal biopsy and cultured *in vitro*. A coeliac patient-derived and Epstein Barr virus (EBV)-transformed HLA-DQ2 homozygous B cell line was used for antigen-presenting cells. Fifty thousand B cells irradiated with 100 Gy were co-incubated overnight at 37 °C with dilutions

of culture medium in RPMI-1640/10% human serum. Forty thousand T cells were added to co-cultures and T cell proliferation measured as the uptake of [^{3}H]-thymidine (1 µCi/well), which was added 20 h prior to harvesting. The cells were harvested after 72 h and [^{3}H]-thymidine incorporation was measured by liquid scintillation counting.

3.7 Determination of TG2 activity (II)

The extracellular transamidation activity of TG2 was determined by live-cell ELISA. Caco-2 cells were cultivated on 96-well plates (Nunclon, Thermo Fisher Scientific, Roskilde, Denmark) and confluent cultures were equilibrated overnight in 1 % FBS medium. A subset of the cultures was incubated with the cell-impermeable TG2 inhibitor R281 for one hour prior to addition of coeliac IgA, non-coeliac IgA or retinoic acid. The cells were incubated with study compounds for five hours at 37 °C. After washing, the cells were incubated with TG2 substrate 0.05 mM monodansylcadaverine (Sigma-Aldrich) for two hours at 37 °C, washed again and fixed with 4 % paraformaldehyde (PFA, Sigma Chemical, St. Louis, MO, USA) for 10 min at room temperature. Fixed cultures were incubated in rabbit anti-dansyl antibody (1:200, Invitrogen) for 30 min at 37°C. The primary antibody was detected with a horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (1:1000, Dako, Glostrup, Denmark) for 30 min at 37 °C. After addition of peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (Slow Kinetic Form, for ELISA, Sigma-Aldrich), the reaction was stopped by 2.5 M H_2SO_4. The absorbance was measured by a Multiscan Ascent spectrophotometer (Thermo Labsystems, Vantaa, Finland) using wavelength 450 nm.

3.8 Determination of actin cytoskeleton rearrangement (III)

Caco-2 cells were cultured on 8-chamber glass slides (BD Biosciences, Eremodegem, Belgium) for 3 – 5 days, starved overnight and pre-treated with either TG2 inhibitor R281 or R283 one hour prior to PT-gliadin, control PT-digested

bovine serum albumin (PT-BSA) or medium only (Table 4). Thereafter, the cell clusters were stained for intracellular F-actin with phalloidin-fluorescein-isothiocyanate (1:300, Sigma Aldrich). The extent of actin cytoskeleton rearrangement was defined by measuring membrane ruffles as a percentage of the cell cluster. The analysis was performed using ImageJ software (National Institutes of Health, USA).

3.9 Immunostaining of Caco-2 cells (III)

For immunofluorescence staining Caco-2 cell culture inserts from the TER assay were fixed with 4 % paraformaldehyde (Sigma-Aldrich) for 10 min. The cell monolayers were permeabilized with 0.05 % Triton X-100 (Sigma-Aldrich) and blocked with 5 % BSA (Sigma-Aldrich). The cells were incubated overnight with antibodies against junctional proteins occludin, claudin-3, ZO-1, β-catenin or E-cadherin (Table 6). They were then incubated with Alexa Fluor -conjugated anti-mouse or anti-rabbit antibody (1:2000; Invitrogen) and mounted with DAPI (4',6-diamido-2phenylindole)-containing VectaShield Mounting Medium (Vector Laboratories Inc., Burlingame, CA, USA).

3.10 Western blot (I, III)

Western blot was performed using the Bio-Rad Mini-Protein Tetra Cell system (Bio-Rad Laboratories, Espoo, Finland). For junction protein analysis the biopsies were cut out of frozen Tissue-Tek and placed in ice-cold H-buffer (150 mM NaCl, 5 mM EDTA, 50 mM potassium phosphate, pH 7.4) containing Complete™ protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). After the homogenization with Ultra Turrax T8 tissuemizer (IKA Labortechnik, Staufen Germany) the biopsy samples were centrifuged at 2500 × *g* for 5 min and supernatants collected. Protein concentrations were determined by Bradford assay and 25 µg of total protein aliquots per lane were loaded on 12 % SDS-polyacrylamide electrophoresis gels. Thereafter, proteins were transferred to nitrocellulose filters (Hybond™-C Extra; Amersham Biosciences Ltd., Little

Chalfont, UK). Non-specific binding was blocked with 5 % milk and the membranes were then incubated with primary antibody (Table 7) overnight. The blots were washed and incubated with horseradish peroxidase (HPR)-conjugated anti-mouse or anti-rabbit antibody (1:2000; DAKO A/S, Copenhagen, Denmark) and then developed with enhanced chemiluminescence (Amersham Biosciences), exposed to autoradiography film (Kodak, New Haven, CT, USA) and scanned for densitometry analysis with 1D image analysis software (Kodak).

For analysis of phosphorylation of ERK1/2 Caco-2 cells were cultivated on 6-well plates for Western blot analysis. Confluent cell monolayers were starved in DMEM supplemented with 0.1 % FBS. The monolayers were pre-treated with either TG2 inhibitor R281 or R283 for one hour prior to addition of PT-gliadin or control PT-BSA. After 30 min of incubation the cells were lysed in SDS-sample buffer containing Complete™ protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and PhosSTOP™ phosphatase inhibitor cocktail (Roche). The detection of ERK1/2 and its phosphorylated form pERK1/2 proceeded as described above.

3.11 Statistical analyses (I – III)

Every independent experiment was performed at least in duplicate and experiments were repeated three or more times. In publication **I** the results are given as means or medians and the ranges are presented as 95 % confidence intervals or standard error of means. Comparisons were made using one-way analysis of variance (ANOVA) followed by appropriate post-hoc tests. Student's *t*-test was used in original articles **I** and **III** and Mann-Whitney *U* test in article **II**. Correlations were studied using Pearson's or Spearman's correlation test depending on the variables (**I**). P-values lower than 0.05 were considered statistically significant.

Table 7. Antibodies used in Western blot

Antigen	Description	Producer	Dilution (host)	Original article
ERK1/2	Extracellular-signal-regulated kinase	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:500 (mouse)	III
pERK1/2	Phosphorylated extracellular-signal-regulated kinase	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:500 (rabbit)	III
Claudin-3	Tight junction protein	Zymed Laboratories, Invitrogen, Camarillo, CA, USA	1:250 (rabbit)	I
Occludin	Tight junction protein	Zymed Laboratories, Invitrogen, Camarillo, CA, USA	1:250 (rabbit)	I
E-cadherin	Adherens junction protein	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:250 (rabbit)	I
γ-tubulin	Microtubule protein	Sigma-Aldrich Co., St. Louis, Missouri, USA	1:8000 (mouse)	III
β-actin	Cytoskeletal actin protein	Sigma-Aldrich Co., St. Louis, Missouri, USA	1:5000 (mouse)	I

4. RESULTS

4.1 Gluten-induced effects on the intestinal barrier

4.1.1 Gluten-induced changes in early developing coeliac disease (I)

Immunohistochemical staining of small-intestinal mucosal biopsies revealed that early signs of coeliac disease are found already before intestinal villus atrophy in patients with early developing coeliac disease. In these patients the expression of epithelial tight junction proteins occludin and claudin-3, the tight-junction-associated protein ZO-1 and adherens junction protein E-cadherin was already decreased compared to controls when villus architecture was still by definition normal (Figure 9). The studies with biopsies derived from patients with early developing coeliac disease showed increased numbers of $CD3^+$, $\alpha\beta^+$ and $\gamma\delta^+$ intraepithelial lymphocytes as well as increased amounts of serum anti-TG2- and anti-endomysial antibodies (Table **I** in original article **I**). Also IgA deposits occurred already in early developing disease (Table I in original article **I**). Patients with early-stage coeliac disease evinced various gastrointestinal symptoms such as abdominal pain and diarrhoea, but mucosal morphology as measured by Vh/CrD was still normal (Table I in original article **I**).

4.1.1 Gluten and gliadin-induced changes in overt coeliac disease (I, III)

The expression of junctional proteins occludin, claudin-3, ZO-1 and E-cadherin was also analysed in biopsies in the active phase of the disease. The expressions of these proteins were diminished compared to control samples as gluten-induced damage progressed from normal villi to overt mucosal atrophy (Figure 9). The expression of most junction proteins correlated negatively with intraepithelial lymphocytosis as

well as serum and mucosal celiac-specific autoantibody levels (Table II in original article **I**). When the correlation between junction protein expression and clinical parameters was studied, a significant negative correlation was found between claudin-3 expression and the occurrence of diarrhoea (Table II in original article **I**). Studies with biopsies from untreated coeliac disease patients showed an increased number of $CD3^+$, $\alpha\beta^+$ and $\gamma\delta^+$ intraepithelial lymphocytes, an increased level of serum anti-TG2 and EmA and the appearance of mucosal IgA deposits (Table I in original article **I**).

In article **III**, the biopsies in organ culture experiments were derived from untreated coeliac disease patients. Incubation of these biopsies with gluten-derived PT-gliadin-containing media induced an increase in $CD25^+$ lymphocytes and $FOXP3^+$ Tregs in the *lamina propria* (Figure 5 in original article **III**). PT-gliadin treatment also raised the number of IL-15^+ cells compared to control cultures. Furthermore, PT-gliadin-supplemented samples showed increased numbers of proliferative Ki-67^+ enterocytes in crypts (Figure 5 in original article **III**). In addition, PT-gliadin increased secretion of anti-TG2 antibodies into culture medium.

4.1.2 Impact of a gluten-free diet on the intestinal barrier (I)

Immunohistochemical staining of coeliac patient-derived intestinal mucosal biopsies showed that the expression of occludin and claudin-3 increased on a gluten-free diet but did not reach the level of non-coeliac controls despite small-bowel mucosal recovery (Figure 9). A similar trend was seen in E-cadherin expression, though the difference in our experimental set-up was not statistically significant. The expression of ZO-1 remained low even in biopsies from patients on a gluten-free diet for one year (Figure 9). Healing of the intestinal mucosa started during the gluten-free diet, as expected. In fact, Vh/CrD was higher than in overt disease, indicating normalization of the mucosa (Table I in original article **I**). Moreover, the number of $CD3^+$, $\alpha\beta^+$ and $\gamma\delta^+$ intraepithelial lymphocytes was decreased after one year of gluten-free diet (Table I in original article **I**). It is noteworthy that serum autoantibodies as well as intestinal mucosal antibody deposits almost disappeared during the gluten-free diet (Table I in original article **I**).

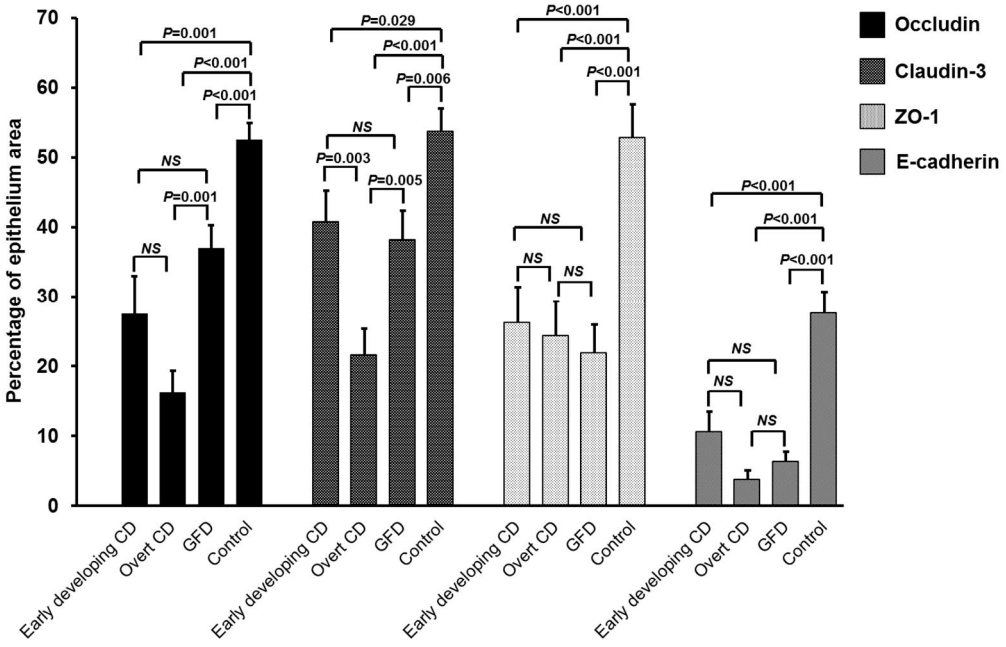


Figure 9. Percentages of epithelial expression of junctional proteins occludin, claudin-3, zonula occludens-1 (ZO-1) and E-cadherin in the immunohistochemically stained small-bowel mucosal biopsy sections derived from coeliac disease (CD) patients and non-CD controls. Coeliac samples are from patients in three different stages of the disease: early developing CD, overt CD and treated CD (gluten-free diet, GFD). Results are given as mean and standard error of mean. *P* values lower than 0.05 were considered statistically significant. *NS*; not significant

4.2 Gliadin-induced effects in Caco-2 cells

TER measurements of PT-gliadin-treated Caco-2 cell monolayers, a model for the intestinal epithelium, revealed increased permeability, shown as decreased recovery of TER values (Figure 1 in original article **II**). In addition, the apical-to-basal flux of a small FITC-labelled dextran molecule was raised compared to control cells, which indicated increased permeability. However, individual gliadin peptides p31-43 and p57-68 had no effect on the permeability measured by TER and FITC-dextran flux (Figure 1 in original article **II**).

One visible PT-gliadin-induced effect in Caco-2 cells was the rearrangement of the actin cytoskeleton, which was seen as an extensive ruffling of the cell membrane

at the edges of cell clusters (Figure 2 on original article **III**). Furthermore, PT-gliadin induced junction protein alterations in Caco-2 cell monolayers. PT-gliadin disturbed the appearance of tight-junction-related proteins ZO-1, occludin and claudin-3, as well as adherens junction proteins E-cadherin and β-catenin (Figure 3 in original article **III**). PT-gliadin-induced phosphorylation of intracellular ERK1/2 was also observed in Caco-2 cells (Figure 4 in original article **II**).

4.3 Coeliac disease-specific antibodies can enhance epithelial permeability (II)

Effects of IgA class antibodies purified from sera from untreated CD patients and non-coeliac controls were studied in Caco-2 cells. Permeability experiments demonstrated that coeliac IgA enhanced the passage of lissamine-labelled gliadin peptides p31-43 and p57-68 through intestinal epithelial cells. Co-incubation of coeliac IgA significantly increased the apical-to-basal flux of lissamine-labelled peptides p31-43 and p57-68 compared to non-coeliac IgA and medium control (Figure 10). Statistically significant coeliac IgA-enhanced passage of lissamine-labelled p31-43 was observed after five hours of treatment, but the same effect with lissamine-labelled p57-68 could already be found at three hours of incubation (Figure 2 in original article **II**). Interestingly, coeliac IgA-treatment increased transcytosis of lissamine-labelled p56-68 more than lissamine-labelled p31-43, and the differences were statistically significant from three hours of incubation (Figure 10B). This would indicate different intracellular trafficking of these peptides. Moreover, coeliac IgA enhanced the translocation of both gliadin peptides without affecting TER and FITC-dextran flux, since they remained at the same level as in control cultures (Figure 1 in original article **II**). Interestingly, non-coeliac IgA significantly enhanced the apical-to-basal translocation of p57-68, but not p31-43, compared to medium control cultures (Figure 10A). However, enhanced passage by IgA derived from untreated coeliac patient was specific for gliadin peptides, since the passage of an irrelevant non-gliadin control, human thyroid peroxidase peptide (hTPO), was not increased (Figure 10A).

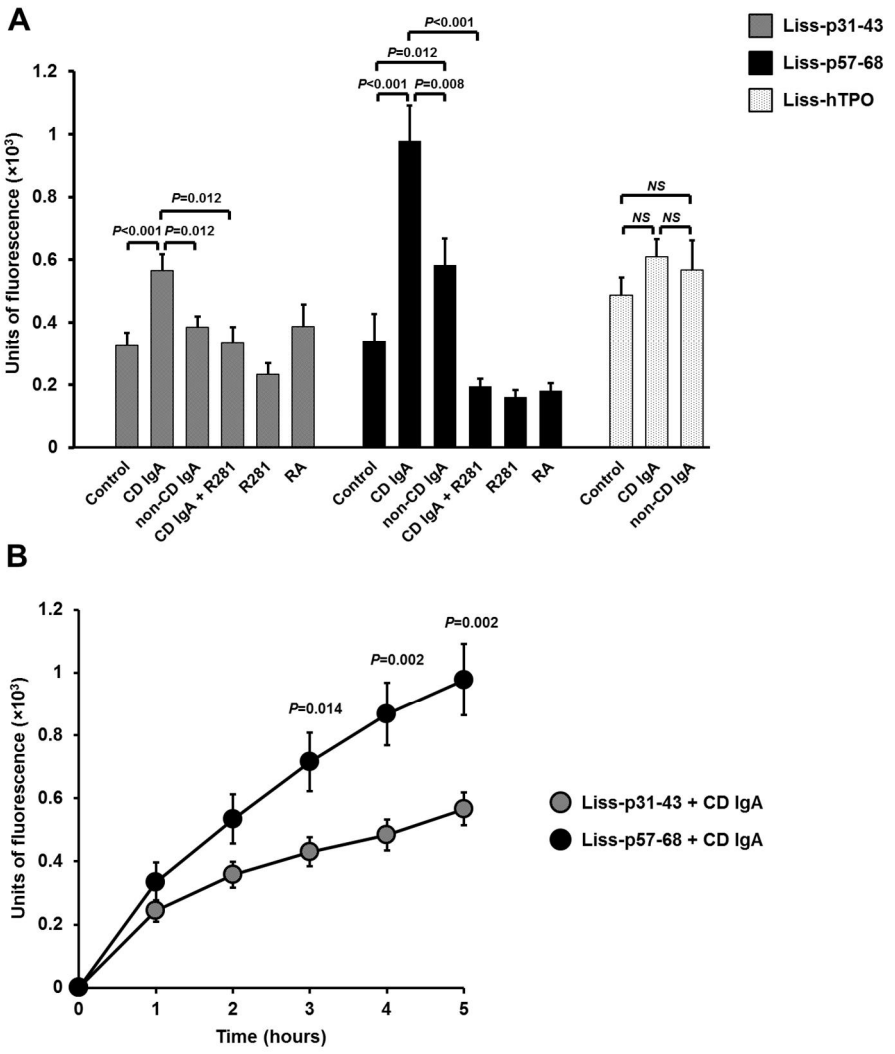


Figure 10. Amount of transcytosed lissamine-labelled gliadin peptides Liss-p31-43 and Liss-57-68 in basal media of Caco-2 cell cultures. Fluorescence was measured after 5 h of incubation in the presence of coeliac (CD) immunoglobulin (Ig) A, non-coeliac (non-CD) IgA, retinoic acid (RA) or medium only (Control). When indicated, transglutaminase 2 inhibitor R281 was added one hour prior to other compounds. Human thyroid peroxidase peptide (hTPO) was used as a lissamine-labelled non-gliadin control (A). Difference between CD IgA-enhanced transcytosis of Liss-p31-43 and Liss-p57-68 (B). The results are presented as mean units of fluorescence ($\times 10^3$) and error bars indicate standard error of mean. *P* values lower than 0.05 were considered statistically significant. Significances between CD IgA and non-CD IgA, IgA + R281 and medium control are shown. *NS*; not significant

The sizes of transcytosed gliadin peptides were analysed with SE-HPLC from the basal medium of translocation assays. Detectable amounts of both p31-43 and p57-68 gliadin peptides passed through the Caco-2 cell monolayer (Figure 3 in original article **II**). An aliquot of medium from translocation experiments with p57-68 was tested for ability to activate T cell proliferation. Results showed that the basal media collected from coeliac IgA-supplemented Caco-2 cultures were able to activate T cell proliferation (Figure 11A). Moreover, TG2 pre-treatment further increased T cell proliferation (Figure 11A). Interestingly, live-cell ELISA with Caco-2 cultures showed that coeliac IgA increased the extracellular TG2 activity similar to retinoic acid, a known activator of TG2 (Figure 11B). Non-coeliac control IgA had no effect on TG2 activity (Figure 11B).

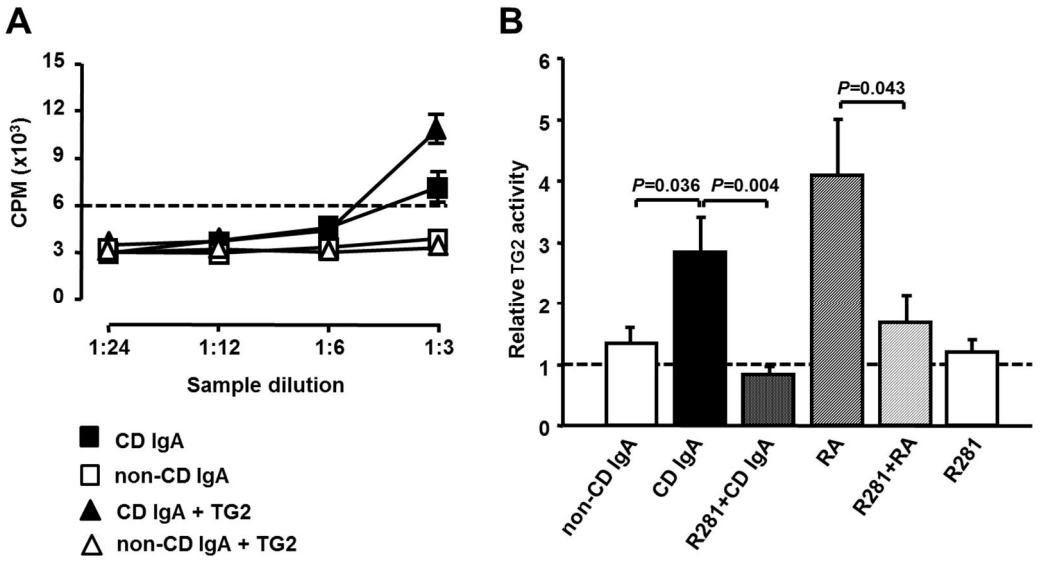


Figure 11. Transcytosed gliadin lissamine-labelled peptide p57-68 in basal media from coeliac (CD) immunoglobulin A (IgA)-treated Caco-2 cultures activated CD patient-derived T cell proliferation, unlike basal media from non-CD IgA-treated cells, and addition of TG2 enhanced the effect (A). T cell proliferation was measured as thymidine incorporation, counts per million (CPM) ($\times 10^3$) and the dotted line corresponds to the threshold of T cell activation (A). CD IgA significantly increased relative TG2 activity, unlike non-CD IgA, and the TG2 inhibitor R281 prevented this enhancement (B). Retinoic acid (RA) was used as positive control and the baseline TG2 activity in Caco-2 cells was set to one (dotted line) (B).

