

Lower Expression of Tight Junction Protein 1 Gene and Increased FOXP3 Expression in the Small Bowel Mucosa in Coeliac Disease and Associated Type 1 Diabetes Mellitus

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Key Words

Coeliac disease • Type 1 diabetes • Tight junction protein 1 • FOXP3 • Regulatory T cells • IgA antibodies • IgG antibodies • Food antigens

Abstract

Background: The role of regulatory T cells expressing FOXP3 in the pathogenesis of coeliac disease (CD) and type 1 diabetes (T1D) has been reported. Recent data have placed special focus on the interplay between the intestinal barrier and immunoregulatory processes. We aimed to determine whether the expression of tight junction protein 1 (TJP1), which reflects small bowel mucosa permeability, is changed in CD and T1D. **Methods:** Transcription levels of TJP1 and FOXP3 genes were evaluated in the small bowel biopsies of 14 children with CD, 12 with CD and coexisting T1D and 40 controls using real-time PCR. Serum IgA and IgG to deamidated gliadin, bovine β -lactoglobulin, bovine α -casein and human tissue transglutaminase (tTG) were determined by ELISA. **Results:** The highest expression of FOXP3 mRNA was seen in patients with CD and T1D compared to patients with CD alone and controls ($p = 0.02$). In contrast, the lowest level of TJP1 mRNA expression was found in patients with CD and T1D ($p = 0.01$). The levels of IgA to deamidated gliadin and

tTG were highest in patients with CD and T1D ($p = 0.0001$ and 0.01 , respectively). The expression of FOXP3 mRNA correlated highly with the level of anti-gliadin IgA ($p = 0.02$) and anti-tTG IgA antibodies ($p = 0.004$). **Conclusion:** The significant decline in TJP1 expression in CD patients, particularly in those with coexisting T1D, was accompanied by an increase in FOXP3 expression. This might reflect an attempt to maintain immune tolerance to counterbalance the loss of mucosal integrity in the small intestine in CD associated with T1D.

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Introduction

Tight junction protein 1 [TJP1; also known as zonula occludens protein 1 (ZO-1)], the first protein discovered at tight junctions in a variety of epithelia [1], is involved in the structural and functional organization of tight junctions in the gut mucosa and might influence small bowel mucosa permeability [2–4].

Coeliac disease (CD) is characterized by an altered immune response to ingested wheat gluten and its component gliadin, which leads to inflammation, villous atrophy and crypt hyperplasia in the small bowel mucosa [5, 6]. CD is a very common chronic gastrointestinal dis-

ease in childhood with a prevalence of 0.5–3% according to international population studies [7, 8]. As confirmed by our own studies, in most cases CD possesses atypical symptoms and could only be detected by a directed search [9].

There is no conclusive knowledge of the interactions between gliadin and intestinal epithelia and the mechanism by which it crosses the epithelial barrier. Gluten peptides may cross the intestinal epithelium either via the paracellular route or via transcytosis of the gliadin after its partial degradation [6, 10]. The role of the gut immune system is also considered in the pathogenesis of another autoimmune disease, type 1 diabetes mellitus (T1D). Dietary gluten as well as cow's milk proteins are thought to be risk factors for T1D [11–13]. In children, CD and T1D are frequently associated. The average prevalence of CD among children with T1D in 26 reports was 4.5% (range 0.97–16.4%) [14, 15]. The prevalence of CD in T1D in Estonia is at least 3.3%. However, the number of CD cases found among T1D patients is dependent on the follow-up time of index patients [16]. In the pathogenesis of both of these autoimmune diseases, the intestinal luminal antigens that have been absorbed through the gut mucosa might play a certain role in triggering consecutive immune events [17, 18]. One of the pre-existing conditions that leads to an autoimmune process is the presentation of an antigen to the mucosal immune system following its paracellular passage from the intestinal lumen to the gut submucosa [2, 17, 19]. This process is usually prevented by tight junctions [2, 17]. Thus, increased intestinal permeability may precede disease by causing delivery of an antigen into the gut immune system, leading to production of IgA antibodies against gliadin [6, 20]. The functional integrity of the small bowel mucosa is impaired in CD, which is even noted in the relatives of patients with CD [21–24]. Antibodies to the autoantigen tissue transglutaminase (tTG) have also been shown to increase intestinal permeability in CD [25]. Duerksen et al. [26] found positive correlation of tTG antibodies with the severity of intestinal lesions and intestinal permeability. Increased permeability of the small intestinal mucosa has also been shown in patients with T1D, which is suggested to be important in the pathogenesis of T1D [27–29].