4.4 TG2 inhibitors can prevent coeliac IgA and gliadin effects (II, III)

Studies with an intestinal epithelial cell model and coeliac patient-derived small-bowel mucosal biopsies demonstrated that the cell-impermeable TG2 active site inhibitor R281 and the cell-permeable inhibitor R283 were able to reduce or even prevent gliadin-induced harmful effects. The impact of these two TG2 inhibitors on coeliac IgA- and PT-gliadin-induced effects in Caco-2 cell cultures is summarized in Table 8. PT-gliadin-induced effects inhibited by R281 in coeliac patient-derived small-bowel mucosal biopsies are summarized in Table 9.

Table 8. Targets of transglutaminase 2 inhibitors R281 and R283 in Caco-2 cell cultures. Arrow down indicates an inhibitory effect

Original article	Effect in Caco-2 cell monolayer	R281	R283
II	Transcytosis of gliadin peptides enhanced by CD IgA	↓	ND
II	TG2 activity induced by CD IgA	↓	ND
III	Epithelial permeability increased by PT-gliadin	↓	↓
III	Rearrangement of actin cytoskeleton induced by PT-gliadin	↓	↓
III	Disruption of junction protein appearance induced by PT-gliadin	↓	↓
III	pERK1/2 expression increased by PT-gliadin	↓	no effect

CD, coeliac disease; IgA, immunoglobulin A; TG2, transglutaminase 2; PT, pepsin-trypsin-digested; pERK1/2, phosphorylated extracellular-signal-regulated kinase 1/2; ND, no data

4.4.1 TG2 inhibitors in an intestinal epithelial cell model

In the gliadin peptide translocation assays TG2 inhibitor R281 was able to counteract the coeliac IgA-enhanced transcytosis of gliadin peptides p31-43 and p57-68 (Figure 10). It was also able to prevent the increased TG2 activity induced by coeliac patient-derived-IgA and retinoic acid. However, this inhibitor had no effect on the basal activity of TG2 (Figure 11B).

Pre-treatment of Caco-2 cell cultures with TG2 inhibitors R281 or R283 prevented the PT-gliadin-induced increase in permeability. TER measurements showed that permeability in the cells treated with both, TG2 inhibitor and PT-gliadin, decreased to the same level as the PT-BSA control (Figure 1 in original article **III**). When PT-gliadin induced actin cytoskeleton rearrangement in Caco-2 cells, the presence of R281 or R283 significantly reduced this (Figure 2 in original article **III**). However, TG2 inhibitors did not completely normalize PT-gliadin-induced ruffling to the PT-BSA control level (Figure 2 in original article **III**). Both TG2 inhibitors also improved the appearance of tight-junction-related proteins ZO-1, occludin and claudin-3, as well as adherens junction proteins E-cadherin and β-catenin disrupted by PT-gliadin in Caco-2 cell cultures (Figure 3 in original article **III**). Moreover, in Caco-2 cells R281 inhibited PT-gliadin-induced phosphorylation of ERK1/2, thus also affecting intracellular signalling cascades (Figure 4 in original article **III**).

4.4.2 The effect of TG2 inhibitors in coeliac patient-derived small-bowel biopsies

Small-intestinal mucosal biopsies were derived from untreated coeliac disease patients and pre-incubated with TG2 inhibitor R281 to study its ability to prevent gliadin-induced effects *ex vivo*. R281 significantly prevented the gliadin-induced increase in the density of $CD25^+$ lymphocytes and $FOXP3^+$ regulatory T cells in the *lamina propria* (Figure 5 in original article **III**). In addition, R281 significantly inhibited the PT-gliadin-induced increase in IL-15^+ cells. In the crypts of biopsies, TG2 inhibitor R281 prevented gliadin-induced proliferation of enterocytes (Figure 5 in original article **III**). In contrast, the secretion of TG2 autoantibodies into culture medium remained elevated in gliadin-supplemented samples despite R281 pre-treatment.

Table 9. Targets of transglutaminase 2 inhibitor R281 in organ cultures of coeliac disease patient-derived small-bowel mucosal biopsies. Arrow up indicates increase induced by PT-gliadin and arrow down indicates inhibition of the increase

Original article	Effect in coeliac patient-derived biopsies	PT-gliadin	PT-gliadin + R281
III	Number of $CD25^+$ lymphocytes	↑	↓
III	Number of epithelial $IL\text{-}15^+$ cells	↑	↓
III	Number of regulatory T cells	↑	↓
III	Proliferation of crypt enterocytes	↑	↓
III	Secretion of coeliac-specific antibodies	↑	no effect

PT, pepsin-trypsin-digested; IL, interleukin

5. DISCUSSION

5.1 Altered intestinal barrier capacity in coeliac disease

A strong relationship between overt coeliac disease and alterations in junction protein expression in the small-intestinal mucosa has been reported in the literature, and these changes have been associated with mucosal damage with villus atrophy (Ciccocioppo et al. 2006, Montalto et al. 2002, Pizzuti et al. 2004, Schumann et al. 2012, Szakal et al. 2010). However, studies concerning changes in the expression of intestinal epithelial junction proteins in early-stage coeliac disease without mucosal damage are currently lacking. The present study demonstrated that the expression of tight junction proteins occludin and claudin-3, a tight-junction-associated ZO-1 and an adherent junction protein E-cadherin, are already decreased in early developing coeliac disease when the mucosal architecture is still normal.

Coeliac disease develops gradually from initial signs of inflammation to severe damage in the flattened small-intestinal mucosa (Marsh 1992). However, many of the coeliac disease-specific indicators are already present in coeliac disease patients before morphology-based diagnosis is possible. In addition to various gastrointestinal symptoms such as abdominal pain and diarrhoea, serum anti-TG2 antibodies and anti-endomysium antibodies, as well as mucosal antibody deposits, are already to be found in early developing coeliac disease (Kaukinen et al. 2001, Kurppa et al. 2009, Paparo et al. 2005, Salmi et al. 2006). Further, the number of intraepithelial lymphocytes is increased in the small-intestinal mucosa in early developing coeliac disease with normal villus-crypt structure, indicating inflammation in the gut prior to mucosal architecture damage (Salmi et al. 2006).

Significant differences in the expression of junctional proteins were found between coeliac patients and non-coeliac controls, but not always between coeliac groups in different phases of the disease, even though there seemed to be a trend in the junctional protein expression pattern. A longitudinal analysis of intestinal junction protein expression in individual coeliac patients showed that there are

variations in the expression levels at each stage of the disease (Figure 3 in original article I). On the other hand, if there are differences in junction protein expression between very-early-stage and overt coeliac disease, after a certain timepoint during the progression of the disease these differences may not necessarily prevail. Previous studies show that in coeliac disease the typically decreased junction protein expression is improved after adoption of a gluten-free diet (Montalto et al. 2002, Pizzuti et al. 2004). We confirm this observation in the present study. However, even if the expression of occludin, claudin-3 and E-cadherin is improved during a gluten-free diet compared to overt coeliac disease, the improvements do not yet reach the control level in one year. This is in line with previous studies demonstrating that increased intestinal permeability and changes in the epithelial barrier do not normalize completely during a short-term gluten-free diet at least in adults (Kemppainen et al. 2005, Smecuol et al. 1997, Vilela et al. 2008). On the other hand, gastrointestinal symptoms disappear on a gluten-free diet. Exclusion of dietary gluten normally removes symptoms of coeliac disease if the diet is strict, and a gluten-free diet can rapidly, usually within days, bring a resolution of symptoms related to diarrhoea in coeliac patients (Murray et al. 2004). However, studies with adult coeliac patients have revealed that despite a strict gluten-free diet some individuals may suffer from persistent gastrointestinal symptoms (Murray et al. 2004, Paarlahti et al. 2013, Paavola et al. 2012).

In the present study, analysis of PT-gliadin-treated intestinal epithelial Caco-2 cells showed that gliadin induces disruption of junctional proteins. This in in accord with previously published *in vitro* findings (Dolfini et al. 2005, Sander et al. 2005). Smecuol and colleagues have observed increased intestinal permeability measured by lactulose/mannitol ratio, and zonulin up-regulation even in dermatitis herpetiformis patients without histologic damage (Smecuol et al. 2005). If up-regulation and release of zonulin occurs already in early-stage coeliac disease, leading to increased intestinal permeability and alterations in epithelial junction proteins, this might contribute to a new understanding of coeliac disease pathogenesis. In this scenario coeliac-inducing gliadin peptides may enter the intestinal epithelium and initiate an immune response in the *lamina propria*. This could lead to progression of mucosal damage and secretion of coeliac-specific antibodies, which in turn may contribute to an increase in intestinal permeability.

5.2 Role of coeliac disease-specific antibodies in the pathogenesis

The role and significance of coeliac disease-specific antibodies has been under debate, since there are controversial theories and study results as to the effects of these antibodies. However, the formation and secretion of coeliac disease antibodies is indisputable. The present findings suggest that coeliac disease-specific IgA plays a role in the pathogenesis by enhancing the translocation of gluten-derived gliadin peptides through the intestinal epithelium and by activating TG2.

5.2.1 Translocation of gliadin peptides

One important stage in the coeliac pathogenesis is the translocation of gliadin peptides across the intestinal epithelium to the *lamina propria*. Previous studies suggest that gliadin peptides can enter the *lamina propria* by either paracellular or transcellular route (Clemente et al. 2003, Schumann et al. 2008). The results of the present study suggest that coeliac patient IgA-class antibodies enhance the translocation of gliadin peptides p31-43 and p57-68 through the intestinal epithelial cell layer. This enhanced transcytosis occurs without affecting paracellular permeability, since neither coeliac patient IgA nor gliadin peptides alone have an effect on permeability measurements. This finding is in line with the observations of Schumann and colleagues, who showed that the immunodominant alpha(2)-gliadin-33-mer peptide crosses the intestinal epithelium via endocytosis in coeliac disease patients (Schumann et al. 2008). In addition, Matysiak-Budnik and colleagues have shown that retrotranscytosis of coeliac SIgA via transferrin receptor (CD71) transports gliadin peptides into the *lamina propria* (Matysiak-Budnik et al. 2008). This suggests a failure in the role of SIgA, which should normally be protective in excluding dietary antigens (Matysiak-Budnik et al. 2008). Moreover, previous results suggest that toxic and immunogenic gliadin peptides are sorted differentially in the endocytotic system in intestinal epithelial cells (Barone et al. 2010, Maiuri et al. 2010, Zimmer et al. 2010). According to previous findings, the toxic gliadin peptides seem to accumulate in intestinal epithelial cells increasing thus cellular

stress and leading to innate response (Barone et al. 2010, Luciani et al. 2010, Zimmer et al. 2010). Therefore also our observation of the differences in the passing rates of p31-43 and p57-68 in the presence of coeliac IgA could be explained by the different trafficking of these two gliadin peptides.

5.2.2 Activation of T cells by translocated gliadin peptides

SE-HPLC analysis revealed that although the majority of gliadin peptides were degraded during translocation from the apical to the basal side of the Caco-2 cell monolayer, a portion of them remained intact. In fact, according to Matysiak-Budnik and colleagues, incomplete degradation of the immunodominant 33-mer and protected transport of toxic p31-49 might explain their effects in active coeliac disease (Matysiak-Budnik et al. 2003). The present study demonstrated that regardless of the partial degradation of p57-68, basal media containing this peptide and collected from coeliac IgA-treated Caco-2 cell cultures were able to induce proliferation of T cells without TG2 pre-treatment. Since the activation of this coeliac patient-derived T cell clone was dependent on deamidated gliadin peptides, the results would suggest that these immunodominant gliadin peptides are present and deamidated during their passage through epithelial cells. Interestingly, regardless of supplementation, apical media from translocation experiments were also able to induce T cell proliferation. This may indicate that the deamidation of gliadin peptide p57-68 by TG2 might occur in epithelial cells, this giving a possible clue as to where this important step in the pathogenesis could take place. Maiuri and colleagues have reported that TG2 is located on the surface of small-intestinal epithelial cells (Maiuri et al. 2005). However, as the concentration of p57-68 in apical medium was markedly higher than that of translocated p57-68 in basal medium, low-level acid-mediated deamidation during the manufacturing process could have been possible.

The present findings demonstrate that, in addition to enhanced transcytosis of gliadin peptides through intestinal epithelial cells, coeliac-patient IgA increases TG2 enzymatic activity. Also retinoic acid, a metabolite of vitamin A and a TG2 activator, increases TG2 activity in Caco-2 cells. However, retinoic acid had no

enhancing effect on the peptide translocation, unlike coeliac patient IgA. Previously, Korponay-Szabó and colleagues demonstrated that coeliac patient-derived affinity-purified antibodies against deamidated gliadin peptides can recognize TG2 (Korponay-Szabó et al. 2008). Thus the increased transepithelial passage of gliadin peptides could be mediated by cross-reactive antibodies which recognise both TG2 and deamidated gliadin peptides. Nevertheless, the ability of coeliac IgA to activate TG2 is disputable, since in previous studies coeliac patient-derived autoantibodies have enhanced (Kiraly et al. 2006, Myrsky et al. 2009, Nadalutti et al. 2014), but also inhibited (Byrne et al. 2010, Esposito et al. 2002) TG2 enzymatic activity. One intriguing aspect in the present study is the origin of patient IgA, which is serum instead of intestinal sample. Serum IgA originating from bone marrow, lymph nodes and spleen is mainly monomeric, while in intestinal secretion IgA synthesized locally by plasma cells in the *lamina propria* is mainly polymeric (Kutteh et al. 1982, Mestecky et al. 1986).

5.3 TG2 inhibitors as a treatment for coeliac disease

TG2 has a central role in the pathogenesis of coeliac disease, since the enzymatically active TG2 deamidates gluten-derived gliadin peptides, thus making them recognizable for the cells of the immune system in genetically susceptible coeliac disease patients. In this light, TG2 inhibitors have been suggested as potential drugs for the treatment of coeliac disease (Sollid and Khosla 2011). The present study demonstrated that two TG2 inhibitors, a cell-impermeable R281 and a cell-permeable R283, were able to prevent gliadin-induced toxic effects in intestinal epithelial cell cultures. Furthermore, R281 selected for *ex vivo* studies also showed inhibition of gliadin-induced effects in small-intestinal mucosal biopsies derived from untreated coeliac disease patients.

5.3.1 TG2 inhibition in the intestinal epithelial cells

The present results would indicate that TG2 inhibitors can block direct gliadin-triggered toxic effects on intestinal epithelial cells independently of T cells.

According to previous studies, gliadin is known to reduce TER and the expression of junction proteins as well as to induce rearrangement of actin filaments and phosphorylation of ERK1/2 signalling protein in intestinal Caco-2 epithelial cells (Barone et al. 2007, Ciccocioppo et al. 2006, Giovannini et al. 1995, Stenman et al. 2009). Here, TG2 inhibitors R281 and R283 evinced protective action against these gliadin-induced effects. Interestingly, in the presence of TG2 inhibitors, TER values are higher than in control cultures. This would indicate that the permeability of Caco-2 monolayers treated with TG2 inhibitors recovered over the baseline. TG2 inhibitors very likely block this direct gliadin-triggered damage to epithelial cells by different mechanisms than in the adaptive-immunity-mediated reactions. TG2 is known to crosslink proteins in the extracellular matrix, an important modulator of the expression of tight junction proteins and epithelial permeability (Griffin et al. 2002, Koval et al. 2010). Since TG2 can catalyse the binding of gliadin peptides to extracellular matrix collagen (Dieterich et al. 2006), this could also have occurred in our Caco-2 cell culture model. Cross-linked gliadin in the matrix could cause structural and functional abnormalities eventually leading to increased epithelial permeability, which is linked to diminished expression of junction proteins. Thus TG2 inhibitors may have prevented gliadin-induced harmful effects by blocking the processing of the extracellular matrix also in Caco-2 cell cultures. The TG2 inhibitor R281 inhibits the coeliac IgA-enhanced transcytosis of gliadin peptides indicating that TG2 participates in the translocation process. Lebreton and colleagues showed similar inhibition of the SIgA-CD71-dependent transport of gliadin peptide p31-49, proposing that TG2 forms complexes with CD71 and SIgA and is thus necessary for endocytosis (Lebreton et al. 2012). This may indicate that the binding of R281 to the active site of TG2 prevents the binding of the IgA-gliadin-complex to TG2, further preventing CD71-mediated endocytosis. On the other hand, the inhibitory effect on the transcytosis of gliadin peptides may also be due to prevention of gliadin peptide deamidation by TG2 and lack of affinity to coeliac IgA.

5.3.2 TG2 inhibition in the immune system

Previous *ex vivo* studies have demonstrated that TG2 inhibitors could be effective in preventing gliadin-induced activation of the adaptive immune system (Maiuri et

al. 2005, Molberg et al. 2001). Maiuri and colleagues have used an organ culture system to study the effects of gliadin peptides in small-bowel mucosal biopsies derived from coeliac patients (Maiuri et al. 2005). They have shown that the cell-permeable TG2 inhibitor R283 prevents the gliadin-peptide-induced increase in $CD3^{+}CD25^{+}$ T cells in the *lamina propria* (Maiuri et al. 2005). Molberg and co-workers have demonstrated that TG2 inhibitors prevent proliferation of intestinal T cells derived from biopsies from coeliac patients, although such an effect has not been detectable in T cells from all patients (Molberg et al. 2001). In the present study, effects of the cell-impermeable TG2 inhibitor R281 in coeliac-patient-derived biopsies support these findings. Generally, Tregs are considered protective and advantageous for immune equilibrium, but in coeliac disease their suppressive effect may be impaired (Hmida et al. 2012, Zanzi et al. 2011), even if their absolute number is increased (Borrelli et al. 2013, Tiittanen et al. 2008, Vorobjova et al. 2011). The present study shows the density of FOXP3-positive Tregs in the *lamina propria* to be reduced by TG2 inhibitor R281. The question remains whether TG2 inhibitor-mediated reduction in the density of Tregs is a favourable effect in the treatment of coeliac disease.

In addition to preventing a direct gliadin-triggered effect on T cells, TG2 inhibitor R281 also abolishes the gliadin-induced increase in proliferating Ki-67-positive crypt epithelial cells. Crypt hyperplasia due to enhanced proliferation of epithelial cells is characteristic of untreated coeliac disease and potential coeliac patients (Barone et al. 2011, Maiuri et al. 2000, Nanayakkara et al. 2013). This process is, at least partly, induced by proinflammatory cytokines secreted by activated T cells in response to ingested gliadin (Barone et al. 2011, Maiuri et al. 2000). Since TG2 is a major factor in T cell activation and downstream secretion of proinflammatory cytokines in coeliac patients, the inhibition of TG2 could block these actions and thereby prevent gliadin-induced crypt epithelial cell proliferation, as shown in the present study.

5.3.2 Challenges in using TG2 inhibitors in the treatment of coeliac disease

TG2 could offer a potential target for non-dietary therapy and drug development in the treatment of coeliac disease (Siegel and Khosla 2007). The TG2 inhibitors used

in the present study were the cell-impermeable R281 and the cell-permeable R283. Both showed promising inhibitory effects on gliadin-induced changes in *in vitro* intestinal models. However, these TG2 inhibitors are not specific for intestinal mucosal TG2. In general, this lack of tissue-directed specificity is a major issue in using TG2 inhibitors. Since TG2 is multifunctional and expressed ubiquitously, the non-specificity may cause side-effects when TG2 inhibitors are used *in vivo*. On the other hand, analyses with different mouse tissues have shown that TG2 inhibition could be compensated by other transglutaminases (Deasey et al. 2013). For example, in the heart and liver this compensation can occur on the transcriptional level, and in the aorta, kidney and cartilage on the functional level (Deasey et al. 2013). However, novel drugs for coeliac disease should be designed for selective delivery to the gastrointestinal system or their action should occur only in the small bowel. For example in liver fibrosis and chirrosis TG2 inhibitors have been envisaged as a potential treatment in which a liposome-based site-specific carrier system could deliver them into the liver (Daneshpour et al. 2011). Nevertheless, the lack of a proper coeliac disease animal model restricts clinical trials of TG2 inhibitors and other alternative drugs *in vivo* (Marietta and Murray 2012, Siegel and Khosla 2007). Further studies of TG2 inhibitors as a treatment for coeliac disease should take into consideration both innate and adaptive immunity as well as other systemic effects.

5.4 Strengths, limitations and future prospects

The present study revealed new aspects regarding alterations in the intestinal barrier and the pathogenesis in coeliac disease. The research focusing on the gluten-induced harmful effects on the gut epithelium proceeded from the patient level to the molecular level. The study reported in article **I** offered an exceptional opportunity to follow the same patients in three different stages of coeliac disease, including early developing disease, which yielded new information on the coeliac pathogenesis. No such research has been done hitherto. In addition, patient samples were important in studies **II** and **III**. Coeliac and non-coeliac serum IgA and small-bowel biopsies from coeliac disease patients brought *in vitro* studies closer to real

life. The variation between individuals was not only a restrictive factor in statistical analyses but also a representative example of individual responses in coeliacs.

This study covered research into the intestinal barrier in coeliac disease from the perspective of both innate and adaptive immunity. The methods chosen were well-tried in earlier studies, albeit here adjusted them to our purposes. Especially organ culture is a suitable method to demonstrate both toxic and immune-mediated gliadin-induced effects in coeliac disease. Also experiments with Caco-2 cells gave representative and comparable results indicating that this cell culture model can be used in coeliac disease research. In addition, the methods were either quantitative or semiquantitative meaning that the results can be repeated.

The limitations in study **I** were for the most part related to samples sizes. The junction protein measurements and the sample size in the early developing coeliac disease group was rather small, only ten patients, which affected the statistical analyses. The variation in the quality of biopsy sections brought challenges in immunohistochemical staining, and some samples were excluded due to detached epithelium.

Correlation analyses between junction protein expression and gastrointestinal symptoms were performed for the whole study cohort in study **I**, including non-coeliac controls. However, non-coeliac control subjects were not asymptomatic, since it is unethical to take biopsies from healthy individuals. Due to the skewed data, one should be cautious in interpretating the correlation analysis between junction protein expression and gastrointestinal symptoms. It would therefore be interesting to expand the sample sizes and study intestinal epithelial junction protein expression in a larger cohort. Expanded sample sizes in each stage of coeliac disease could enable the exclusion of symptomatic non-coeliacs from correlation analyses between intestinal junction protein expression and gastrointestinal symptoms. In addition to the connection between claudin-3 expression and diarrhoea, this could also reveal correlations between other junction proteins and gastrointestinal symptoms. Another issue in study **I** was the duration of the gluten-free diet. Undeniably, one year is a rather short time. Thus long-term follow-up studies with the same patients would be interesting, albeit challenging to put into practise.

As noted above, the problem in study **II** concerning the role of coeliac IgA in the coeliac disease pathogenesis is the source of IgA. Total IgA was extracted and purified from sera and not from intestinal secretions. Intestinal dimeric IgA may act

in the same way as serum monomeric IgA, but distinctions cannot be excluded. This notwithstanding, the present study demonstrated that the simultaneous treatment of epithelial Caco-2 cells with both coeliac IgA and gliadin peptides enhanced peptide transcytosis through intestinal epithelial Caco-2 cells. Since total IgA from coeliac patients contains not only anti-TG2 and AGA, but also other antibodies, it would be interesting to test whether these specific antibodies are also able to enhance transcytosis of gliadin peptides and increase TG2 activity. More detailed studies could explain the mechanism of this effect. In intestinal epithelial cells, an analysis by confocal microscopy could shed light on the co-localization of gliadin peptides and endosomes, which might reveal details of transcytosis.

In the present study, the intestinal epithelial permeability experiments focused on measurements of TER (**II, III**), as well as the flux of FITC-dextran (**II**) and lissamine-labelled gliadin peptides (**II**) in Caco-2 cells. Also gliadin-induced effects and their prevention by TG2 inhibitors were demonstrated in Caco-2 cell monolayers. This human colorectal adenocarcinoma cell line is widely used as a model for the intestinal epithelium, since Caco-2 cells differentiate spontaneously, expressing many morphological and biochemical characteristics of the small intestine (Sambuy et al. 2005). Nevertheless, although Caco-2 cells have been used in coeliac disease studies, it must be borne in mind that the cells are cancer cells and not from a coeliac patient. To study the effects of coeliac IgA and TG2 inhibitors further, *ex vivo* and *in vivo* studies would be needed. Lack of an authentic coeliac animal model limits *in vivo* research, but at least intestinal permeability tests could be extended to *ex vivo* experiments with biopsies by using an Ussing chamber or another more advanced system. Such an approach could demonstrate coeliac-induced effects on permeability and intestinal barrier function on the organ level including the impact of immune cells.

The present study introduces a number of further details in the complex pathogenesis of coeliac disease. However, the first trigger of the disease remains unknown. Why can symptoms appear and coeliac disease develop in genetically susceptible individuals at any age regardless of the time of gluten intake? On the other hand, why do only some HLA-DQ2/8-positive individuals develop the disease? Is there an unidentified internal or external factor which suddenly causes an undesirable response to gluten? Probably the activation of the immune system is somehow involved in this. Undoubtedly, impairment of the intestinal barrier

function is a consequence of gluten response, but it may also be a reason for the development of coeliac disease. Future studies may find more early markers for the disease, and perhaps even the first trigger, which could be useful in the development of diagnosis and treatment.

SUMMARY AND CONCLUSIONS

The present study considered coeliac disease from the perspective of processes in the small-intestinal epithelial mucosa. Coeliac patient-derived small-bowel mucosal biopsies and intestinal epithelial Caco-2 cell monolayers were used to demonstrate gluten-induced effects in the intestinal epithelial barrier function.

Small-bowel mucosal lesions with villus atrophy and crypt hyperplasia are prominent characteristics for the diagnosis of coeliac disease. However, various gastrointestinal symptoms and coeliac-specific autoantibodies are already present before visible mucosal damage in the small intestine. Experiments with biopsies derived from patients in the early stage of coeliac disease revealed disruptions in the expression of epithelial junction proteins, although the villus architecture was still normal by definition. Later these patients were diagnosed with coeliac disease, evincing intestinal villus atrophy and crypt hyperplasia, elevated coeliac-specific autoantibodies, increased numbers of IELs and a number of gastrointestinal symptoms. In addition, intestinal epithelial junction protein expression remained decreased during the active phase of the disease. However, one year on a gluten-free diet brought an improvement in junction protein expression and other measured indicators of coeliac disease.

Coeliac-specific antibodies are already present in the intestinal mucosa and serum in early-stage disease. The present findings suggest that these coeliac-specific antibodies play a role in the pathogenesis of the disease by promoting the translocation of gluten-derived toxic and immunogenic gliadin peptides through the intestinal epithelium. Further, translocated immunogenic gliadin peptides are able to activate coeliac patient-derived T cells, this implying that the peptides could be deamidated during the IgA-enhanced transcytosis process. Related to this, coeliac IgA was found also to activate TG2 in intestinal epithelial cells.

The enzyme activity of TG2 could be prevented by TG2 inhibitors R281 and R283. These inhibitors reduced or prevented gliadin-induced effects in intestinal epithelial cells and coeliac patient-derived small-bowel mucosal biopsies. In view of

the central role of TG2 in the pathogenesis, TG2 inhibitors are being considered as a potential non-dietary therapy for coeliac disease.

In conclusion, signs of coeliac disease exist already before visible morphological small-bowel mucosal damage. Gluten-induced disruption of intestinal epithelial cell junctions may be one of these first signs. Impairment of the intestinal barrier enables the cross of gluten-derived gliadin peptides through the epithelium thus enhancing the immune reaction induced by deamidated gliadin peptides. This deamidation by TG2 leads to the production of coeliac-specific antibodies that, in their part, contribute to the pathogenesis further by enhancing the transcytosis of gliadin peptides through intestinal epithelium. However, inhibition of the deamidation activity of TG2 can prevent harmful coeliac-related effects induced by these gliadin peptides.

The present study demonstrated the processes leading to the disruption of intestinal barrier function in coeliac disease and revealed new aspects for the pathogenesis in intestinal mucosal epithelium. These results could also offer opportunities for the development of new therapies for coeliac disease.

ACKNOWLEDGEMENTS

This study was carried out at the School of Medicine, University of Tampere, and at the Departments of Internal Medicine, Paediatrics, and Gastroenterology and Alimentary Tract Surgery, Tampere University Hospital.

The work was financially supported by the Academy of Finland, the Sigrid Juselius Foundation, the Competitive State Research Financing of the Expert Responsibility Area of Tampere University Hospital, Elna Kaarina Savolainen's Legacy Allocated for the Development of Cancer Treatment, the Pediatric Research Foundation, the Research Fund of the Finnish Celiac Society, the SalWe Research Program for Intelligent Monitoring of Health and Wellbeing (Tekes – the Finnish Funding Agency for Technology and Innovation), the Pirkanmaa Regional Fund of the Finnish Cultural Foundation, the European Commission IAPP grant TRANSCOM, the Hungarian Scientific Research Fund, the Competitive Research Funding of Tampere University Hospital. The Tampere Graduate Program in Biomedicine and Biotechnology (TGPBB) provided travel grants.

I am grateful to Professor Markku Mäki, who offered this possibility of being part of the coeliac disease study group. I also thank him for being a member of my thesis committee and for teaching me how to survive in the academic and scientific world.

I wish to express my gratitude to my supervisors, Docent Katri Lindfors and Professor Katri Kaukinen, whose guidance, support and patience were very important to me. Visits to your office had a positive effect and saved my day every time.

I also want to thank the other member of my thesis committee, Jarkko Venäläinen, Ph.D., whose positive and supportive attitude motivated me to continue with new enthusiasm after our annual thesis committee meetings.

I thank the external reviewers, Docent Maria Vittoria Barone and Docent Aki Manninen, who kindly reviewed the manuscript and made constructive comments

towards improving this thesis. Many thanks go to Robert MacGilleon, M.A., who kindly revised the English language of my articles and this thesis.

My thanks go to my co-authors and collaborators Shuo-Wang Qiao, M.D., Ph.D., Zhongqing Jiang, MSc, Jussi Loponen, Ph.D., Hannu Salovaara, Ph.D., J. Arturo Garcia-Horsman, Ph.D., Russell Collighan, Ph.D., Alexandre Mongeot, Ph.D., Martin Griffin, Ph.D., Pekka T. Männistö, Ph.D., Zhuo Wang, Ph.D., Ilma Kopronay-Szabó, M.D., Ph.D. and Päivi Saavalainen, Ph.D., who contributed to this study.

Special thanks go to my co-authors and colleagues in Tampere, Essi Myrsky, Ph.D., Outi Koskinen, M.D., Ph.D., Kalle Kurppa, M.D., Ph.D. and Docent Pekka Collin, for work-related, and especially work-unrelated, discussions and activities. Warmest thanks to students and interns under my supervision, Rami Kivistö, M.Pharm., Mikko Oittinen, B.Sc. and Jennifer Culligan, B.Sc., for contributing to this study and being helpful as extra hands for laboratory experiments.

I am grateful to my co-author Kaija Laurila, M.Sc., and the “Keli-ladies”, Mrs. Soili Peltomäki, Mrs. Mervi Himanka, Ms. Suvi Anttila and Mrs. Anne Heimonen for helping me with patient samples. Heartfelt thanks go to Mr. Jorma Kulmala for assistance with cell culturing. I could not have succeeded without your valuable contribution.

I extend warmest thanks to my long-standing ”officemates” Laura Airaksinen, M.Sc., Suvi Kalliokoski, M.Sc., Cristina Nadalutti, Ph.D., and Minna Hietikko, M.Sc. You were able to cheer me up day after day. I want to thank also Alina Popp, M.D., Katriina Heikkilä, M.D., Ph.D., Ana-Marija Sulić, Ph.D., Heidi Kervinen, B.Sc., Sergio Caja, Ph.D., Emily McKinney, B.Sc., Keijo Viiri, Ph.D., Anniina Ukkola, M.D., Ph.D. and other present and former members of Coeliac Disease Study Group. In addition, my thanks go to Heidi Kontro, M.Sc., and Kaisa Teittinen, Ph.D., Toni Grönroos, M.Sc., Susanna Teppo, M.Sc. and other members of Docent Olli Lohi’s “HemoRes” group for laboratory companionship as well as extra-curricular activities.

I wish to thank Aija-Leena Lehtinen, B.A., Ms. Kaija Kaskela and Mrs. Zoe Virmaa for their help with office issues. Special thanks go to Mrs. Marja-Terttu Oksanen for nursing and coffee room chat.

My fellow students and friends in Kuopio, Taina Vuorio, M.Sc. and Karoliina Soininen, M.Sc., thank you for these years and shared experiences since 2002. I was lucky to find friends like you. Biochemistry rules!

I am truly grateful to my family for everything. My dear parents, Terhi and Martti, you urged me to educate myself since I was a little girl and supported me in my choices. My dear siblings, Mikko and Jaana, thank you for your encouraging words to your big sister. Tuovi-mummu ja Aaro-pappa, lämmin kiitos teille kannustuksesta ja mielenkiinnosta työtäni kohtaan. Kyselitte aina, että missä vaiheessa väitöskirja on ja olenko muistanut pitää lomaakin.

My dear husband and soulmate, Anssi Pelkonen, Ph.D., you pushed me to finish this work. We shared the same phase of life and survived together. You won our thesis competition but I had an excuse that brings a light to our days now. Little Akseli, thank you for appearing to our live and teaching mum how to put things in an order of importance.

Tampere, June 2014

Tiina Rauhavirta

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ORIGINAL PUBLICATIONS (I – III)

Accepted Manuscript

Impaired epithelial integrity in the duodenal mucosa in early stages of celiac disease

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PII: S1931-5244(14)00211-4

DOI: 10.1016/j.trsl.2014.02.006

Reference: TRSL 799

To appear in: *Translational Research*

Received Date: 13 September 2013

Revised Date: 25 February 2014

Accepted Date: 25 February 2014

Please cite this article as: Rauhavirta T, Lindfors K, Koskinen O, Laurila K, Kurppa K, Saavalainen P, Mäki M, Collin P, Kaukinen K, Impaired epithelial integrity in the duodenal mucosa in early stages of celiac disease, *Translational Research* (2014), doi: 10.1016/j.trsl.2014.02.006.

Impaired epithelial integrity in the duodenal mucosa in early stages of celiac disease

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Abstract

The small-bowel mucosal damage characteristic of celiac disease, develops from normal villus morphology to inflammation and finally to villus atrophy with crypt hyperplasia. Patients with early-stage celiac disease may already suffer from abdominal symptoms before the development of villus atrophy. Although epithelial junctional integrity is compromised in overt disease, the appearance of such changes in early phases of the disorder is not known. We investigated whether alterations in epithelial junction protein expression occur already in early-stage celiac disease with normal mucosal morphology, and whether this correlates with inflammation indicators and clinical symptoms. The study involved 10 patients with early-stage and 10 patients with overt villus atrophy that were followed yearly according to the study protocol. As controls, 20 non-celiac subjects were included. The expression of junction proteins (occludin, claudin-3, zonula occludens (ZO)-1, E-cadherin) was studied in small-intestinal biopsies using immunohistochemistry and Western blot. The correlation between junctional proteins and mucosal morphology, autoantibodies, the number of intraepithelial lymphocytes (IELs) and gastrointestinal symptoms was assessed. The expression of all junction proteins was already decreased in early-stage celiac disease when compared to non-celiac controls, ($P<0.05$). Junction protein expression correlated positively with mucosal villus morphology and negatively with the number of IELs, the intensity of small-intestinal autoantibody deposits and serum autoantibodies. The expression of claudin-3 showed a negative correlation with diarrheal score ($R=-0.314$, $P=0.04$). These findings show that the mucosal epithelial integrity is disrupted already in early-stage celiac disease before the disorder progresses to full-blown enteropathy.

Running head

Gut epithelium in early-stage celiac disease

Abbreviations

ELISA, enzyme-linked immunosorbent assay; EmA, endomysial antibodies; GSRS, Gastrointestinal Symptom Rating Scale; HLA, human leukocyte antigen; IEL, intraepithelial lymphocyte; IgA, immunoglobulin A; TG2, transglutaminase 2; Vh/CrD, villus height crypt depth ratio; ZO-1, zonula occludens-1

Introduction

In the healthy individual the epithelial layer lining the small bowel mucosa is fairly impermeable to macromolecules, allowing only nutrients and relatively small molecules to gain access from the intestinal lumen to the *lamina propria*. However, the epithelial barrier integrity is compromised in various intestinal disorders, including celiac disease, a dietary gluten-induced immune-mediated enteropathy occurring in genetically susceptible individuals expressing the human leukocyte antigen (HLA) molecules DQ2 or DQ8.[1] The small-intestinal mucosal inflammation, crypt hyperplasia and villus atrophy in untreated celiac patients is associated with increased mucosal permeability due to decreased expression of epithelial junction proteins, such as occludin, claudins, E-cadherin and β-catenin.[2-5] This makes for increased passage of gluten-derived gliadin peptides to the *lamina propria* and a subsequent downstream inflammatory immune response, including the production of disease-specific autoantibodies against endomysium and transglutaminase 2 (TG2).[6] Upon withdrawal of gluten from the diet the mucosal inflammation abates and the autoantibodies disappear parallel to the normalization of mucosal epithelial barrier function.[1]

Fasano and colleagues have suggested that the mechanism leading to this epithelial barrier defect could involve a molecule called zonulin already in early phases of celiac disease.[7] In celiac patients the typical gluten-induced small-intestinal mucosal damage develops gradually from normal villus structure to mild mucosal inflammation and eventually to totally flat mucosa with crypt hyperplasia.[8] Interestingly, many signs of the disease are already perceptible in the early phases of the disease when the small-bowel mucosal morphology is still normal. Such indicators include the presence of disease-specific endomysial- (EmA) and TG2-targeted antibodies in serum, small-bowel mucosal disease-specific antibody deposits and lymphocytosis.[8-10] In addition, patients may already suffer from anemia and various

gastrointestinal symptoms such as abdominal pain and diarrhea before the mucosa deteriorates.[8, 11, 12] This implies that the symptoms in celiac disease are not solely a result from small-bowel villus atrophy. Altogether, early developing celiac disease offers the possibility to understand the sequence of events leading to the full blown disease.

There are no studies addressing the expression of epithelial junction proteins in the small-bowel mucosa of celiac patients with early developing disease, when the mucosal villus morphology is still normal. With this in mind, we undertook to establish whether the expression of intestinal mucosal junction proteins is altered in this stage of celiac disease, and whether such altered junction protein expression is gluten-dependent and correlates with mucosal inflammation and symptoms.

Materials and methods

Patients and study design

The study cohort comprised 20 EmA-positive adult patients who underwent upper gastrointestinal endoscopy in Tampere University Hospital due to clinical suspicion of celiac disease. Follow-up studies were done according to planned protocol at yearly intervals (Fig 1). 10 patients had normal villus structure (Marsh I) compatible with early developing celiac disease.[12] Of these, seven developed villus atrophy (Marsh IIII) during the follow-up when they continued one year on a gluten-containing diet, and later after one year on a gluten-free diet, the mucosal morphology reverted to normal. The remaining three patients with early developing celiac disease (Marsh I) and 10 with overt small-bowel villus atrophy (Marsh III) started a gluten-free diet directly after the first biopsy (Fig 1), and after one year, clinical, serological and histological recovery was evident in all. 20 EmA-negative subjects, who had been investigated because of dyspepsia and had no relatives with celiac disease, served as

non-celiac controls. All had normal small-bowel mucosal morphology while on a normal gluten-containing diet.

A minimum of six small-bowel mucosal forceps biopsy samples were taken from the distal duodenum upon upper gastrointestinal endoscopy from celiac disease patients representing early and overt stages of the disease and from controls. Further, at each visit a clinical examination was carried out, serum and whole blood samples were drawn and gastrointestinal symptoms assessed by structured and validated Gastrointestinal Symptom Rating Scale (GSRS) questionnaires.[13] The study protocol was approved by the Ethical Committee of Tampere University Hospital. All subjects gave their written informed consent.

Small- bowel mucosal morphology, inflammation and TG2-specific immunoglobulin (Ig)A-deposits

Three small-bowel mucosal forceps biopsy samples were fixed in formalin, processed and stained with hematoxylin and eosin. Small-intestinal mucosal morphology was studied under light-microscopy from several well-oriented biopsy sections to avoid misinterpretation due to patchiness. Poorly oriented sections were discarded and, if necessary, the samples were re-dissected until they were of good quality. The villus structure was further evaluated in detail by measuring the villus height crypt depth ratio (Vh/CrD); a ratio less than 2.0 was regarded as compatible with villus atrophy (Marsh III) and overt celiac disease.[12]

Three small-bowel mucosal biopsy samples were snap-frozen and embedded in optimal cutting temperature compound (OCT, Tissue-Tec, Miles Inc., Elkhart, IN) and stored at -70 °C. Immunohistochemical staining was employed to detect $CD3^+$, $\alpha\beta^+$ and $\gamma\delta^+$ intraepithelial lymphocytes (IELs) in the small-intestinal mucosal biopsies and positive IELs were counted as previously described.[14] The reference values were set at 37 cell/mm for $CD3^+$ IELs, at 25 cell/mm for $\alpha\beta^+$ IELs and for 4.3 cell/mm for $\gamma\delta^+$ IELs.[14]

Intestinal mucosal TG2-targeted autoantibody deposits were detected using direct immunofluorescence as previously described.[15, 16] The deposits were further graded from negative (0) to strong positive (3) on the basis of their intensity in the villus-crypt area, as described elsewhere.[16]

Celiac serology and HLA genotype

Serum IgA-class EmA was determined by an in-house indirect immunofluorescence method with human umbilical cord as a substrate.[17] A serum dilution of 1: ≥5 was considered positive. Positive sera were further diluted 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000, and 1:4000.

Serum IgA-class anti-TG2 antibodies were detected by enzyme-linked immunosorbent assay (ELISA, Celikey®, Phadia, Freiburg, Germany) according to the manufacturer's instructions; values >5.0 U were considered to be positive.

Celiac-type HLA was determined using either the DELFIA Celiac Disease Hybridization Assay (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland) or the SSP DQB1 low resolution kit (Olerup SSP AB, Saltsjöbaden, Sweden) according to the manufacturer's instructions.

Immunohistochemistry for epithelial junction proteins

For immunohistochemistry staining of epithelial junction proteins, 5-µm-thick sections were cut from paraffin blocks of small-bowel biopsies derived from 10 early developing, 17 overt and 20 treated celiac disease patients and 20 non-celiac controls (Fig 1). Thereafter the sections were deparaffinised in xylene and rehydrated in descending alcohol series. This was followed by antigen retrieval for claudin-3 by boiling in 0.01 M citrate buffer and for occludin, ZO-1 and E-cadherin by enzymatic antigen retrieval with protease from *Streptomyces griseus* (Sigma-Aldrich). Non-specific antibody binding sites were blocked with

goat normal serum (1:20; Vector Laboratories inc., Burlingame, CA) in 5 % milk – 5 % bovine serum albumin for 30 min. Subsequently, the sections were incubated with a primary antibody, either rabbit anti-E-cadherin (1:50; Santa Cruz Biotechnology), rabbit anti-occludin (1:500; Invitrogen), or rabbit anti-ZO-1 antibody (1:100; Invitrogen) for one hour at room temperature, or with rabbit anti-claudin-3 (1:100; Invitrogen) at +4 °C overnight. After washing in phosphate buffered saline solution, the sections were incubated with a biotinylated anti-rabbit antibody (1:100; Vector Laboratories) for 30 min. Endogenous peroxidase was blocked by 0.3 % H_2O_2, immunodetection was done using an avidin-biotin complex method and the sections were counterstained with Harris hematoxylin. The specimens with detached epithelia or poor quality were excluded from the final analyses. The expression of every epithelial junction protein was analyzed from microscope images (Olympus BX60F5, Olympus Optical co. LTD, Japan) using ImageJ software (National Institutes of Health, USA). For measurements the epithelium was demarcated and the threshold set to visualize epithelial junctions (Fig 2A). Results were given as a percentage of junctions of the whole measured epithelium area.

Western blot

Protein expression of occludin, claudin-3, and E-cadherin were assessed by Western blot from frozen biopsies available from five early developing, seven untreated and five treated celiac patients as well as six controls. Biopsies were cut out of frozen Tissue-Tek and placed in ice-cold H-buffer (150 mM NaCl, 5 mM EDTA, 50 mM potassium phosphate, pH 7.4) containing Complete™ protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The biopsies were homogenized with Ultra Turrax T8 tissuemizer (IKA Labortechnik, Staufen, Germany) and centrifuged at 2500×g for 5 min. Supernatants were collected and the protein concentration determined by Bradford assay. Thereafter 25 µg of total protein aliquots per

lane were loaded on 12 % SDS-polyacrylamide electrophoresis gels. Proteins were transferred to nitrocellulose filters (Hybond™-C Extra; Amersham Biosciences Ltd., Little Chalfont, UK) and non-specific binding was blocked with 5 % non-fat milk for 1 h at room temperature. Subsequently, the membranes were incubated overnight with rabbit anti-occludin antibody (1:250), rabbit anti-claudin-3 antibody (1:250), rabbit anti-E-cadherin antibody (1:250) or mouse anti-β-actin antibody (1:5000). The blots were washed and incubated with secondary horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse antibody (1:2000; DAKO A/S, Copenhagen, Denmark) for 2 h at room temperature. After washing, the blots were developed with enhanced chemiluminescence (Amersham Biosciences), exposed to autoradiography film (Kodak, New Haven, CT) and scanned for densitometry analysis with 1D image analysis software (Kodak). The expression of epithelial junction proteins was normalized to β-actin expression as earlier.[5] The calculated values are derived from four independent experiments performed in duplicate.

Clinical evaluation

Abdominal complaints were evaluated by structured GSRS questionnaire widely used in celiac disease.[18-20] The 15 items in the questionnaire cover five different gastrointestinal symptoms: diarrhea, indigestion, constipation, abdominal pain and gastroesophageal reflux. Ratings are based on a seven-grade Likert scale, where higher scores indicate more severe symptoms. In this study GSRS total scores and diarrheal sub-scores were determined from patients according to study design (Fig 1). GSRS data from two celiac patients and twelve non-celiac controls were not available. Subjects' weight and height were measured and body mass index computed as kg/m^2. Blood hemoglobin levels were measured by standard laboratory methods.