In addition to the micro- and macro-anatomical changes demonstrated in CD and T1D, there is an increasing body of evidence showing that substantial immunological changes take place in the small intestinal mucosa in both diseases. Among them, the role of regulatory T cells (Treg), most frequently characterized by expression of CD4+ and FOXP3+ by intestinal lympho-

cytes, has been reported in the pathogenesis of both T1D and CD [30–33]. There is evidence that intestinal Treg together with thymus-derived Treg collaborate to reinforce intestinal homeostasis in order to suppress immune responses toward dietary antigens and self-antigens [32].

Recent data have placed special focus on the interplay between the intestinal barrier and immunoregulatory processes. It has been hypothesized that immunoregulatory mechanisms will be more strongly activated in the case of impaired intestinal barrier function to prevent inappropriate inflammatory responses [3]. Dysfunction of epithelial tight junctions causes mucosal immune activation by challenging CD4+ lymphocytes in the intestinal lamina propria [6, 34], as well as leading to Treg generation [35]. Elevated intestinal permeability leads to an increase in the number of dendritic cells in the intestinal lamina propria [3], which in turn, being conditioned after contact with epithelial cells, are able to promote Treg differentiation [36].

In our previous study [37], we found higher expression of FOXP3 mRNA, as well as a higher number of CD25+/CD4+/FOXP3+ cells, in small bowel biopsy specimens of children with CD, particularly in those with coexisting T1D, compared with FOXP3 expression in normal small bowel mucosa. However, the association of this phenomenon with intestinal permeability and development of CD-related autoantibodies remained unclear.

In the present study, we aimed to determine whether small bowel tight junction integrity, characterized by TJP1 expression (as TJP1 mRNA and ZO-1 protein in tissue samples), is changed in patients with CD and coexisting T1D and whether it is associated with FOXP3 expression in the intestinal mucosa and development of antibodies against dietary antigens.

Subjects and Methods

Study Subjects

Sixty-six patients (33 females, 33 males; mean age 8.7 ± 6.0 years) who were admitted to the Children's Clinic of Tartu University Hospital in the periods 1998–2000 and 2005–2006 and who underwent small bowel biopsy were studied. All patients were recruited at the time of the diagnosis of CD. A duodenal biopsy was taken either using the Watson capsule (26 cases) or by gastroduodenoscopy (40 cases). In the former procedure, the biopsy sample was divided into 2 portions; one half was stored for morphological examination and the other half was immediately quick-frozen in Tissue Tek OCT Compound (Sakura Finetek, Finland) for real-time (RT)-PCR and stored at -80°C . One biopsy sample taken by gastroduodenoscopy was used for morphological examination, another was stored in RNAlater (Ambion Inc., Austin, Tex., USA) at -25°C for further RT-PCR and the third one was

Table 1. Study groups, state of the small bowel mucosa and the studies employed**a** State of the small bowel mucosa in the different study groups

Study group	n	Mean age years	Males/females	Normal mucosa	Villous atrophy
CD without T1D	14	3.5 ± 4.2	9/5	0	14
CD with T1D	12	9.5 ± 2.9	5/7	0	12
Controls	40	10.4 ± 6.4	19/21	36	4
Total	66	8.5 ± 0.8	33/33	36	30

b Studies employed

Study group	n	TJP1 mRNA expression studied	FOXP3 mRNA expression studied	ELISA antibodies to			
				deamidated gliadin IgA and IgG	α-casein IgA and IgG	β-lacto-globulin IgA and IgG	tTG IgA, IgG and IgM
CD without T1D	14	14	14	13	5	10	6
CD with T1D	12	11	7	12	10	10	10
Normal mucosa	36	33	22	32	31	25	29
Controls with PVA or SVA	4	4	4	4	4	4	0
Total	66	62	47	61	50	49	45

PVA = Partial villous atrophy (Marsh II); SVA = subtotal villous atrophy (Marsh IIIa).

snap frozen, embedded in Tissue Tek OCT Compound (Sakura Finetek) and stored at -80°C.