Statistical analyses

Statistical analyses were performed using Predictive Analysis SoftWare (PASW)® Statistics 18 (SPSS Inc., Chicago, IL). Every test was two-sided and $p < 0.05$ was considered statistically significant. Comparisons were made using one-way analysis of variance (ANOVA) followed by appropriate post-hoc tests. Correlations were studied using either Pearson's or Spearman's correlation test depending on the variables in question.

Results

Celiac disease patients and controls

The study groups were comparable in respect to sex and age (Table I). All EmA-positive celiac disease patients in both the early developing and overt celiac disease groups were HLA-DQ2 or -DQ8-positive, whereas 25 % of the non-celiac controls were negative for these haplotypes. By definition, small-bowel mucosal villus morphology measured by Vh/CrD was normal in all patients with early developing celiac disease. However, similar to overt celiac disease, the densities of $CD3^+$, $\alpha\beta^+$ and $\gamma\delta^+$ IELs as well as serum and mucosal autoantibody levels were already increased in the early phase of the disease (Table I). Untreated celiac disease patients tended to suffer more from gastrointestinal complaints than non-celiac controls when assessed by GSRS scores (Table I). After one year on gluten-free diets all patients in the early developing and overt celiac disease groups were in clinical, serological and histological remission (Table I).

Expression of junction proteins

Immunohistochemical analysis revealed that in comparison to non-celiac controls, in celiac disease patients the expression of small-bowel mucosal epithelial tight junction proteins occludin and claudin-3, the tight-junction-associated protein ZO-1 and adherens junction

protein E-cadherin was already decreased before the development of villus atrophy (Fig 2 and Supplementary Fig 1). The expression of junctional proteins remained diminished as gluten-induced damage progressed from normal villi to overt mucosal atrophy, and a significant decrease from early developing celiac disease to overt disease was found in claudin-3 expression (Fig 2B – E). Subsequently, on a gluten-free diet the expression of occludin, and claudin-3 significantly increased compared to overt stage of the disease but did not reach the level of non-celiac controls despite small-bowel mucosal recovery (Fig 2B and C). The expression of E-cadherin showed similar trend, albeit the difference was not statistically significant (Fig 2E). Small-bowel mucosal epithelial ZO-1 expression was already diminished in early developing celiac disease, being comparable to that in overt celiac disease (Fig 2D). Furthermore, the expression of ZO-1 remained low even after one year on a gluten-free diet (Fig 2D). A longitudinal comparison of individual patients, who were studied in all three phases of celiac disease, shows similar trends than the analyses of the whole group, even though all differences were not statistically significant here either (Fig 3). Also the immunoblot analysis of whole small-bowel mucosal biopsy lysates revealed that the expression of occludin, claudin-3 and E-cadherin was significantly decreased in early developing and overt celiac disease compared to the non-celiac control group (Fig 4A – D). According to Western blot analysis, the expression of occludin, claudin-3 and E-cadherin normalized to control level on a gluten-free diet.

Correlations between junction protein expression and the clinical profile

When determined by immunohistochemical staining, the expression of occludin, claudin-3 and E-cadherin correlated significantly with Vh/CrD (Table II). In addition, the expression of most junction proteins also negatively correlated with intraepithelial lymphocytosis as well as serum and mucosal celiac-specific autoantibody levels (Table II). Interestingly, when the correlation between the junction protein expression and clinical parameters were studied, a

significant negative correlation was found between claudin-3 expression and the occurrence of diarrhea (Fig 5, Table II). In contrast, there was no association between the expression of junction proteins and total GSRS score (Table II), body mass index or hemoglobin levels (data not shown).

Discussion

The current study increases the knowledge about gluten-dependent small-bowel mucosal epithelial barrier disruption in celiac disease. According to our results, the epithelial integrity is already impaired in the early stage of celiac disease, when the mucosal villus morphology is still normal, but serum celiac disease-specific autoantibodies are positive and mucosal TG2-targeted autoantibody deposits as well as lymphocytosis are detectable. Regardless of the variation between individuals, the longitudinal analyses of celiac patients demonstrate a trend in the junctional protein expression in three different phases of the disease. This analysis revealed that in some patients in early-stage coeliac disease the expression of junction proteins appears already as low as in overt disease. On the other hand, in the majority of individuals the junctional protein expression, which was already lower than the control level in early-stage disease, was further decreased in overt coeliac disease. Our findings support the idea that the process leading to mucosal damage is promoted by epithelial barrier disruption in which the zonulin pathway might play a role.[7] In line with such a view, Smecuol and colleagues[21] have shown that intestinal permeability is increased in conjunction with zonulin upregulation in patients suffering from a skin manifestation of celiac disease, dermatitis herpetiformis, irrespective of the degree of small-bowel mucosal damage. Altogether, compromised epithelial barrier function is probably an early event during the gluten-driven pathogenesis of celiac disease. However, *in vivo* intestinal permeability studies will be needed to solve out the functional significance of our findings.

The pathogenesis of gluten-induced gastrointestinal symptoms in celiac disease has remained unclear, as they obviously extend beyond small-bowel mucosal villus atrophy.[12, 22] Interestingly, we showed here that the epithelial integrity was disturbed in celiac patients before the disease had progressed to full-blown villus atrophy and the expression of the tight junction protein claudin-3 appeared to correlate negatively with the occurrence of diarrhea. Correlation was not found between diarrhea and other junctional proteins. However, a rigid interpretation of the correlation analyses may be restricted due to skewed data. This might have been contributed by the fact that the non-celiac controls included in the study were not healthy controls. They were suffering from dyspepsia or other gastrointestinal symptoms as it is unethical to endoscopy completely healthy individuals. Thus, correlations between junction protein expression and GSRS scores may not be as explicit as they might have been if completely asymptomatic controls could have been included in the study. Moreover, although unlikely, the results might have been influenced by possible patchiness of the mucosal lesion.[23] For all the above mentioned reasons, further studies are needed to confirm the possible correlation between junctional protein expression and gastrointestinal symptoms.

It is of note that in irritable bowel syndrome, where the small-bowel mucosal villus morphology is normal, the severity and duration of gastrointestinal symptoms has been related to increased intestinal permeability and the expression level of tight junction proteins.[25, 26] Furthermore, a low-grade mucosal inflammation may be present in patients suffering from irritable bowel syndrome and this has been held to play a role in symptom development.[27, 28] In the present study, the early-stage celiac patients had mucosal inflammation, which was related to the level of expression of epithelial junction proteins. Our findings thus imply that symptom generation in celiac disease, similarly to irritable bowel syndrome, might reflect disruption of small-bowel mucosal integrity and an inflammatory state.

According to our own and previous studies epithelial junction protein expression in celiac disease improves during a gluten-free diet.[2, 3, 29] It would nonetheless appear that during a one year's diet the expression does not fully normalize to the level of non-celiac controls. It is well established that complete recovery of the small-bowel mucosal villus architecture takes more than a year, and further, intraepithelial lymphocytosis may persist for years.[30-32] In our study cohort, such persisting lymphocytosis was also clearly observed in treated celiac disease patients. Moreover, there was a relationship between IEL counts and junctional proteins, pointing to an important role of mucosal IELs in epithelial integrity. Further studies are warranted to show whether epithelial junctional integrity fully normalizes in celiac disease after a longer gluten-free diet.

Speculations

Altogether, our findings increase the understanding of early-stage celiac disease and its progression to full-blown enteropathy. We conclude that the disruption of mucosal epithelial integrity is an early phenomenon during the pathogenic process and is presumably of clinical relevance, since the compromised epithelial barrier might be related to abdominal symptoms.

Acknowledgements

Conflicts of interest: All authors have read the journal's authorship agreement and policy on disclosure of potential conflicts of interest, and have nothing to disclose.

This study and the Celiac Disease Study Group have been financially supported by the Academy of Finland, the Sigrid Juselius Foundation, the Competitive State Research Financing of the Expert Responsibility Area of Tampere University Hospital (grant numbers 9R034 and 9R018), Elna Kaarina Savolainen's fund allocated for the development of cancer treatment, Pediatric Research Foundation, the Finnish Celiac Society, the SalWe Research

Program for Intelligent Monitoring of Health and Wellbeing (Tekes – the Finnish Funding Agency for Technology and Innovation grant 648/10), the Pirkanmaa Regional Fund of the Finnish Cultural Foundation, and the European Commission IAPP grant TRANSCOM (Contract number PIA-GA-2010-251506).

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Figure legends

Fig 1. Study protocol. CD, celiac disease; GFD, gluten-free diet

Fig 2. Expression of junction proteins in the small-bowel mucosal epithelium of celiac disease (CD) patients and non-CD controls presented as percentage of epithelium area analyzed by immunohistochemical staining. An example of quantitative analysis of junction protein expression (A) and the expression of occludin (B), claudin-3 (C), zonula occludens-1 (ZO-1) (D) and E-cadherin (E) in patients with early developing CD, overt CD, treated CD (gluten-free diet, GFD), and in non-CD controls. Results are given as a mean ± standard error of mean (SEM). *P* values lower than 0.05 were considered statistically significant. NS; not significant

Fig 3. Longitudinal comparison of small-bowel mucosal junction protein expression in individual celiac patients in early developing celiac disease (CD), overt CD and treated CD (gluten-free diet, GFD). Expression of occludin (A), claudin-3 (B), ZO-1 (C) and E-cadherin (D) is presented as percentage of epithelium area analyzed by immunohistochemical staining. Interval between each study points is one year. A dotted line represents the average junction protein expression level in non-celiac controls. *P* values are from comparison between celiac patient groups and values lower than 0.05 were considered statistically significant. Only significant *P* values are indicated.

Fig 4. Expression of junction proteins in whole small-bowel mucosal biopsies of celiac disease (CD) patients and non-CD controls by Western blot. Representative immunoblots (A) and densitometric analyses of occludin (B), claudin-3 (C), and E-cadherin (D) in small-bowel

mucosa of patients with early developing CD (n = 4), overt CD (n = 7) and treated CD (gluten-free diet, GFD) (n = 5), and non-CD controls (n = 6). Junction protein expression levels are presented relative to β-actin expression. The experiment was repeated six times and results are given as mean ± standard error of mean (SEM). *P* values are from comparison with control and values lower than 0.05 were considered statistically significant. Only significant *P* values are indicated.

Fig 5. Correlation between claudin-3 protein expression in the small-bowel epithelium defined by immunohistochemical staining and severity of diarrhea defined by Gastrointestinal Symptom Rating Scale. The analysis included 43 samples from individuals from whom both GSRS diarrhea and claudin-3 expression results were available. Data were correlated by using parametric Pearson correlation test. CD, celiac disease; GFD, gluten-free diet

Supplementary Fig 1. Morphology of small-bowel mucosal epithelium and immunohistochemical staining of tight junction proteins occludin and claudin-3, tight-junction-associated protein zonula occludens (ZO)-1 and adherens junction protein E-cadherin in the small-bowel mucosal epithelium of celiac disease (CD) patients and non-celiac controls. Celiac samples are from patients in different stages of the disease: early developing CD, overt CD and treated CD after one year of gluten-free diet (GFD).

Table I

Characteristics of patients and controls

	Early developing CD gluten, normal villi (*n* = 10)	CD gluten, atrophy (*n* = 17)	GFD normal villi (*n* = 20)	Non-CD controls normal villi (*n* = 20)
Female; n (%)	8 (80)	15 (88)	15 (75)	12 (60)
Age; median (range), years	55 (19 – 67)	53 (18 – 68)	54 (19 – 69)	43 (18 – 71)
DQ2 or DQ8 positive; *n* (%)	10 (100)	17 (100)	20 (100)	15 (75)
Vh/CrD; mean (95 % CI), ratio	2.8 (2.5 – 3.0)	0.9 (0.5 – 1.2)	2.8 (2.5 – 3.1)	3.4 (3.1 – 3.7)
$CD3^+$ IELs ; mean (95 % CI), cells/mm	64 (55 – 74)	62 (54 – 71)	54 (43 – 65)	31 (25 – 37)
$\alpha\beta^+$ IELs ; mean (95 % CI), cells/mm	41 (32 – 50)	39 (34 – 44)	32 (24 – 41)	22 (18 – 27)
$\gamma\delta^+$ IELs ; mean (95 % CI), cells/mm	23.2 (18.1 – 28.2)	22.4 (18.0 – 26.8)	21.6 (17.9 – 25.3)	4.5 (2.4 – 6.7)
Mucosal IgA deposits present; (%)	10 (100)	17 (100)	14 (70)	2 (10)

TG2-ab; mean (range), U/ml	21.2 (3.3 – 101.0)	43.5 (2.9 – 101.0)	2.4 (0.0 – 7.7)	0.5 (0.0 – 2.9)
EmA; median (range), titer	1:50 (1:5 – 1:1000)	1:100 (1:5 – 1:2000)	0 (0 – 1:100)	0
GSRS total; mean (range), score	2.5 (1.4 – 4.2)	2.9 (1.4 – 4.9)	2.1 (1.3 – 4.0)	2.2 (1.2 – 3.1)
GSRS diarrhea; mean (range), score	2.0 (1.0 – 4.7)	3.1 (1.0 – 7.0)	1.9 (1.0 – 5.0)	1.5 (1.0 – 2.3)
Body mass index; mean (95 % CI), kg/m^2	25.4 (22.9 – 27.8)	24.1 (22.1 – 26.1)	24.5 (22.3 – 26.6)	*NA*
Hemoglobin; mean (95 % CI), g/l	142 (137 – 147)	140 (133 – 146)	142 (137 – 146)	*NA*

CD, celiac disease; Vh/CrD, villus height crypt depth ratio; CI, confidence interval; IELs, intraepithelial lymphocytes; IgA, immunoglobulin A; TG2-ab, anti-transglutaminase 2 antibodies; EmA, endomysial antibodies; GSRS, Gastrointestinal Symptom Rating Scale; *NA*, data not available

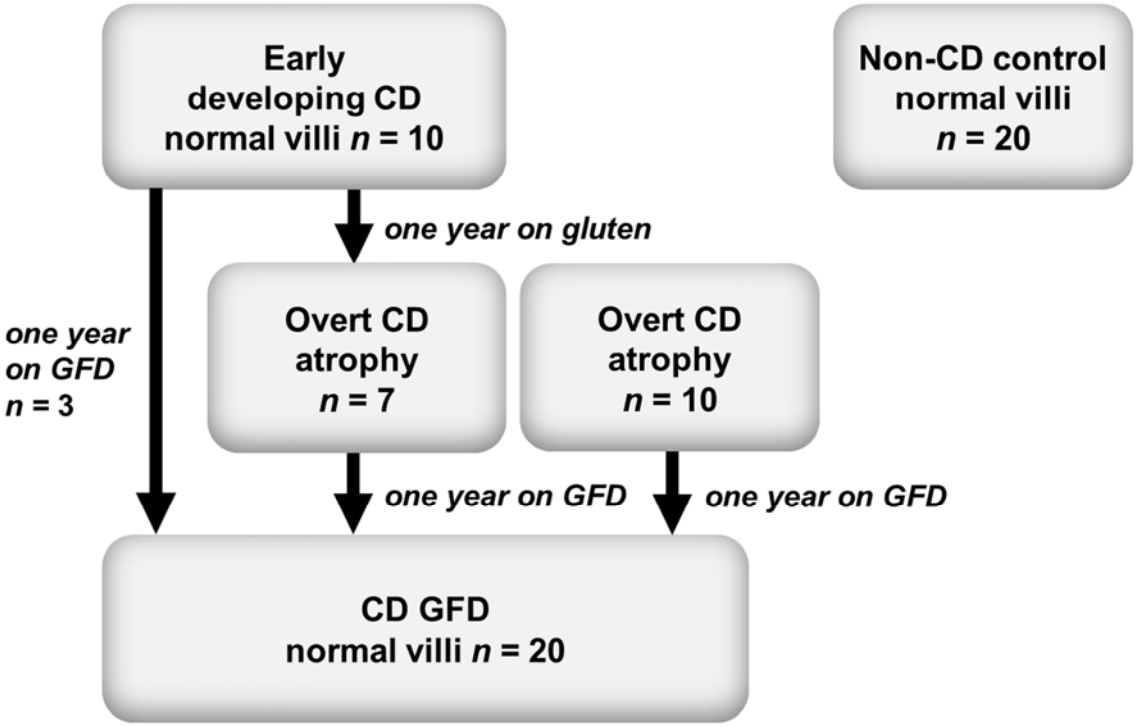
Table II

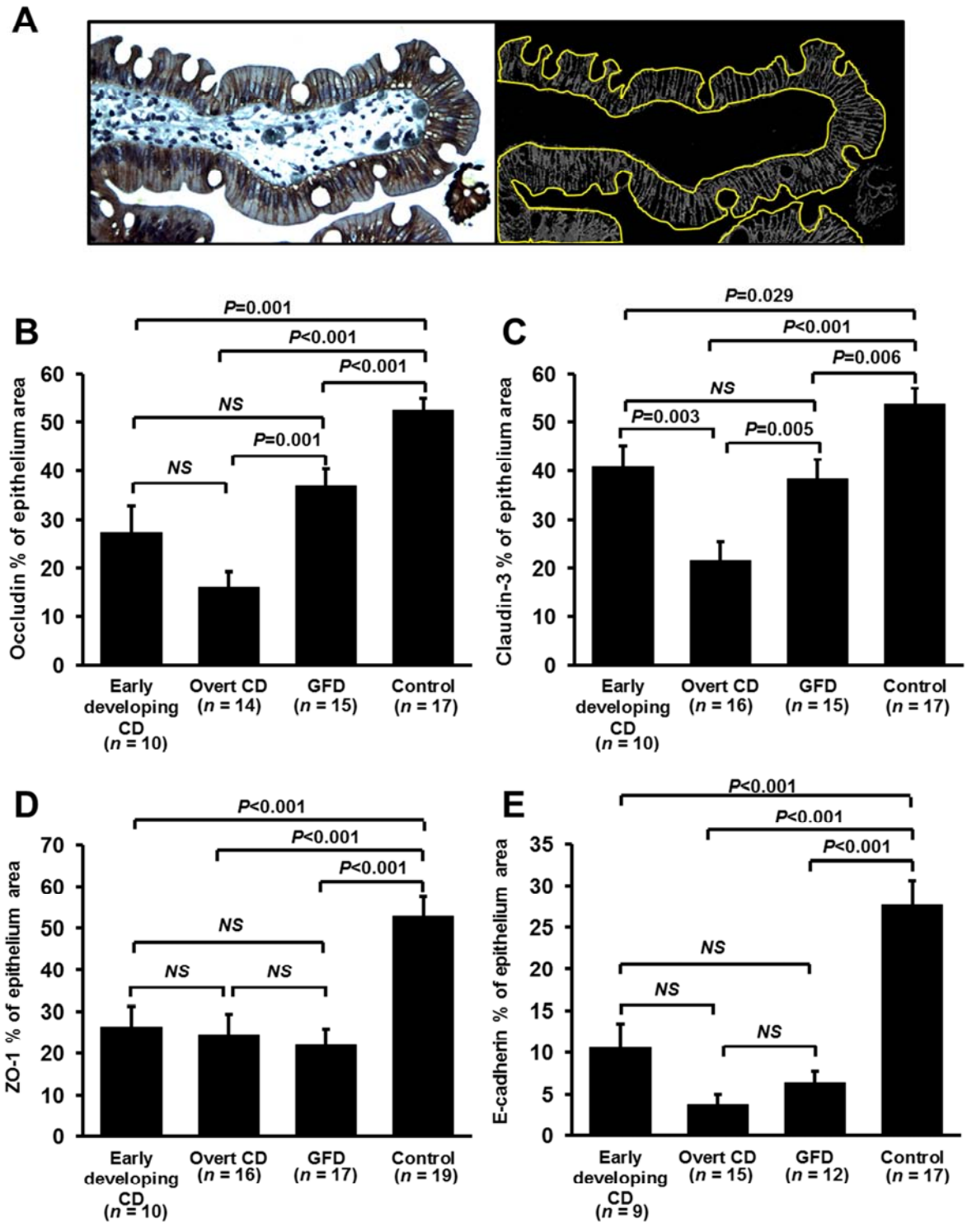
Correlations between immunohistochemically defined small-intestinal mucosal epithelial junction protein expression and celiac disease indicators. High Vh/CrD indicates less mucosal damage. Epithelial junction protein expression in intestinal mucosal biopsies was. Coefficients (R) of parametric variables were defined by Pearson correlation and a non-parametric serum EmA by Spearman's rho correlation. *P* values lower than 0.05 were considered statistically significant.