The diagnosis of CD was established on the basis of the criteria of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition [38]. Morphologically, villous atrophy was classified according to the Marsh classification [39, 40].

CD without T1D was diagnosed in 14 patients (mean age 3.5 ± 4.2 years; villous atrophy according to Marsh: IIIa in 4 cases, IIb in 10), and CD associated with T1D was diagnosed in 12 patients (mean age 9.5 ± 2.9 years; in all 12 patients, villous atrophy IIb according to Marsh). Forty patients (mean age 10.4 ± 6.4 years) with other diagnoses (functional dyspepsia, Down's syndrome, allergy to cow's milk protein, retardation of psychomotor development, allergic dermatitis, duodenal ulcer, erosive gastritis) formed the control group. Four of the patients with other diagnoses had partial or subtotal villous atrophy, while the remaining 36 patients without CD had normal small bowel mucosa (Marsh classification 0; table 1a).

The study was approved by the Ethics Review Committee for Human Research of the University of Tartu. All studied children and/or their parents gave their written consent.

Real-Time PCR

Total RNA was isolated from the biopsy samples using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The amount of RNA obtained was determined by measuring absorption at 260 and 280 nm using NanoDrop (ND-1000 Spectrophotometer, Kisker Biotech,

Germany). The quality of RNA was checked by agarose gel electrophoresis using ethidium bromide staining according to the *RNeasy Mini Handbook* instructions (Qiagen, June 2001, p 92). When the 28s ribosome RNA band was present with an intensity approximately twice that of the 18s RNA band, the quality of RNA proved suitable for further use. In addition, the purity of RNA was estimated by measurement in a spectrophotometer as the ratio of absorbance of RNA at 260 and 280 nm, which should be 1.9–2.1.

cDNA was prepared using a cDNA transcription Kit (Applied Biosystems, USA) according to the manufacturer's protocol. Genomic DNA was eliminated by additional DNAase I treatment (Roche Diagnostics, Germany) before cDNA synthesis. Random Hexamers (Applied Biosystems) were used for prime first-strand synthesis, and the reaction was carried out in a total volume of 20 µl.

The transcription level of the TJP1 (ZO-1) gene was evaluated in 62 small bowel biopsy samples (table 1b) using SYBR Green RT-PCR Gene Expression Analysis according to the manufacturer's instructions (SuperArray Bioscience Corporation, USA). A total of 1 µl of cDNA probe was used for each duplicate measurement of human TJP1 (Hs.510833, SuperArray Bioscience Corporation), and 1 µl of cDNA was used for measurement of the housekeeping gene β-actin (Hs.520640, SuperArray Bioscience Corporation).

The home-made exogenous cDNA calibrator sample was prepared from the Caco-2 cell line (American Tissue Culture Collection), cultivated for 21 days at a concentration of 5 × 10⁶ cells in 120 µl and used as the interassay standard to which all other samples were compared for each run. Interassay variation was

5%. The Caco-2 cell line is known to express high levels of TJP1 mRNA [41].

The ABI Prism 7000 sequence detector was programmed to 10 min at 95°C (enzyme activation), followed by 40 thermal cycles of 15 s at 95°C (denaturation) and 1 min at 60°C (annealing/extension). Amplification was performed in a total volume of 25 µl. Each measurement was carried out in duplicate.

The quantitative value obtained from the SYBR Green run is the cycle threshold (Ct) value, which indicates the number of PCR cycles at which the amount of the amplified target molecule exceeds a predefined threshold value. The comparative threshold method was used to quantitate gene transcription in the samples. The Ct of human β-actin was subtracted from the target gene Ct. The obtained difference was taken as the ΔCt value. The ΔCt of the calibrator was then subtracted from the ΔCt of the sample. The obtained difference was called the ΔΔCt value, and the results were expressed in relative units based on the calculation of $2^{-\Delta\Delta Ct}$, which yielded the relative amount of the target gene normalized to the endogenous control (β-actin) and relative to the calibrator, multiplied by 1,000.

The transcription level of the FOXP3 gene (Hs00203958_m1), as a characteristic of Treg, was evaluated in the same way, but in 15 biopsies, using the TaqMan Gene Expression assay (Applied Biosystems) as described and published previously [37].