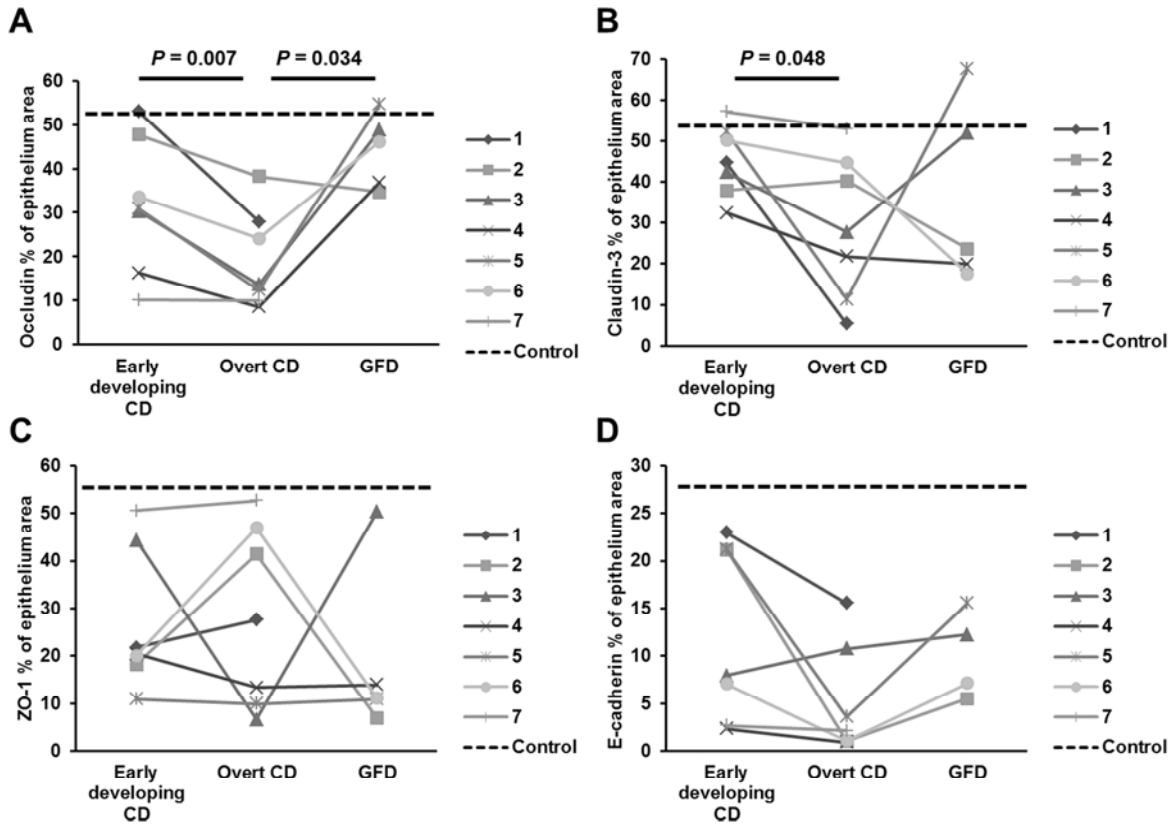
	Occludin		Claudin-3		ZO-1		E-cadherin	
	R	*P* value	R	*P* value	R	*P* value	R	*P* value
Vh/CrD	0.535	<0.001***	0.627	0.005**	0.305	0.019*	0.533	0.005**
$CD3^+$ IELs	-0.295	0.032*	-0.418	0.001***	-0.458	<0.001***	-0.338	0.016*
$\alpha\beta^+$ IELs	-0.273	0.048*	-0.423	0.001***	-0.357	0.006**	-0.222	0.121
$\gamma\delta^+$ IELs	-0.336	0.014*	-0.357	0.006**	-0.465	<0.001***	-0.497	<0.001***
Mucosal IgA deposits	-0.570	<0.001***	-0.568	<0.001***	-0.499	<0.001***	-0.463	0.001***
Serum TG2-ab	-0.436	0.001***	-0.518	<0.001***	-0.223	0.090	-0.348	0.011*
Serum EmA	-0.708	<0.001***	-0.526	<0.001***	-0.404	0.002**	-0.604	<0.001***
GSRS total	-0.183	0.257	-0.099	0.527	0.204	0.202	-0.095	0.580
GSRS diarrhea	-0.222	0.169	-0.314	0.040*	0.028	0.861	-0.177	0.302

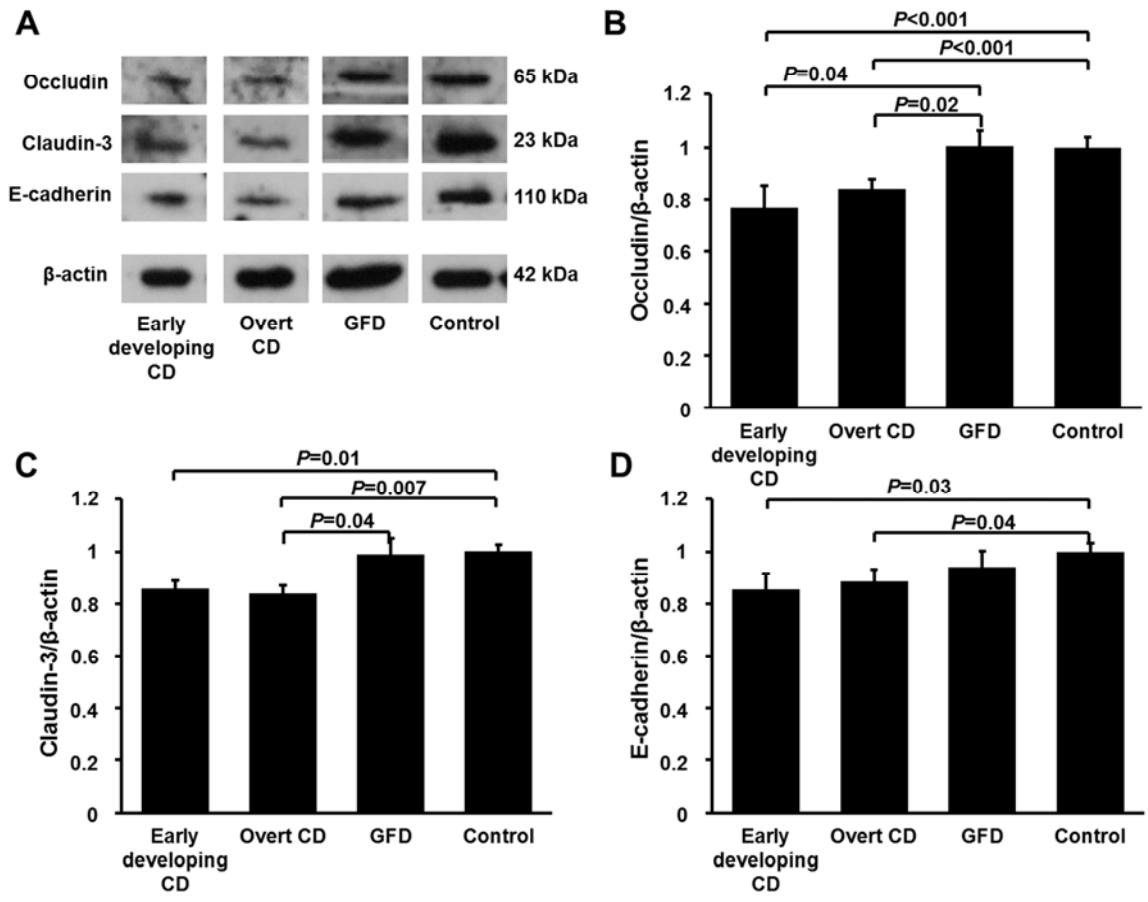
ZO-1, zonula occludens-1; Vh/CrD, villus height crypt depth ratio; IELs, intraepithelial lymphocytes; IgA, immunoglobulin A; TG2-ab, anti-transglutaminase 2 antibodies; EmA, endomysial antibodies; GSRS, Gastrointestinal Symptom Rating Scale .*NS*, not statistically significant

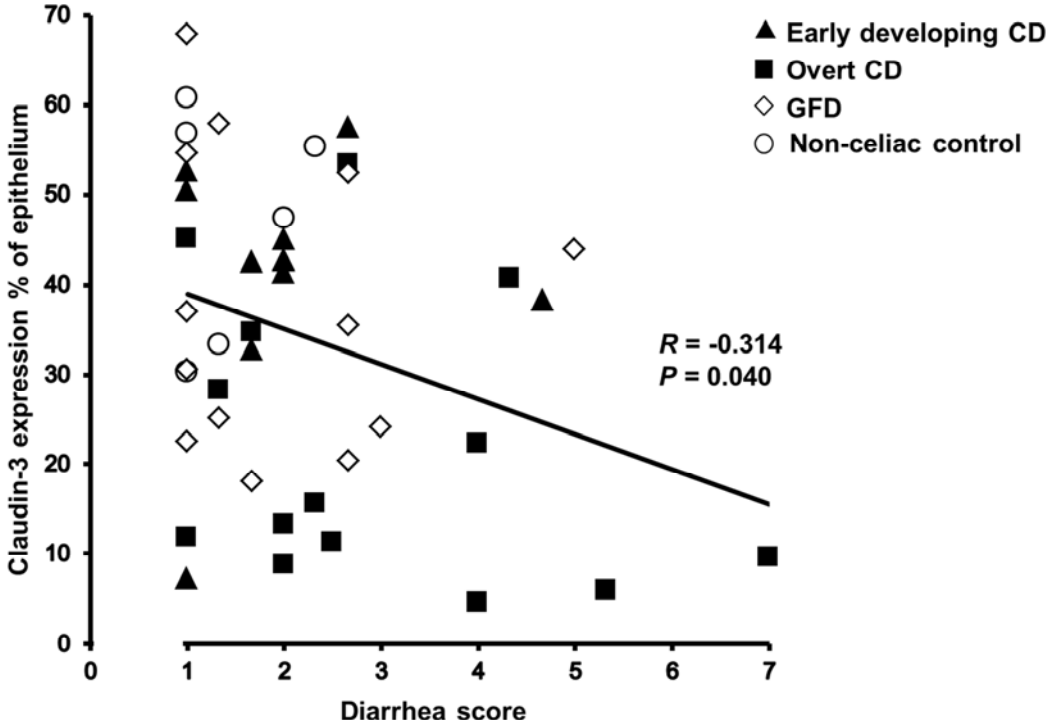
* ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001




A
B
Occludin % of epithelium area
P=0.001
P<0.001
P<0.001
NS
P=0.001
NS
Early developing CD (n = 10)
Overt CD (n = 14)
GFD (n = 15)
Control (n = 17)
C
Claudin-3 % of epithelium area
P=0.029
P<0.001
P=0.006
NS
P=0.003
P=0.005
Early developing CD (n = 10)
Overt CD (n = 16)
GFD (n = 15)
Control (n = 17)
D
ZO-1 % of epithelium area
P<0.001
P<0.001
P<0.001
NS
NS
NS
Early developing CD (n = 10)
Overt CD (n = 16)
GFD (n = 17)
Control (n = 19)
E
E-cadherin % of epithelium area
P<0.001
P<0.001
P<0.001
NS
NS
NS
Early developing CD (n = 9)
Overt CD (n = 15)
GFD (n = 12)
Control (n = 17)





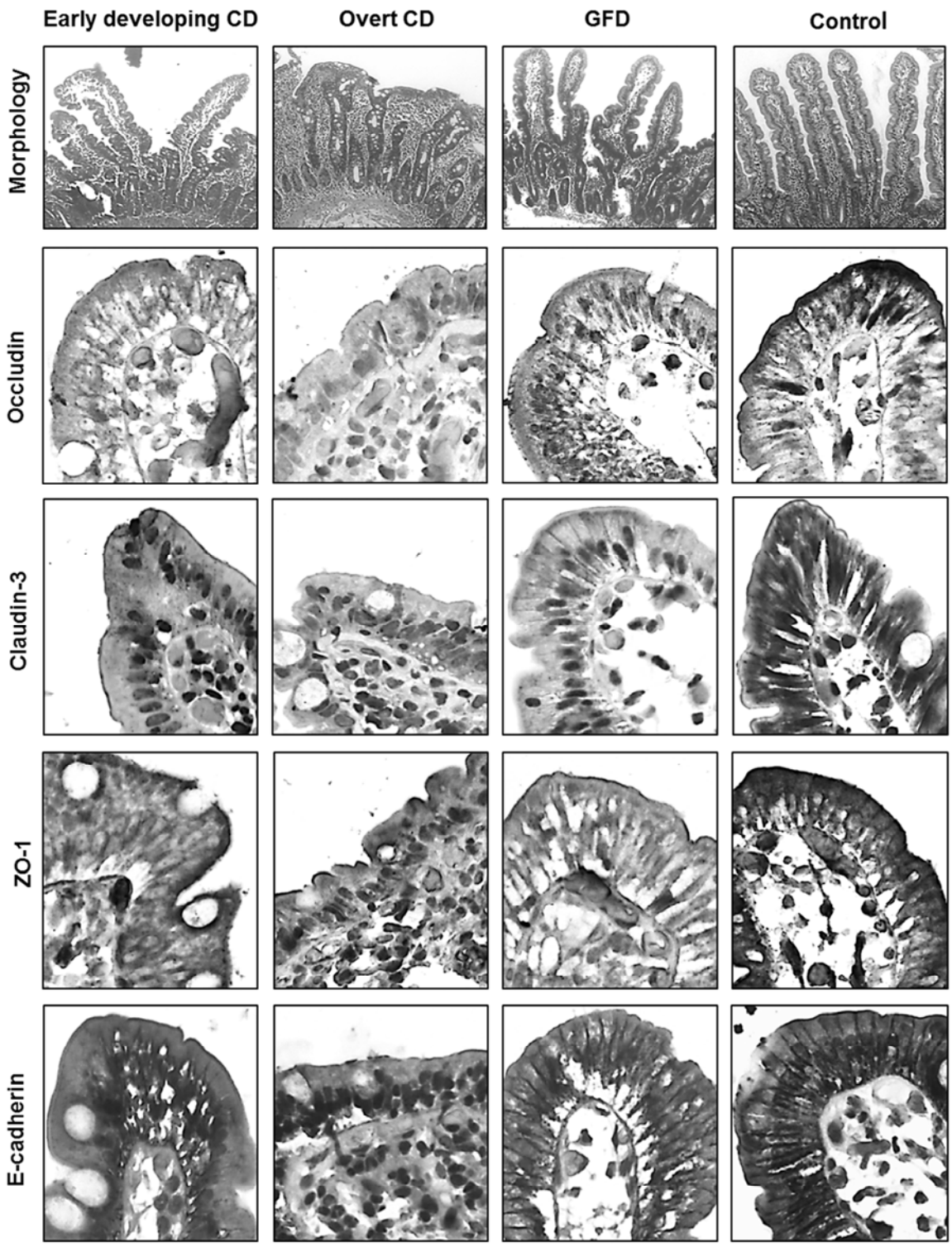


Background

In celiac disease the small-intestinal inflammation and damaged morphology are associated with increased mucosal permeability due to decreased expression of epithelial junction proteins. However, it is not known whether the junctional changes appear already in early phases of the disorder, when the small-bowel mucosal morphology is still normal.

Translational Significance

Our results suggest that mucosal epithelial integrity is disrupted already in early-stage celiac disease before damaged morphology and further advances as the disease progresses to full-blown enteropathy. These findings could be clinically relevant since the compromised epithelial barrier was related to abdominal symptoms.


Early developing CD
Overt CD
GFD
Control
Morphology
Occludin
Claudin-3
ZO-1
E-cadherin

Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2010.04317.x

Epithelial transport and deamidation of gliadin peptides: a role for coeliac disease patient immunoglobulin A

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Summary

In coeliac disease, the intake of dietary gluten induces small-bowel mucosal damage and the production of immunoglobulin (Ig)A class autoantibodies against transglutaminase 2 (TG2). We examined the effect of coeliac patient IgA on the apical-to-basal passage of gluten-derived gliadin peptides p31–43 and p57–68 in intestinal epithelial cells. We demonstrate that coeliac IgA enhances the passage of gliadin peptides, which could be abolished by inhibition of TG2 enzymatic activity. Moreover, we also found that both the apical and the basal cell culture media containing the immunogenic gliadin peptides were able to induce the proliferation of deamidation-dependent coeliac patient-derived T cells even in the absence of exogenous TG2. Our results suggest that coeliac patient IgA could play a role in the transepithelial passage of gliadin peptides, a process during which they might be deamidated.

Keywords: coeliac disease, deamidation, IgA, permeability, transglutaminase 2

Introduction

In coeliac disease patients carrying the disease-predisposing DQ2 or DQ8 HLA types the ingestion of wheat gluten and other cereal prolamins initiates a pathological process which leads to massive small-bowel mucosal inflammation, crypt hyperplasia, villous atrophy and clinical symptoms. The development of the small-bowel mucosal lesion is paralleled by the appearance of disease-specific immunoglobulin class A (IgA) antibodies targeted against transglutaminase 2 (TG2) and gluten-derived deamidated gliadin peptides in patient serum [1,2]. In addition to circulating in the blood, both conventional anti-gliadin antibodies and anti-TG2-autoantibodies are found in intestinal secretions [3,4]. Moreover, in untreated coeliac disease patients the anti-TG2-autoantibodies are already found deposited in the small-bowel mucosa below the epithelial basement membrane before the development of villous atrophy and crypt hyperplasia [5,6]. However, the role of these coeliac disease-specific antibodies in the pathogenesis is controversial, although experimental evidence from *in vitro* studies shows that antibodies derived from coeliac patients inhibit the differentiation [7] and induce the proliferation of intestinal epithelial cells [8], activate monocytes [9], inhibit angiogenesis [10] and increase vascular permeability [11].

In contrast to the role of the disease-specific antibodies, it is accepted widely that the pathogenesis of coeliac disease involves components of both innate and adaptive immunity. These immunological cascades are provoked by specific gliadin peptide populations resulting from their incomplete

cleavage during gastrointestinal proteolysis [12]. So-called toxic gliadin peptides such as p31–43 evoke an innate immunity response probably mediated by interleukin (IL)-15 [13], leading to epithelial cell damage [14]. Conversely, the entry of the immunogenic gliadin peptides such as 33-mer and its shorter fragment p57–68 into the lamina propria initiates an adaptive immunity response [15]. TG2, the target of the disease-specific autoantibodies, plays a role in this cascade by deamidating immunogenic gliadin peptides, thus increasing their affinity to DQ2 molecules on antigen-presenting cells [16]. Further, this cascade leads to T cell activation in the lamina propria and secretion of a legion of proinflammatory cytokines and eventually to small-bowel mucosal damage [17].

Despite increased insight into the pathogenesis of coeliac disease, a number of crucial questions remain unanswered. Cytokines involved in coeliac disease disrupt the intestinal epithelial barrier and increase the passage of gliadin peptides through the epithelium [18,19], but the process by which gliadin peptides traverse the intestinal epithelium to the lamina propria prior to substantial small-bowel mucosal remodelling with epithelial barrier defects and inflammation remains obscure. In addition, the exact location at which the deamidation of immunogenic gliadin peptides takes place is not clear. Because coeliac disease patients have gluten-induced antibodies on both sides of the epithelium, in the jejunal juice and in the basement membrane below the small-bowel mucosal epithelium, we hypothesize that these might contribute to the transepithelial passage of gliadin peptides. To test this hypothesis we studied whether the presence of IgA derived from coeliac patients would increase the translocation of gliadin peptides p31–43 and p57–68 across a monolayer of Caco-2 cells, a model used widely in physiopathological studies concerning intestinal epithelial barrier function [20,21]. We also addressed the question of whether deamidation of the immunogenic peptides might occur during contact with the intestinal epithelium.

Materials and methods

Reagents

Gliadin was extracted from wheat flour (Raisio Oyj, Raisio, Finland) and digested using pepsin and trypsin (PT) as described previously [20]. PT-gliadin was used at a concentration of 1 mg/ml. Gliadin peptides p31–43 and p57–67, both unlabelled and lissamine-labelled, as well as the lissamine-labelled control peptide p537–548 of human thyroid peroxidase (hTPO), were ordered from Inbios (Naples, Italy) (Table 1). hTPO peptide has been used previously as an irrelevant control peptide for both the toxic and the immunodominant gliadin peptides [13]. All peptides were administered at a concentration of 0·5 mg/ml unless indicated otherwise. Native and deamidated p57–68 peptides (Table 1) were used as negative and positive controls, respectively, in T cell activation experiments. Peptides were synthesized by multiple peptide synthesis on Wang resin using a robotic system (Syro MultiSynTech, Bochum, Germany) and Fmoc/OtBu chemistry. The identity of the peptides was confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, and purity was analysed by reverse-phase high performance liquid chromatography (HPLC). The TG2 activity-inducing agent retinoic acid [22] was purchased from Sigma Aldrich (St Louis, MO, USA) and used in experiments at a concentration of 15 μM. A cell impermeable TG2 active site inhibitor, R281 [23,24], was dissolved at 0·1 M in H_2O (stock solution). The inhibitor was diluted further in Hanks' balanced salt solution (Gibco, Invitrogen, the Netherlands) and used in experiments at a final concentration of 200 μM and added apically 1 h prior to the purified serum IgA.

Table 1. Sequences of synthesized gliadin and control peptides.

Peptide name	Sequence
Gliadin peptide p31–43	LGQQQPFPPQQPY
Gliadin peptide p57–68	QLQPFPQPQLPY
Deamidated gliadin peptide p57–68	QLQPFPQP*E*LPY
Human thyroid peroxidase p537–548	PLIRPLLARPAK

Patients and serum IgA autoantibodies

Coeliac antibody-positive serum samples from 12 IgA-competent coeliac patients on a gluten-containing diet and three autoantibody-negative non-coeliac controls were used in the study. All coeliac sera were positive for anti-TG2 and endomysial antibodies, whereas all control sera were negative. Total IgA fractions were purified using affinity chromatography as described previously [10]. IgA was used in the experiments at a concentration of 1 μg/ml, based on earlier studies [10,11]. IgA was administered to the cell cultures at the same time as PT-gliadin or different gliadin peptides.

The study protocol was approved by the Ethics Committee of Tampere University Hospital, Tampere, Finland. All individuals involved gave their written informed consent.

Cell culture

Caco-2 cells (passage 25–81, American Type Culture Collection, HTB-37; Rockville, MD, USA) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco Invitrogen, Paisley, UK) supplemented with 20% fetal bovine serum (FBS), 1% non-essential amino acids, sodium bicarbonate, penicillin (all from Gibco Invitrogen) and sodium pyruvate (Sigma Aldrich). The cells were grown at 37°C a 5% CO_2 atmosphere and passaged twice a week upon reaching 80% confluence.

Barrier function measurements

Caco-2 cells were plated at a density of 1·7 to 2×10^5 cells/cm^2 on semipermeable Millicell®-PCF culture inserts (pore size 0·4 μM, 12 mm diameter (Millipore, Carrigtwohill, Ireland) on 24-well plates. Cells were maintained until steady-state transepithelial electrical resistance (TER) was 1000 to 1500 Ω/cm^2. TER was measured using a Millicell® ERS volt-ohm meter (Millipore). Confluent monolayers were equilibrated overnight in DMEM supplemented with 1% FBS, non-essential amino acids, sodium pyruvate, sodium bicarbonate and penicillin. Thereafter, the cultures were treated with the different study compounds for up to 5 h and measurements were made at 0, 1, 3 and 5 h. The TER was given as the percentage of initial TER values to normalize for variation in absolute values between individual monolayers. Translocation of 4 kDa fluorescein isothiocyanate (FITC) dextran (Sigma-Aldrich) was evaluated by administration of the compound (1·0 mg/ml) to the apical chamber. Samples from the basal chamber were taken at the above-mentioned time-points and the amount of fluorescence measured by Wallac Victor2™ (Perkin Elmer, Turku, Finland) using 485 and 535 nm as the excitation and emission wavelengths, respectively. All experiments were performed in triplicate at least three independent times.

Peptide translocation assay

For the peptide translocation assay cells were cultured as above until TER reached a steady-state 1000 to 1500 Ω/cm^2. Thereafter, the lissamine-labelled gliadin and control peptides were added apically to the cultures. Samples of the basal media were taken at 0, 1, 3 and 5 h and fluorescence was measured using Wallac Victor2™ (excitation 485 nm and emission 590 nm). To control for the integrity of the monolayer TER was remeasured after completion of the experiment. The experiments were performed independently in triplicate at least three times.

Size-exclusion (SE) HPLC analysis of lissamine-labelled gliadin peptides

The intactness of translocated gliadin peptides was determined by SE-HPLC after translocation assays. One volume of each basal medium containing translocated lissamine-labelled peptides was dissolved in one volume of the running buffer [0·1% sodium dodecyl sulphate (SDS), 20% acetonitrile, 50 mM sodium phosphate, pH 6·9] and centrifuged (10 min, 11 000 ***g***). The supernatants were analysed using a Superdex Peptide 10/300 GL column (GE Healthcare Biosciences AB, Uppsala, Sweden) with the HP 1050 series HPLC system (Hewlett Packard, Waldbronn, Germany) equipped with an HP 1046A fluorescence detector (Hewlett Packard). As controls, intact lissamine-labelled gliadin peptides not subjected to translocation assays were analysed by dissolving 1·0 mg of each peptide initially in 200 μl 60% ethanol and then diluting further with 32 ml of the running buffer. The injection volume was 100 μl, flow rate 0·5 ml/min, and the analysis made at room temperature. The fluorescence detector utilized excitation at 485 nm and emission at 590 nm. The results were analysed using ChemStation software (ChemStation for LC 3D systems; Agilent Technologies, Waldbronn, Germany).

T cell proliferation assay

The ability of apical and basal culture supernatant to stimulate T cells was tested with a T cell proliferation assay *in vitro*. The CD4$^+$ T cell clone TCC387·9 cultured *in vitro* from a coeliac intestinal biopsy, as described previously [25], was used. This clone recognizes DQ2-α-I gliadin epitope with the 9-mer core region PFPQPELPY in a strict deamidation-dependent manner. In some cases the basal supernatant was pretreated with 100 μg/ml of recombinant human TG2 in the presence of 2 mM Ca^{2+} for 90 min at room temperature. A human leucocyte antigen (HLA)-DQ2 homozygous Epstein–Barr virus (EBV)-transformed B lymphoblastoid cell line derived from a coeliac disease patient (CD114) was used for antigen-presenting cells. Fifty thousand B cells irradiated with 100 Gy were co-incubated overnight at 37°C on a 96-well plate with twofold dilutions of culture supernatant in triplicate in RPMI-1640/10% human serum. Forty thousand TCC387·9 cells were added to each well on day 1. T cell proliferation was measured by the uptake of [^{3}H]-thymidine (1 μCi/well) added 20 h prior to harvesting. The cells were harvested after 72 h onto glass-fibre paper and [^{3}H]-thymidine incorporation was measured by liquid scintillation counting.

Determination of TG2 activity

The extracellular TG2 transamidation activity was determined by live-cell enzyme-linked immunosorbent assay (ELISA). Caco-2 cells were grown to confluence on 96-well plates (Nunclon, Thermo Fisher Scientific, Roskilde, Denmark). Confluent cultures were incubated overnight in medium containing 1% FBS and subsequently with a cell-impermeable TG2 active site inhibitor, R281 [24], for 1 h at 37°C. Retinoic acid, coeliac IgA or non-coeliac IgA were added to the media and the cells incubated for 5 h at 37°C. Thereafter, cells were washed carefully and incubated with TG2 substrate monodansylcadaverine (0·5 mM, Sigma-Aldrich) for 2 h at 37°C. After washing they were fixed with 4% paraformaldehyde (PFA; Sigma Chemical, St Louis, MO, USA) for 10 min at room temperature and then incubated with rabbit anti-dansyl antibody (1:200; Gibco Invitrogen) for 30 min at 37°C. The primary antibody was detected with a horseradish peroxidase-conjugated anti-rabbit antibody (1:1000; Dako, Glostrup, Denmark) for 30 min at 37°C.

Finally, peroxidase substrate, 3,3′,5,5′-tetramethylbenzidine (Slow Kinetic Form, for ELISA; Sigma-Aldrich) was added and the reaction stopped by 2·5 M H_2SO_4. The absorbance at 450 nm was measured by a Multiscan Ascent spectrophotometer (Thermo Labsystems, Vantaa, Finland). To check the confluence of the monolayers, cells were stained with crystal violet.

Gliadin peptide ELISA

Gliadin peptides p31–43 and p57–68 were deamidated by acid/heat treatment in 0·015 N HCl (pH 1·8) at 37°C for 3 h as described earlier [26]. Maxisorp™ microtitre plates (Nunc) were coated for 30 min at 37°C with 10 µg/ml of either native peptides (non-deamidated) or acid/heat deamidated gliadin peptides diluted in bicarbonate buffer pH 9·6. After washings with Tris-buffered saline, pH 7·4, containing 0,05% (v/v) Tween 20 (TBS) serum samples (1:100) were added and incubated at 37°C for 30 min. Bound antibodies were detected with horseradish peroxidase-conjugated rabbit anti-human IgA (1:2000; Dako) and 100 µl of 3,3′,5,5′-tetramethylbenzidine substrate (Sigma-Aldrich). The absorbance was read at 450 nm after stopping the reaction with 50 µl 1 M H_2SO_4.

Statistics

Statistical analyses were performed using the Mann–Whitney *U*-test. Data are presented as mean ± standard error of the mean (s.e.m.). A *P*-value < 0·05 was considered statistically significant.

Results

Coeliac disease IgA enhances the passage of gliadin peptides through epithelial cells

As expected, PT-gliadin increased permeability of the confluent Caco-2 intestinal epithelial cell monolayers, which is shown as inhibited recovery of TER to baseline level and increase of apical-to-basal flux of 4 kDa FITC-dextran (Fig. 1a and c) [21]. No such effects appeared in cultures supplemented with single gliadin peptides, p31–43 and p57–68, nor in cultures with no supplementation (Fig. 1a and c). Similarly, IgA derived from either untreated coeliac disease patients or healthy individuals (non-coeliac disease patient) had no effect on the recovery of TER and 4 kDa FITC-dextran permeability (Fig. 1b and d). However, when studying the transport of gliadin peptides across the epithelium in apical-to-basal direction, coeliac patient IgA enhanced the passage of both lissamine-labelled gliadin peptides, p31–43 and p57–68, when compared to the relevant control cultures (Fig. 2a and b). Coeliac IgA enhanced the translocation of both peptides without inhibiting the recovery of TER (data not shown). The flux of p31–43 increased significantly after 5 h in the presence of coeliac patient IgA, whereas that of p57–68 was already increased significantly after 3 h (Fig. 2a

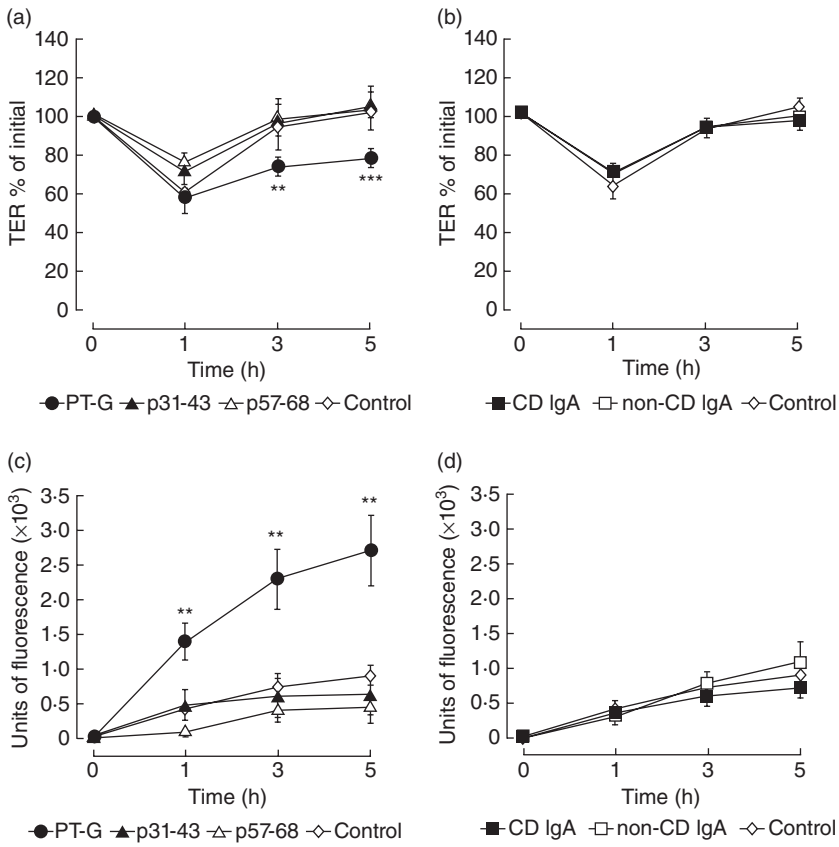

Fig. 1. Effects of pepsin–trypsin-digested gliadin (PT-G), gliadin peptides p31–43 and p57–68 and immunoglobulin (Ig)A on the paracellular permeability of Caco-2 cells. Transepithelial resistance (TER) was measured at appropriate time-points after treatment with PT-G and gliadin peptides p31–43 and p57–68 (a) or coeliac (CD) and non-coeliac (non-CD) IgA (b). The apical-to-basal flux of 4 kDa fluorescein isothiocyanate (FITC)-dextran, shown as units of fluorescence (×10^3), was determined both in samples treated with PT-G and gliadin peptides (c) or CD and non-CD IgA (d). In all experiments the control indicates those performed in medium only. Error bars indicate standard error of the mean. A *P* value < 0·05 was considered significant. $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$, when comparing PT-G to control.

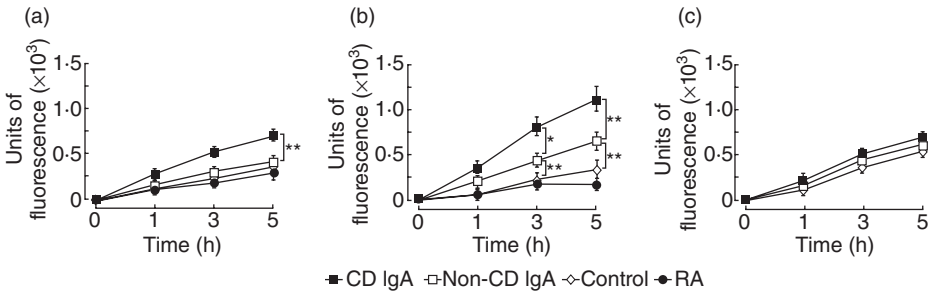

Fig. 2. The apical-to-basal translocation of lissamine-labelled gliadin peptides p31–43 and p57–68. Confluent Caco-2 cell layers on Millicell culture inserts were treated with lissamine-labelled gliadin peptides p31–43 (a) and p57–68 (b) in the presence of coeliac (CD) and non-coeliac (non-CD) immunoglobulin (Ig)A, and retinoic acid (RA). Human thyroid peroxidase peptide (hTPO) was used as a lissamine-labelled control (c). In all experiments the control indicates experiments performed in medium only. The results are expressed as mean units of fluorescence (×10³). Error bars indicate standard error of mean. A P value < 0·05 was considered significant. *P < 0·05; **P < 0·01, when comparing CD IgA to non-CD IgA and non-CD IgA to control.

and b). Coeliac patient IgA enhanced the flux of both gliadin peptides approximately twofold after 5 h (Fig. 2a and b). Interestingly, IgA derived from non-coeliac subjects also enhanced significantly the apical-to-basal flux of p57–68 when compared to cultures with no supplementation (Fig. 2b). IgA derived from untreated coeliac patients did not enhance the passage of an irrelevant control peptide, hTPO, through the epithelial layer (Fig. 2c).

Intactness of translocated gliadin peptides and immunogenicity of p57–68

The basal media were collected after gliadin peptide translocation assays and analysed with SE-HPLC to evaluate the stability of gliadin peptides p31–43 and p57–68. SE-HPLC control experiments, performed with intact native lissamine-labelled gliadin peptides diluted in cell culture medium, showed both p31–43 and p57–68 eluting at 10–12 ml (Fig. 3a and b). Analysis of the basal medium from the p31–43 gliadin peptide translocation assays revealed a peak eluting at the same volume as the intact lissamine-labelled peptide and an additional peak eluting at 20–22 ml (Fig. 3a). Based on the experimental column calibration with synthetic non-labelled prolamin peptides (9-mer, 19-mer, 33-mer), the peaks eluting after 18 ml correspond to free lissamine or lissamine attached to single amino acid rather than to peptide-associated lissamine. However, the peak eluting at the volume of the intact p31–43 was higher in the

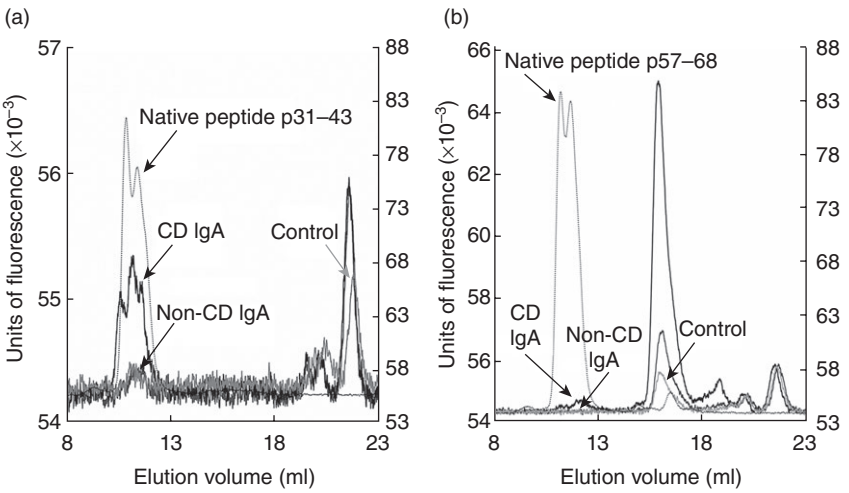

Fig. 3. Size-exclusion high-performance liquid chromatography (SE-HPLC) analysis of gliadin peptides p31–43 (a) and p57–68 (b) after coeliac disease (CD) immunoglobulin (Ig)A-enhanced epithelial translocation. In the SE-HPLC assay the native lissamine-labelled gliadin peptides (dotted lines in a and b) were eluted between 10 and 12 ml. In basal culture media containing gliadin peptides and CD IgA (black lines in a and b) peaks eluted at the same volume than native gliadin peptides, whereas in the presence of non-coeliac (non-CD) IgA (dark grey lines in a and b) and in the absence of any IgA (control, light grey lines in a and b) the peaks at this volume were considerably lower (a) or completely absent (b). Peaks after the volume of the intact gliadin peptides were also detected. Scales for fluorescence of lissamine-labelled gliadin peptides from CD IgA and non-CD IgA samples are on the left y-axes and scales for fluorescence of the lissamine-labelled native gliadin peptide are on the right y-axes.

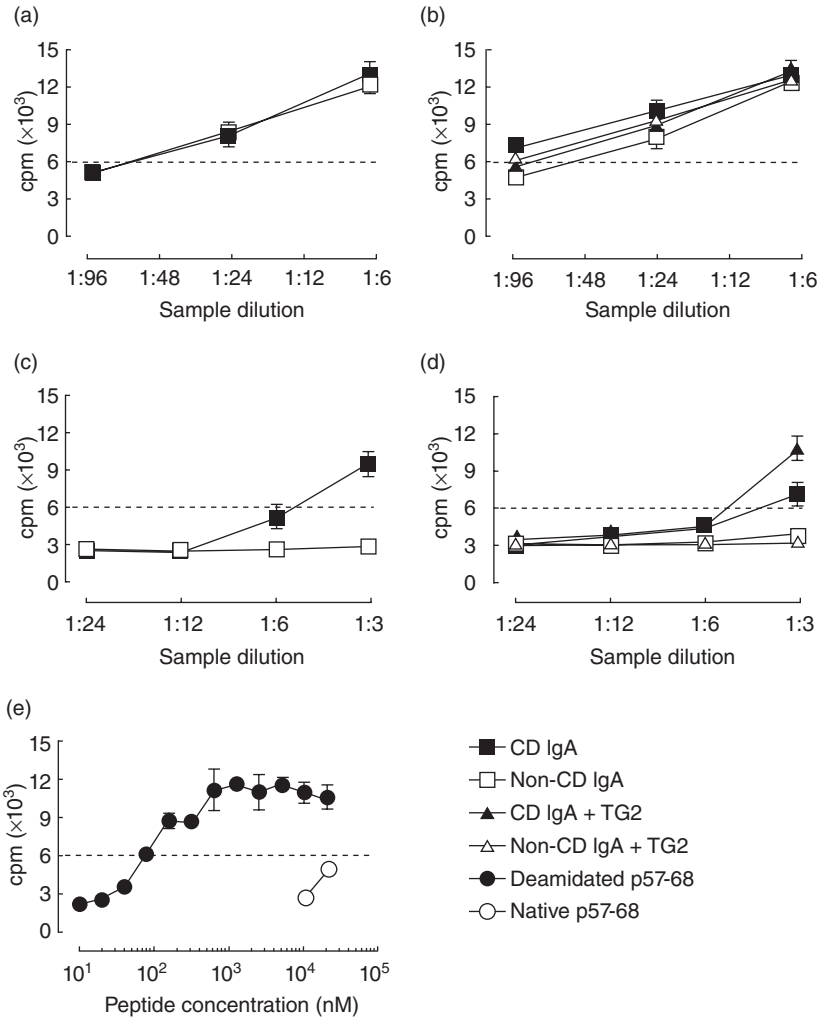

Fig. 4. Gliadin peptide p57–68 containing culture media from the translocation assay induces coeliac disease patient-derived T cell proliferation measured as thymidine incorporation, counts per million (CPM) ($\times 10^3$). The p57–68-containing apical culture media induced T cell proliferation in the presence of both coeliac (CD) and non-coeliac (non-CD) immunoglobulin (Ig)A (a). Transglutaminase 2 (TG2) pre-treatment of the apical culture media did not increase T cell proliferation compared to samples without TG2 pretreatment (b). Basal culture media containing the epithelially translocated gliadin peptide p57–68 in the presence of CD IgA activated T cell proliferation, unlike basal media from non-CD IgA-treated cells (c). Addition of TG2 increased the proliferation of T cells induced by p57–68 in the case of CD IgA basal media but not non-CD basal media (d). Only deamidated p57–68, but not native non-deamidated p57–68 was able to induce thymidine incorporation (e). The dotted line corresponds to the threshold of T cell activation (CPM).

basal media collected from experiments with coeliac patient IgA than in those with non-coeliac IgA or no IgA. SE-HPLC analysis of basal media derived from p57–68 translocation experiments demonstrated that the peak at the volume of the intact lissamine-labelled peptide was virtually absent in media derived from cultures supplemented with non-coeliac IgA or no IgA (control), but detectable in basal media obtained from translocation assays with coeliac IgA supplementation (Fig. 3b). In contrast, a peak eluting between 15 and 18 ml was evident in all tested p57–68 samples and this peak was especially high in the basal media collected from the experiments performed with coeliac patient IgA (Fig. 3b). This peak corresponds in size to ~1200 Da compound, but as the lissamine label as such has a molecular weight of 800 Da it is evident that the remaining peptide is clearly smaller than 9 amino acids, probably a 4-mer. No peaks eluting before the intact peptides were detected in experiments performed with either p31–43 or p57–68.

A subset of both apical and basal media from experiments performed with p57–68 was tested further for their ability to activate T cells derived from coeliac disease patients. In order to address the deamidation of the immunogenic p57–68 gliadin peptide, we selected a T cell clone which is dependent for activation on TG2-mediated deamidation (Fig. 4e) [27]. Interestingly, the majority (75%) of apical media containing p57–68 induced proliferation of the T cell clone without TG2 pretreatment and regardless of the presence of a control subject or an untreated coeliac patient IgA (Fig. 4a). In addition, TG2 pretreatment had no effect on media-induced T cell proliferation (Fig. 4b). Furthermore, basal media collected from cultures supplemented with untreated coeliac patient IgA were able to activate coeliac patient T cell proliferation in all cases where the concentration of the basally translocated peptide exceeded the detection limit of the T cell proliferation assay (Fig. 4c). One basal medium sample derived from an experiment performed with supplementation of coeliac patient IgA was tested further for T cell activation in the presence of TG2. We found that the T cell clone could be activated without TG2 pretreatment, although pretreatment increased T cell proliferation slightly (Fig. 4d).

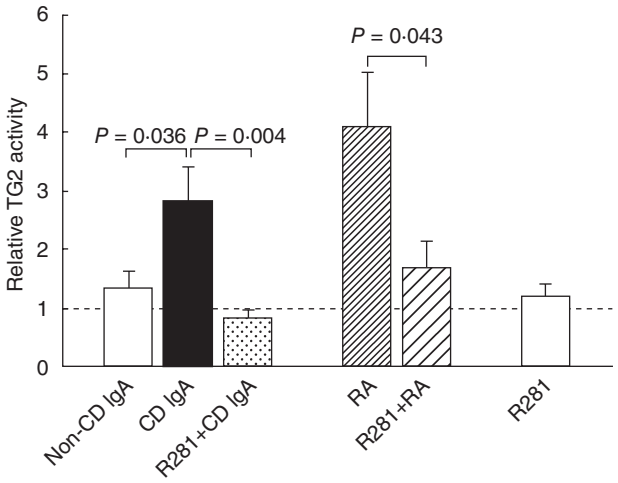

Fig. 5. Relative Caco-2 intestinal epithelial cell extracellular transglutaminase 2 (TG2) activity. The baseline activity in Caco-2 cells without any treatment was considered one (dotted line). Coeliac (CD) immunoglobulin (Ig)A and retinoic acid (RA) significantly increased TG2 activity, in contrast to non-coeliac (non-CD) IgA. The site-directed irreversible TG2 inhibitor R281 prevented the enhancement of the enzymatic activity of TG2 but had no effect on the baseline activity. Error bars indicate standard error of mean. A P value < 0·05 was considered significant.

Coeliac disease patient IgA-induced TG2 activity is involved in the enhanced gliadin peptide translocation

Live-cell ELISA to determine the extracellular transamidation activity of TG2 showed that IgA derived from coeliac patients but not from non-coeliac control subjects increased the extracellular activity of TG2 in Caco-2 cells similarly to retinoic acid (Fig. 5), a known activator of TG2 [22]. This increased TG2 activity could be abolished by R281, a site-directed inhibitor of TG2 (Fig. 5). However, the TG2 inhibitor R281 had no effect on the baseline activity (Fig. 5). In the gliadin peptide translocation assays, R281 was able to counteract increased passage of the gliadin peptides p31–43 and p57–68 provoked by the untreated coeliac patient IgA (Fig. 6a and b). The TG2 inhibitor R281 alone did not affect gliadin peptide translocation when compared to control (Fig. 6a and b). Interestingly, although retinoic acid increased TG2 activity similarly to coeliac patient IgA (Fig. 5), it was not able to enhance the transepithelial passage of gliadin peptides (Fig. 2a and b).

Discussion

Intestinal epithelial barrier function is at the centre of the stage in coeliac disease research, as it has not been shown conclusively how gliadin peptides translocate across the epithelium to the lamina propria. In this study we showed that untreated coeliac patient IgA enhances the passage of gliadin peptides p31–43 and p57–68 across the epithelium. Furthermore, basal media containing the translocated p57–68 peptides were able to induce proliferation of coeliac patient T cells even without pretreatment with TG2. In addition, we demonstrated that untreated coeliac patient IgA increases the cross-linking activity of TG2 and inhibition of TG2 abolishes the enhanced transepithelial passage of gliadin peptides induced by coeliac IgA.

We showed that coeliac patient IgA increased the passage of p31–43 and p57–58 gliadin peptides significantly without affecting the recovery of TER or 4 kDa FITC-dextran flux, which are generally regarded as markers of paracellular permeability [21]. This finding would suggest that the specific gliadin peptides use a transepithelial route to pass through the epithelium, which is in line with previously published work [19]. Moreover, recent studies suggest that toxic and immunogenic gliadin peptides (p31–49 and p56–68, respectively) are sorted differentially in the endocytotic compartment of intestinal epithelial cells [28,29]. Such differential processing of the two gliadin peptides by intestinal epithelial cells might explain slight differences in rate of the passages of p31–43 and p57–68 through the epithelium in the presence of coeliac patient IgA.

Our SE-HPLC results revealed two peaks in the basal medium containing p31–43. The first peak eluted at the same volume as the intact peptide and the second at a greater volume, suggesting that these samples might contain the intact lissamine-labelled p31–43 and free lissamine-label. In the case of p57–68, in accord with earlier studies [30], our SE-HPLC results implied that this peptide is partly degraded but that a portion of it is transported across the epithelium intact in the presence of coeliac disease IgA. Possibly the differential endosomal sorting of the gliadin peptides [28,29] could account for the p31–43 passing through the

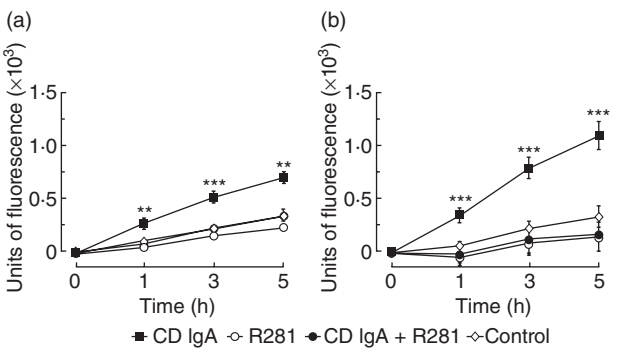

Fig. 6. The effect of transglutaminase 2 (TG2) inhibitor R281 on the apical-to-basal translocation of lissamine-labelled gliadin peptides p31–43 (a) and p57–68 (b). The site-directed irreversible TG2 inhibitor R281 prevented the increased gliadin peptide flux induced by coeliac (CD) immunoglobulin (Ig)A. In all experiments the control indicates experiments performed in medium only. The results are expressed as mean arbitrary units (AU) of fluorescence. Error bars indicate standard error of the mean. A P value < 0·05 was considered significant. $^{**}P < 0·01$; $^{***}P < 0·001$, when comparing CD IgA to CD IgA + R281.

epithelial cell layer largely intact and the p57–68 being partly degraded, as also earlier reported [19]. Regardless of the partial degradation, the basal media from the p57–68 translocation assays were still able to activate a T cell clone derived from an untreated coeliac disease patient, demonstrating the presence of epithelially translocated immunogenic peptides. Our results are also in accord with those published by Matysiak-Budnik and coworkers [31] using small-intestinal biopsy specimens from untreated coeliac disease patients mounted in an Ussing chamber. They demonstrated that the toxic gliadin peptide p31–49 is transported largely intact across the tissue, whereas the immunogenic peptides 33-mer and p57–68 are processed to shorter peptide products during the transport [31]. Subsequently, authors reported that in untreated coeliac disease the apical-to-basal transcytosis of gliadin peptides across the small-bowel epithelium is mediated by secretory anti-gliadin IgA via binding to the transferrin receptor [31]. However, we were not able to demonstrate co-localization of the lissamine-labelled gliadin peptides with CD71 in Caco-2 cells (data not shown).

It has been reported that QPFXXQXPY (X denoting any amino acid) in alpha-gliadin is a motif recognized preferably by coeliac patient serum antibodies [32]. This motif is present as such in gliadin peptides p31–43, and particularly homologously in p57–68. However, our ELISA experiments revealed that coeliac patient serum IgA does not bind specifically to either of these peptides (data not shown), which would imply that the enhanced transport of the gliadin peptides across the epithelial cell layer may not involve the binding of coeliac IgA to native gliadin peptides. Distinct gliadin peptides are, however, subjected to deamidation by TG2 [33], and coeliac patients have antibodies against deamidated gliadin peptides [29]. Therefore, the gliadin peptides are deamidated by TG2 possibly on the apical surface of the Caco-2 cells, this enhancing their antigenicity to coeliac IgA. This notwithstanding, we were unable to demonstrate binding of coeliac patient serum IgA to p31–43 or p57–68 after their acid/heat-deamidation. It is conceivable that in order to bind to coeliac IgA, the deamidated peptides need to adopt a distinct three-dimensional conformation [29], which is not promoted by the experimental conditions in ELISA or other methodological factors. As heat/acid treatment causes the release of lissamine label we were not able to perform an ELISA to address the question whether lissamine-labelled gliadin peptides p31–43 and p57–68 bind to coeliac patient IgA mounted on plates. However, based on the SE-HPLC results, after translocation to the basal side of the epithelium, the lissamine-labelled peptides are not bound to IgA.

Korponay-Szabó and co-workers [34] demonstrated that affinity-purified antibodies against deamidated gliadin peptides from untreated coeliac disease patients are able to recognize TG2. According to our results, the coeliac IgA-induced increase in the transepithelial passage of gliadin peptides could be inhibited by enzymatic inhibition of TG2. Further, the TG2 activity induced by retinoic acid did not alone enhance gliadin peptide translocation. Because both coeliac patient IgA and retinoic acid increased the activity of TG2 but only coeliac IgA enhanced the transepithelial passage of gliadin peptides, the increased TG2 activation may not be the only effective factor in this context. The increased transepithelial passage of gliadin peptides seen in our experiments might actually be mediated by cross-reactive antibodies which recognize both TG2 and deamidated gliadin peptides. Furthermore, it must be noted that serum IgA is mainly monomeric, whereas IgA in intestinal secretion is mainly polymeric [35], and therefore the results might be influenced by the source of the IgA used in our study.

We found that 75% of all the apical media from gliadin peptide p57–68 translocation experiments, regardless of the supplementation, were able to induce proliferation of the coeliac patient deamidation-dependent T cell clone even without TG2 pretreatment. These findings would suggest that the gliadin peptide p57–68 is deamidated by the TG2 of Caco-2 cells. Maiuri and co-workers [36] reported that TG2 is located on the surface of small-intestinal epithelial cells, this conception being based on immunofluorescence stainings with a 6B9 antibody, although the specificity of the antibody has since been questioned [37]. Recently, Hodrea and co-workers [38] found TG2 expressed and enzymatically active on the surface of monocytes. As we detected increased TG2 activity in Caco-2 cells treated with coeliac IgA, TG2 on the apical surface of Caco-2 intestinal epithelial cells could deamidate the gliadin peptides, modifying them to be recognizable by coeliac patient antibodies against deamidated gliadin peptides. However, as the concentration of the p57–68 in the apical chamber was extremely high, we cannot exclude the possibility that the T cell proliferation induced by apical cell culture media was actually due to a low-level acid-mediated deamidation of the peptide during their manufacturing process. Regardless of this, our studies showed that the basal media collected from the translocation assays in the presence of coeliac patient IgA were able to induce proliferation of coeliac patient T cells even without TG2 pretreatment. This would imply strongly that the immunodominant peptides are deamidated during their contact with the epithelial cells. This is an important point in the pathogenetic cascade of coeliac disease and sheds light on the central issue of where the deamidation of the immunodominant gliadin peptides could occur.

We conclude that IgA derived from coeliac disease patients seems to enhance the apical-to-basal passage of gliadin peptides. As the autoantibodies are present in intestinal secretions and also deposited below the epithelium in the small-intestinal mucosa of coeliac patients even prior to the development of the manifest mucosal lesion, the antibodies might allow the passage of gliadin peptides to the lamina propria already in the early phase of the disease. In addition, our results would suggest that immunogenic peptides might be deamidated during their contact with intestinal Caco-2

epithelial cells. Similarly, it is conceivable that in the coeliac patient small-bowel mucosa the deamidation of immunogenic gliadin peptides by TG2 could occur during their interaction with the intestinal epithelial cells.

Acknowledgements

The Coeliac Disease Study Group has been supported financially by the Academy of Finland, the SalWe Research Program for Intelligent Monitoring of Health and Wellbeing (Tekes – the Finnish Funding Agency for Technology and Innovation grant 648/10), the Pirkanmaa Regional Fund of the Finnish Cultural Foundation, the Sigrid Juselius Foundation, the Paediatric Research Foundation, the Competitive Research Funding of Tampere University Hospital, the Research Fund of the Finnish Coeliac Society, the Hungarian Scientific Research Fund (OTKA K61868) and the European Commission IAPP grant TRANSCOM (Contract number PIA-GA-2010-251506).

Disclosure

The authors confirm that there are no conflicts of interest to disclose.

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J Clin Immunol (2013) 33:134–142
DOI 10.1007/s10875-012-9745-5

ORIGINAL RESEARCH

Are Transglutaminase 2 Inhibitors Able to Reduce Gliadin-Induced Toxicity Related to Celiac Disease? A Proof-of-Concept Study

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Received: 10 April 2012 / Accepted: 16 July 2012 / Published online: 10 August 2012

Abstract
Purpose Celiac disease is an autoimmune-mediated enteropathy characterized by adaptive and innate immune responses to dietary gluten in wheat, rye and barley in genetically susceptible individuals. Gluten-derived gliadin peptides are deamidated by transglutaminase 2 (TG2), leading to an immune response in the small-intestinal mucosa. TG2 inhibitors have therefore been suggested as putative drugs for celiac disease. In this proof-of-concept study we investigated whether two TG2 inhibitors, cell-impermeable R281 and cell-permeable R283, can prevent the toxic effects of gliadin in vitro and ex vivo.

Methods Intestinal epithelial Caco-2 cells were treated with peptic-tryptic-digested gliadin (PT-gliadin) with or without TG2 inhibitors and thereafter direct toxic effects (transepithelial resistance, cytoskeletal rearrangement, junction protein expression and phoshorylation of extracellular-signal-regulated kinase 1/2) were determined. In an organ culture of celiac-patient-derived small-intestinal biopsies we measured secretion of TG2-autoantibodies into the culture medium and the densities of CD25- and interleukin (IL) 15-positive cells, forkhead box P3 (FOXP3)-positive regulatory T cells (Tregs) and Ki-67-positive proliferating crypt cells.
Results Both TG2 inhibitors evinced protective effects against gliadin-induced detrimental effects in Caco-2 cells but the cell-impermeable R281 seemed slightly more potent. In addition, TG2 inhibitor R281 modified the gluten-induced increase in CD25- and IL15-positive cells, Tregs and crypt cell proliferation, but had no effect on antibody secretion in celiac-patient-derived biopsies.
Conclusions Our results suggest that TG2 inhibitors are able to reduce certain gliadin-induced effects related to responses in vitro and ex vivo.

Keywords Celiac disease · gliadin · transglutaminase 2 inhibitor · small intestine

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Introduction

Celiac disease is an autoimmune-mediated enteropathy in genetically susceptible individuals expressing the disease-

predisposing human leukocyte antigen (HLA) molecules DQ2 or DQ8. In celiac disease patients the ingestion of wheat gluten and similar rye and barley proteins initiate a pathological process which leads to massive small-intestinal mucosal inflammation, crypt hyperplasia, villous atrophy and clinical symptoms. The development of the small-intestinal mucosal lesion is paralleled by the appearance of disease-specific immunoglobulin class A (IgA) antibodies targeted against transglutaminase 2 (TG2) and gluten-derived deamidated gliadin peptides in patient serum [1, 2]. Thus far, a strict and lifelong gluten-free diet is the only treatment for celiac disease, but such a restrictive diet is known to be burdensome [3]. As a diet completely devoid of gluten is almost impossible to maintain, novel alternative therapy options are under intensive research.

One suggested approach is to use TG2 inhibitors to prevent the development of the gluten-induced small-intestinal mucosal damage in celiac disease [4]. TG2 is a multifunctional enzyme ubiquitously expressed in mammalian cells and involved in various biological processes, functioning in both the intra- and extracellular space [5]. In the cytosol, TG2 functions mainly as a G-protein and at the cell surface as a co-receptor involved in adhesion [6]. In the extracellular environment, TG2 promotes the crosslinking of extracellular matrix proteins by catalyzing the crosslinking of peptide-bound glutamine residues to peptide-bound lysine residues, this process resulting in proteolytically resistant ε-(γ-glutamyl)lysine isopeptide bonds [7]. In the celiac pathogenesis the role of TG2 is undisputed, as the enzyme deamidates specific glutamine residues in gliadin peptides. This deamidation creates epitopes which bind to DQ2 with increased affinity, thus leading to enhanced T cell activation paralleled by secretion of celiac disease-specific TG2-targeted autoantibodies [8]. The small-intestinal mucosal adaptive immune response is controlled by regulatory T cells (Tregs) whose expansion has been shown in peripheral blood and in intestinal biopsy specimens from celiac patients [9, 10]. In addition to the activation of the adaptive immune system, it is widely accepted that the pathogenesis of celiac disease also involves factors of innate immunity, as distinct gliadin components are able to induce direct cytotoxic epithelial cell damage resulting in compromised epithelial barrier function with increased permeability [11, 12]

Although the contribution of TG2 to the adaptive immune activation in celiac disease is well known, its involvement in the innate immune mechanism is only poorly understood. The present study was designed to test the ability of TG2 inhibitors to prevent both the toxic and immune-mediated responses induced by gliadin. This proof-of-concept study was thus undertaken to assess whether TG2 inhibitors could reduce gliadin-induced harmful effects in widely used in vitro models such as the human intestinal Caco-2 cell line [12, 13] and organ culture [14, 15].

Materials and Methods

Reagents

Gliadin was purchased from Sigma-Aldrich (G3375). For cell culture experiments gliadin was digested using pepsin (P-6887, Sigma-Aldrich, Seelze, Germany) and trypsin (T-7418, Sigma-Aldrich) as previously described [16]. To abolish the harmful effects exerted by pepsin and trypsin enzymes, the samples were heat-inactivated at >95 °C for 10 min. Bovine serum albumin (BSA, A8806, Sigma-Aldrich) was treated similarly and served as a negative control for peptic-tryptic (PT) treatment as previously [12, 17].

Cell-impermeable TG2 active site inhibitor R281 and cell-permeable R283 [18, 19] were dissolved in H_2O at a concentration of 0.1 M (stock solution). The inhibitors were diluted further in Hank's balanced salt solution (Gibco, Invitrogen, the Netherlands) and used in experiments at a final concentration of 200 μM. Concentrations of R281 and R283 were based on previous studies, [20–22]. We pretested also concentrations of 500 μM and 1 mM in Caco-2 cells but the higher concentrations were even toxic. Inhibitors were added 1 h prior to gliadin administration to both Caco-2 cells and the small-intestinal organ culture system.

Cell Culture

Caco-2 cells (American Type Culture Collection, HTB-37, Rockville, MD, USA) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco Invitrogen, Paisley, UK) supplemented with 20 % fetal bovine serum (FBS), non-essential amino acids, sodium bicarbonate, penicillin (all from Gibco Invitrogen) and sodium pyruvate (Sigma Aldrich). The cells were grown at 37 °C in a 5 % CO_2 atmosphere and divided twice a week upon reaching 80 % confluence. Experiments were performed with Caco-2 cells passaged 25 – 71 times; similar scales have been used also earlier in Caco-2 experiments [23, 24]. Each experiment with different treatments was always performed with Caco-2 cells of the same passage thus making the comparisons between the treatment groups valid.

Determination of Actin Membrane Ruffling

To evaluate cytoskeleton actin rearrangement, Caco-2 cells were plated onto eight-chamber glass slides (BD Biosciences, Erembodegem, Belgium) and cultured for 3–5 days before being equilibrated overnight in 1 % culture medium. The cells were incubated for 24 h in the presence of PT-gliadin (1 mg/ml), PT-BSA (1 mg/ml) or medium only. To visualize actin cytoskeleton rearrangement the cells were stained for intracellular F-actin with phalloidin-fluorescein isothiocyanate (1:300, Sigma-Aldrich). Ruffling of the cell

membrane indicating the extent of actin cytoskeleton rearrangement was quantified by measuring the cellular edge covered by membrane ruffles as a percentage of the total length of the cell cluster. The measurements were made with ImageJ software from 20 different cell clusters from each sample group.

Barrier Function Measurements

For transepithelial electrical resistance (TER) measurements, Caco-2 cells were plated on semipermeable Millicell®-PCF Culture inserts (pore size 0.4 μm, diameter 12 mm, Millipore, Carrigtwohill, Co. Cork, Ireland) on 24-well plates. TER was measured using a Millicell®ERS volt-ohm meter (Millipore) and cells were allowed to grow until steady-state TER reached 1,000 to 1,500 Ω/cm^2. Confluent monolayers were equilibrated overnight in DMEM supplemented with 1 % FBS, non-essential amino acids, sodium pyruvate, sodium bicarbonate and penicillin. Thereafter, the cultures were treated with study compounds for up to 5 h and measurements were made at 0, 1, 3 and 5 h. The TER was given as the percentage of initial TER values to normalize the variation in absolute values between individual monolayers.

Junction Protein Expression in Caco-2 Cells

After completion of TER measurements, Caco-2 cell culture inserts were fixed for 10 min with 4 % paraformaldehyde (Sigma-Aldrich) for immunofluorescence staining. After repeated washing with PBS, the cells were permeablized with 0.05 % Triton X-100 (Sigma-Aldrich). Thereafter, the culture inserts were blocked with 5 % BSA (Sigma-Aldrich) followed by overnight incubation with mouse anti-occludin antibody (1:100; Zymed Laboratories, Invitrogen, Camarillo, CA, USA), rabbit anti-claudin-3 (1:100; Zymed Laboratories, Invitrogen, Camarillo, CA, USA), mouse anti-zonula occludens 1 (ZO-1, 1:300; Invitrogen, Camarillo, CA, USA), mouse anti-β-catenin (1:400; BD Transduction Laboratories) or rabbit anti-E-cadherin (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Subsequently, the inserts were labeled with secondary Alexa Fluor 488 or Alexa Fluor 568 conjugated rabbit anti-mouse or goat anti-rabbit antibody (1:2,000; Molecular Probes, Leiden, the Netherlands). Labeled cells were mounted with DAPI-containing VectaShield Mounting Medium (Vector Laboratories Inc., Burlingame, CA, USA). Staining was visualized with an Olympus BX60F5 microscope (Olympus CO Ltd, Tokyo, Japan).

Phosphorylation of ERK1/2 in Caco-2 Cells

Western blot was applied using Bio-Rad Mini-Protein Tetra Cell system (BioRad Laboratories, Espoo, Finland). Caco-2 cells were plated on 6-well plates and confluent monolayers were equilibrated overnight in DMEM supplemented with 0.1 % FBS, non-essential amino acids, sodium pyruvate, sodium bicarbonate and penicillin. Thereafter the cultures were treated with study compounds for 30 min and lysed in SDS-sample buffer (250 mM Tris–HCl pH 6.8, 4 % SDS, 20 % glycerol, 10 % β-mercaptoethanol, and 0.005 % bromophenolblue) with Complete™ protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and PhosSTOP™ phosphatase inhibitor cocktail (Roche). Twenty μl aliquots per lane were loaded on 12 % SDS-polyacrylamide electrophoresis gels. Proteins were transferred to nitrocellulose filters (Hybond C-extra; Amersham Biosciences Ltd., Little Chalfont, UK) and non-specific binding was blocked with 5 % non-fat powdered milk for 1 h at room temperature. Subsequently, the membranes were incubated overnight with mouse anti-extracellular-signal-regulated kinase 1/2 (anti-ERK1/2) antibody (1:500; Santa Cruz Biotechology, Santa Cruz, CA, USA), rabbit anti-phosphorylated-ERK1/2 (anti-pERK1/2) antibody (1:500; Santa Cruz) or mouse anti-γ-tubulin antibody (1:8,000; Sigma-Aldrich). The blots were washed and incubated with secondary horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit antibody (1:2,000; DAKO A/S, Copenhagen, Denmark) for 2 h at room temperature. After washing, the blots were developed with enhanced chemiluminescence (Amersham Life Sciences), exposed to autoradiography film (Kodak, New Haven, CT, USA) and scanned for densitometry analysis with 1D image analysis software (Kodak). Quantification of pERK/ERK was based on densitometric analysis of the Western blots and the data were normalized to the level of the untreated control. The calculated values are derived from four independent experiments, each repeated twice.

Small-Intestinal Mucosal Biopsies and Organ Culture

In organ culture studies we used small-intestinal mucosal biopsies derived from untreated celiac patients with small-bowel mucosal TG2-targeted autoantibody deposits to ensure a response with gliadin [15]. Small-intestinal mucosal biopsies were taken upon upper gastrointestinal endoscopy from eight different untreated celiac disease patients (median age 49.5 years, range 31–63 years, females 50 %) evincing subtotal villous atrophy with crypt hyperplasia and positive serum TG2 autoantibodies. The study protocol was accepted by the Ethical Committee of Tampere University Hospital and written informed consent was obtained from all patients. From each eight participant three biopsies were subjected to organ culture: one received medium only, one PT-gliadin and one PT-gliadin with the TG2 inhibitor R281. Organ culture was maintained for 24 h in RPMI-1640 medium (Invitrogen-Gibco, Paisley, Scotland, UK) containing 15 % FBS (Invitrogen-Gibco), 100 μg/ml streptomycin (Invitrogen-Gibco), 100 U/ml penicillin (Invitrogen-Gibco),

4 mM L-glutamine (Invitrogen-Gibco), 50 μg/ml insulin (Sigma-Aldrich Co, St. Louis, Missouri, USA) and 10 mM HEPES buffer (Invitrogen-Gibco) as previously described [14]. After incubation the culture media were collected and the biopsies snap-frozen at optimal cutting temperature (OCT, Tissue-Tek, Sakura Finetek Europe, Holland). Both samples were stored at -20 °C until analyzed.

TG2-antibody and interferon (IFN) α levels were measured from organ culture supernatants by enzyme-linked immunosorbent assay (ELISA, Celikey®, Phadia, Freiburg, Germany and BioVendor, Brno, Czech Republic, respectively), according to manufacturers' instructions in undiluted samples.

CD25-, forkhead box P3 (FOXP3)-, Ki-67- and interleukin (IL) 15-positive cells were stained in 5-μm-thick cryostat biopsy sections. The sections were fixed in ice-cold acetone and incubated with 5 % goat normal serum (Vector Laboratories Inc., Burlingame, USA) Thereafter the samples were incubated with a primary antibody, anti-CD25 (1:25; Imgenex, San Diego, CA, USA), anti-FOXP3 (1:400; Abcam, Cambridge, UK) or anti-Ki-67 (1:100; Novus Biologicals), at +37 °C for 30 min, anti-IL-15 (1:50; R&D Systems) at +4 °C overnight. Bound CD25 and IL-15 primary antibodies were detected by Alexa-conjugated goat anti-mouse IgG (1:2,000; Invitrogen), FOXP3 antibody by goat anti-rabbit IgG (1:2,000; Invitrogen) and Ki-67 antibody by biotinylated anti-rabbit antibody (1:100; Vector Laboratories, Inc. Burlingame, CA, USA) at +37 °C for 30 min. Incubation with the biotinylated antibody was followed by treatment with peroxidase-conjugated streptavidin (BioGenex, San Ramon, CA). The densities of small-intestinal mucosal CD25-positive T cells in the *lamina propria* and IL-15-positive cells on the epithelial level and in the *lamina propria* were calculated and presented as the number of cells in a total area of one mm^2. The number of FOXP3-positive lymphocytes and Ki-67-positive crypt enterocytes were counted and the results expressed as percentages of the total number of *lamina propria* cells and crypt epithelial cells, respectively.

Statistical Analysis

Results are given as mean±standard error of mean (SEM). Statistical comparisons were made using one-way analysis of variance (ANOVA) followed by two-tailed Student's *t*-test. P-values lower than 0.05 were considered statistically significant.

Results

TG2 Inhibitors R281 and R283 Reduce Toxic Gliadin-Induced Effects in Caco-2 Cells

PT-gliadin increased the permeability of the Caco-2 cell monolayer, which was seen as a decrease in TER. The TER values in cultures treated with PT-gliadin did not recover to the control level in 5 h (Fig. 1a and b). Pre-treatment with TG2 inhibitors R281 (Fig. 1a) and R283 (Fig. 1b) abolished the toxic effect of PT-gliadin and the permeability rose to the same level as the PT-BSA control.

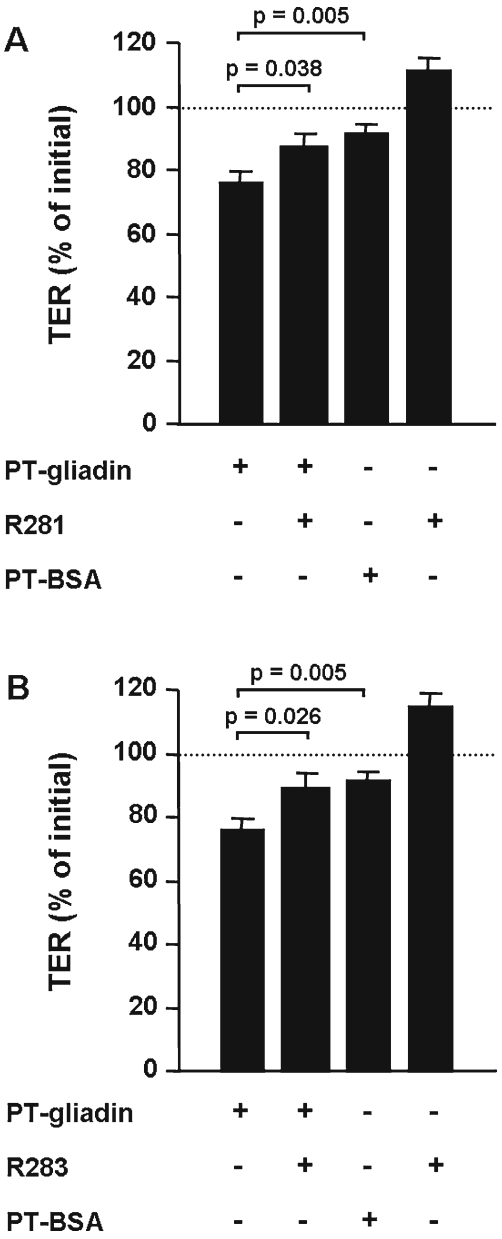

Fig. 1 Transglutaminase 2 (*TG2*) inhibitors R281 and R283 reduce the barrier-breaking effect of gliadin in Caco-2 cells. Transepithelial resistance (*TER*) was measured after 5 h' treatment with pepsin-trypsin-digested (*PT*) gliadin. The TG2 inhibitors R281 **a** and R283 **b** were added to the cultures 1 h prior to PT-gliadin. The dotted line indicates controls values derived from experiments with cells cultured with medium only. Bars represent mean values and error bars standard error of mean. Data are derived from three independent experiments performed in triplicate. Statistical analysis was performed by Student's two-tailed *t* test and $p<0.05$ was considered significant. BSA; bovine serum albumin

One observable effect of gliadin in Caco-2 cells is the rearrangement of the actin cytoskeleton, which is seen as extensive membrane ruffling at the edges of cell clusters. TG2 inhibitors R281 and R283 significantly reduced this toxic effect (Fig. 2a and b), while the pre-treatment with the TG2 inhibitors did not completely normalize the gliadin-induced extent of ruffling to the level of the PT-BSA control (Fig. 2a).

PT-gliadin disrupted the appearance of the tight junction-related proteins ZO-1, occludin and claudin-3, as well as adherent junction proteins E-cadherin and β-catenin (Fig. 3). Incubation with TG2 inhibitors reduced the effect of PT-gliadin on all the proteins, albeit less so for occludin (Fig. 3). Further, PT-gliadin increased the expression of pERK1/2 in Caco-2 cells and TG2 inhibitor R281 was able to abolish this effect (Fig. 4). As R281 performed equally well or even slightly better than R283 in the Caco-2 cell culture model, we selected the cell-impermeable R281 for further ex vivo studies in small-intestinal mucosal biopsies derived from celiac patients.

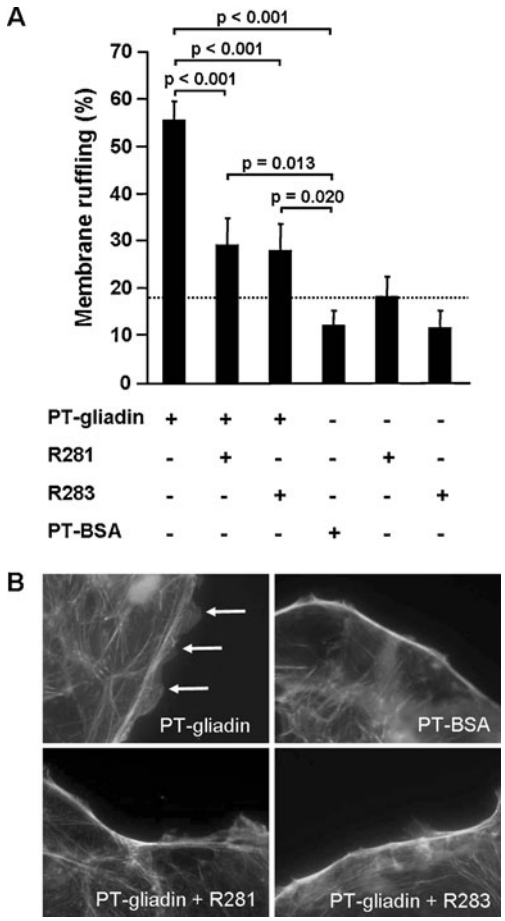

Fig. 2 Transglutaminase 2 (*TG2*) inhibitors R281 and R283 reduce gliadin-induced rearrangement of the actin cytoskeleton in Caco-2 cells. **a** The extent of actin cytoskeleton rearrangement in different treatment groups is presented as percentage of Caco-2 cell edge covered by membrane ruffles. The dotted line represents the level of membrane ruffling in Caco-2 cells treated with medium only. Data are derived from two independent experiments performed in duplicate, and are given as mean values and standard error of mean. Statistical analysis was performed by Student's two-tailed *t* test and $p<0.05$ was considered significant. **b** Representative images of actin membrane ruffling (*arrows*) in Caco-2 cells. PT; pepsin-trypsin digested, BSA; bovine serum albumin

TG2 Inhibitor R281 Reduces Gliadin-Induced Effects in Celiac Disease Patient-Derived Small-Intestinal Mucosal Biopsies

A 24-hour incubation of celiac patient-derived small-intestinal mucosal biopsies in PT-gliadin induced a significant increase in the density of CD25-positive lymphocytes and FOXP3-positive cells in the *lamina propria* (Fig. 5a and b). Moreover, PT-gliadin significantly increased the number of Ki-67-positive proliferating enterocytes in crypts compared to control samples (Fig. 5c). TG2 inhibitor R281 was able to prevent all these gliadin-induced changes. Number of IL-15-positive cells per mm^2 showed a parallel response to PT-gliadin treatment (686±90 in control and 1,048±136 in gliadin-supplemented cultures, $p=0.004$). R281 significantly decreased the gliadin-induced expression of IL-15 (641±129 in PT-gliadin+R281 cultures, $p=0.005$ when compared PT-gliadin treatment). The number of IL-15 expressing cells did not significantly differ in control cultures and those administered with R281 and PT-gliadin ($p=0.585$). R281 was not able to prevent gliadin-induced secretion of TG2 antibodies to the culture medium and IFNα levels were undetectable in these cultures (data not shown).

Discussion

Due to the central role of TG2 in the pathogenesis of celiac disease, TG2 inhibitors have been suggested as putative drugs for the treatment of celiac disease [4]. In this study we demonstrated that two different TG2 inhibitors, the cell-impermeable R281 and the cell-permeable R283, attenuated the toxic effects of gliadin in intestinal epithelial cell culture experiments. Moreover, R281, selected for further studies, abolished most of the gliadin-induced effects in small-intestinal mucosal biopsies derived from untreated celiac disease patients.

Previous ex vivo studies have indicated that TG2 inhibitors could be effective in preventing the gliadin-induced activation of the adaptive immune system [21, 25]. Maiuri and co-workers [21] have shown that the cell-permeable TG2 inhibitor R283 abolishes the gliadin-peptide-induced increase in $CD3^+CD25^+$ T cells in the *lamina propria* in an organ culture system of celiac-patient-derived small-intestinal mucosal biopsies. In addition, TG2 inhibitors have proved to prevent proliferation of intestinal T cells derived from biopsies from celiac patients, although such an effect has not been detectable in T cells from all patients [25]. Our

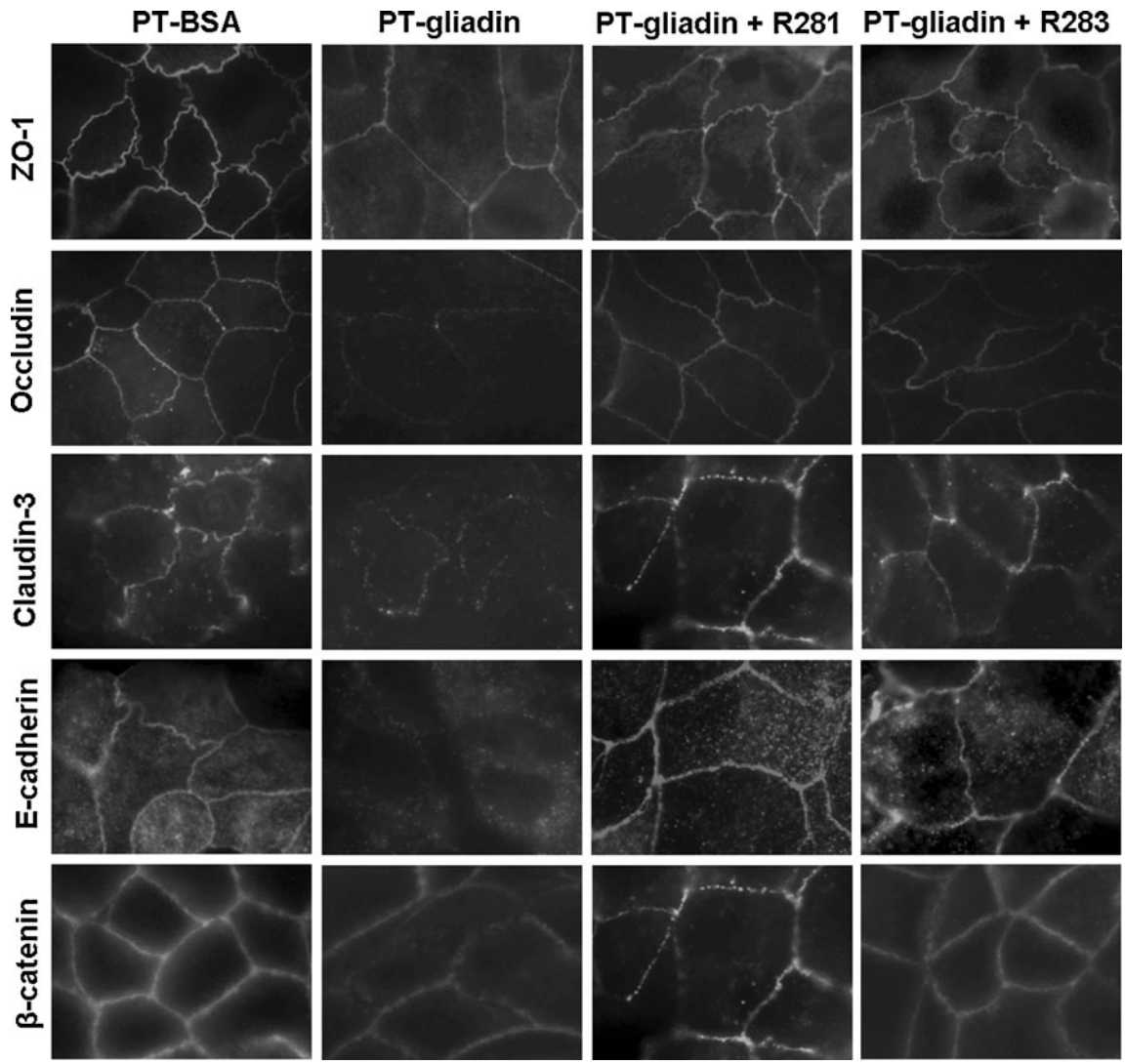

Fig. 3 The effect of pepsin-trypsin-digested (*PT*) gliadin and transglutaminase 2 (*TG2*) inhibitors R281 and R283 on the appearance of cell junction proteins in a Caco-2 cell monolayer determined by immunofluorescence staining. PT-gliadin disrupted the appearance of both tight junction-related proteins ZO-1, occludin and claudin-3, and adherent junction proteins E-cadherin and β-catenin. TG2 inhibitors reduced the effect of PT-gliadin. BSA; bovine serum albumin. Magnification×100

results using the cell-impermeable TG2 inhibitor R281 in an organ culture of celiac-patient-derived biopsies thus support previous conceptions. In this study, we extended these findings by showing that TG2 inhibition can also counteract the gliadin-induced increase in the density of FOXP3-positive Tregs in the *lamina propria*. Generally, Tregs are considered protective and advantageous for immune equilibrium, but in celiac disease their suppressive effect may be impaired [26] even if their absolute number is increased [27, 28]. This raises the question whether the TG2 inhibitor-mediated reduction in the density of FOXP3-positive cells is a desired and favorable effect in the treatment of celiac disease.

In addition to preventing direct gliadin-triggered effects on T cells, TG2 inhibition also abolished the gliadin-induced increase in the number of proliferating Ki-67-positive crypt epithelial cells. Crypt hyperplasia due to enhanced proliferation of epithelial cells is characteristic of untreated celiac disease, and this process is at least partly induced by proinflammatory cytokines secreted by activated T cells in response to ingested gliadin [29, 30]. Since TG2 is a major determinant in T cell activation and downstream secretion of proinflammatory cytokines in celiac patients, it is conceivable that in our model the inhibition of TG2 blocked these actions and thereby prevented gliadin-induced crypt epithelial cell proliferation.

Although TG2 inhibition was able to abolish the effects of gliadin in organ cultures of celiac-patient-derived small-intestinal mucosal biopsies, it did not affect the gliadin-triggered secretion of TG2 antibodies into the culture media. Since this was a proof-of-concept study, we did not optimize the concentration or the incubation times of the TG2 inhibitor,

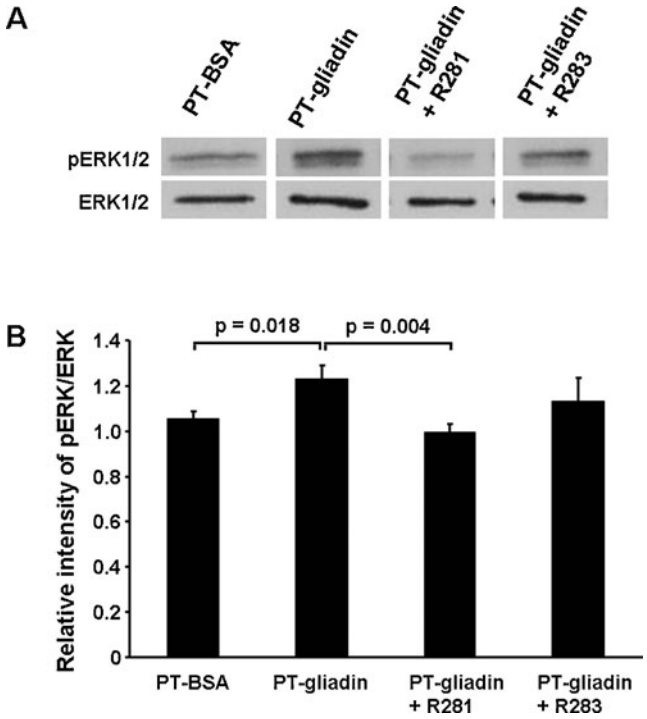

Fig. 4 Immunoblot representing the expression of extracellular-signal-regulated kinase 1/2 (ERK1/2) and its phosphorylated form (pERK1/2) in Caco-2 cell lysates after 30 min of incubation with pepsin-trypsin-digested (PT) gliadin. Transglutaminase 2 (TG2) inhibitor R281, but not R283, reduced the gliadin-induced up-regulation of pERK1/2. **a** A representative Western blot showing pERK1/2 up-regulation in response to PT-gliadin. **b** Quantification of pERK/ERK based on densitometric analysis of the immunoblots ($n=8$). Data are derived from four independent experiments performed in duplicate. Bars represent the mean value after normalization to control and error bars indicate standard error of mean. BSA; bovine serum albumin. Statistical analysis was performed by Student's two-tailed t test and $p<0.05$ was considered significant

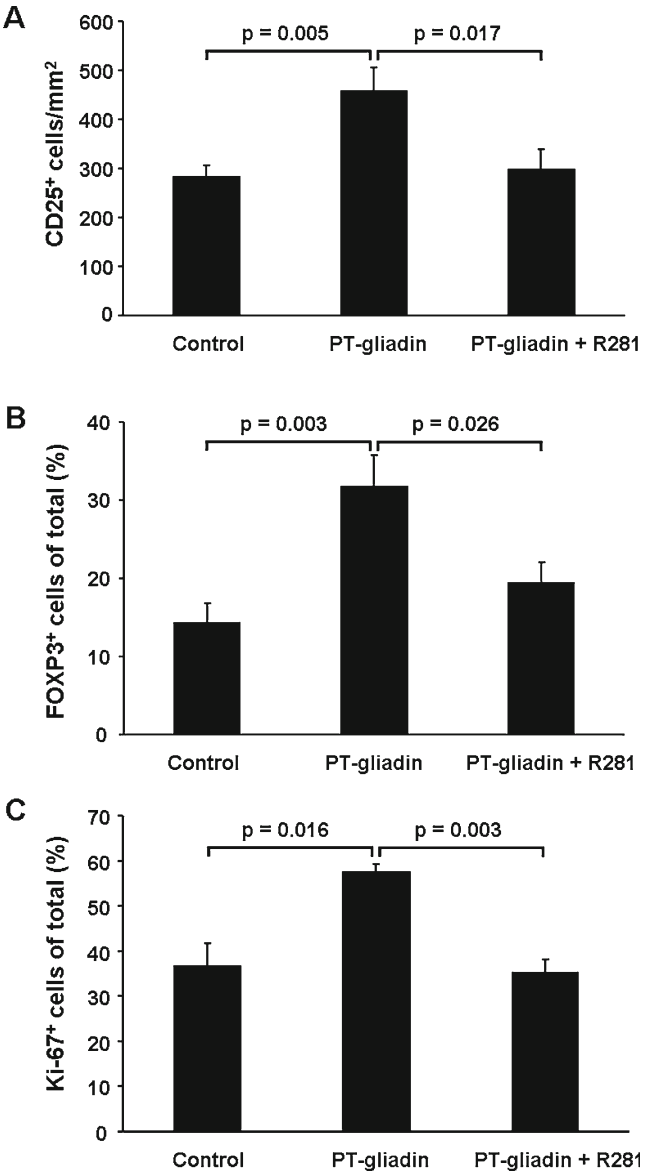

Fig. 5 Transglutaminase 2 (*TG2*) inhibitor R281 reduced the harmful gliadin-induced effects in small-intestinal mucosal biopsies derived from untreated celiac disease patients. **a** The total number of mucosal *lamina propria* CD25-positive T cells was significantly increased by pepsin-trypsin-digested (*PT*) gliadin and TG2 inhibitor R281 prevented this effect. **b** The percentage of FOXP3-positive regulatory T cells was significantly higher in biopsies treated with PT-gliadin compared to controls and R281 abolished this effect **c** The percentage of proliferative Ki-67-positive enterocytes was increased by PT-gliadin in the crypts of biopsies. R281 treatment inhibited this increase. Bars represent mean values and standard error of mean. Statistical analysis was performed by Student's two-tailed t test and $p<0.05$ was considered significant

and our results do not exclude the possibility of a positive readout in fully optimized conditions. It is notable that in earlier studies, the TG2 inhibitor R283 was not able to prevent the gliadin-induced increase in the number of CD8-postive lymphocytes and apoptotic epithelial cells [21]. Further studies with TG2 inhibitors are thus needed to address their potential as a future celiac disease drug.

In addition to adaptive-immune-related effects we sought to establish whether TG2 inhibitors could block the direct T cell-independent toxic effects of gliadin on intestinal epithelial cells. Gliadin is known to reduce TER and the expression of junction proteins as well as to induce rearrangement of actin filaments and phosphorylation of ERK1/2 in intestinal Caco-2 epithelial cells [12, 23, 31, 32]. Both TG2 inhibitors in our study showed protective effects against gliadin-induced insults, but the cell-impermeable R281 appeared slightly more potent. Interestingly, in the presence of TG2 inhibitors TER values were higher than in control cultures treated with medium only indicating that permeability of cultures treated with TG2 inhibitors were recovered over the baseline. The mechanism by which these TG2 inhibitors block direct gliadin-triggered damage to epithelial cells is obviously different from that involved in the adaptive-immunity-mediated reactions. TG2 is known to crosslink several proteins in the extracellular matrix [7] which is an important modulator of the expression of tight junction

proteins and epithelial permeability [33]. TG2 has also been reported to catalyze the binding of gliadin peptides to extracellular matrix collagen [34], and it is conceivable that this could also have occurred in our Caco-2 cell culture model. The cross-linked gliadin in the matrix could lead to structural and functional abnormalities eventually resulting in increased epithelial permeability coupled with decreased expression of junction proteins. Thus in the Caco-2 cell culture the TG2 inhibitors might have prevented gliadin-induced harmful effects by blocking the processing of the extracellular matrix.

TG2 offers a potential target for drug development in celiac disease. However, it is known that a major issue with TG2 inhibitors is their lack of tissue-directed specificity potentially causing serious side-effects when used in vivo. Therefore, novel drugs should be designed for exclusive delivery to gastrointestinal system or their action should happen selectively and locally in the small bowel. Another noteworthy aspect is that gliadin can increase permeability and cytoskeleton rearrangement also by binding to a chemokine receptor CXCR3, which induce zonulin release [35]. Thus, TG2 inhibition would not inhibit gliadin interaction with CXCR3 and thus not be sufficient in preventing all gliadin-induced harm in the context of celiac disease.

Conclusion

Our results in this proof-of-concept study suggest that the TG2 inhibitors R281 and R283 are able to reduce gliadin-induced harmful effects in a Caco-2 intestinal epithelial cell line. Furthermore, R281 prevents gliadin-induced effects in small-intestinal mucosal biopsy specimens derived from celiac disease patients. These findings are promising as a proof of concept in an effort to develop new TG2 inhibitor-based therapies for celiac disease.

Acknowledgements The Celiac Disease Study Group has been financially supported by the Academy of Finland, the SalWe Research Program for Intelligent Monitoring of Health and Wellbeing (Tekes—the Finnish Funding Agency for Technology and Innovation grant 648/10), the Pirkanmaa Regional Fund of the Finnish Cultural Foundation, the Sigrid Juselius Foundation, the Competitive Research Funding of Tampere University Hospital, the Research Fund of the Finnish Celiac Society, the European Commission IAPP grant TRANSCOM (Contract number PIA-GA-2010-251506) and Elna Kaarina Savolainen's Legacy Allocated for the Development of Cancer Treatment.

Conflict of interest The authors declare no conflict of interest.

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