Immunofluorescence Staining for TJP1 (ZO-1)

Immunofluorescence staining for TJP1 (ZO-1) protein on frozen sections of intestinal mucosa was performed in 14 snap frozen biopsy specimens of small bowel mucosa (7 CD patients with coexisting T1D with villous atrophy and 7 patients with normal small bowel mucosa) according to Pizzuti et al. [42]. Biopsy specimens were embedded in Tissue Tek OCT Compound (Sakura Finetek) and stored at -80°C.

The 4-µm frozen sections cut with a cryostat microtome were mounted on SuperFrost Plus slides (Menzel GmbH & Co KG, Braunschweig, Germany), air dried overnight and used immediately. The sections were fixed in 4% paraformaldehyde for 10 min at room temperature, followed by permeabilization with 0.01% Triton X-100 (Serva Feinbiochemica, Heidelberg, Germany) in Tris buffer for 30 min at room temperature.

The sections were incubated with polyclonal rabbit antibodies to ZO-1 (Zymed Laboratories, Invitrogen Corporation, Carlsbad, Calif., USA) at a dilution of 1:100 (2.5 µg/ml) in Tris buffer for 2 h at 37°C in a humid chamber under coverslips. After washing with Tris, a sheep antibody against rabbit IgG (whole molecule) Fab' fragment conjugated with Cy3 (Sigma-Aldrich, St. Louis, Mo., USA) at a dilution of 1:100 was used as the secondary antibody (incubation for 1 h at room temperature). After the next washing in Tris buffer, the slides were mounted in Tris-glycerol solution. A negative control was performed by omitting the primary antibody (incubation with Tris buffer). The slides were examined under an Olympus VX50 fluorescence microscope, using objectives with a magnification of ×20 and ×40 and an eyepiece at ×10. All sections were studied with the investigator blinded to the RT-PCR data and scored according to the intensity of staining from 0 to 5 (absence of staining was scored as 0, weak staining intensity as 1, mild as 2, moderate as 3, marked as 4 and strong intensity as 5). The corresponding haematoxylin-eosin slides were reviewed for confirmation of histology from the same frozen biopsy specimens. ZO-1-positive staining appeared at the apical

part of the epithelial cell layer of the intestinal mucosa as a crenelated or linear pattern with dot-like accentuation.

Enzyme-Linked Immunosorbent Assay

Serum IgA and IgG antibodies to deamidated gliadin (GAF-3X) were detected in 61 sera by ELISA using the Euroimmun kit (Medizinische Labordiagnostika AG, Lübeck, Germany).

Serum IgG and IgA antibodies to bovine β-lactoglobulin (Sigma-Aldrich) in 49 sera and to bovine α-casein (Sigma-Aldrich) in 50 sera were measured using ELISA. The wells of microtitre plates (Nunc Polysorp, Denmark) were coated with bovine β-lactoglobulin at a concentration of 1 µg/ml, or with bovine α-casein at a concentration of 2 µg/ml in PBS, and incubated overnight at 4°C. After washing with PBS-Tween (0.05%), the plates were blocked with 2% normal horse serum in PBS for 1 h at 37°C. Sera were diluted to 1:20 in 1% horse serum-PBS, applied to the plate in triplicate (2 coated wells and 1 well without the antigen) and incubated for 1 h at 37°C. After washing, 100 µl of horseradish peroxidase-conjugated rabbit anti-human IgG (Dako, Glostrup, Denmark; dilution 1:4,800) and rabbit anti-human IgA (Dako; dilution 1:4,000) were applied to the wells, followed by incubation for 1 h at 37°C. After washing, 100 µl of tetramethylbenzidine liquid substrate (Sigma-Aldrich) was applied to the plates and incubated for 10 min at room temperature. The reaction was stopped with 2 M H₂SO₄, and optical density was measured immediately at 450 nm. The absorption value in the well without the antigen was subtracted from the absorption values of the antigen-coated wells, which yielded the absorbance value of the studied sera. Three reference sera with a high level of the studied antibodies were used as positive controls (donated by Prof. E. Savilahti, Helsinki, Finland).

IgA, IgG and IgM antibodies against tTG were detected in 45 sera by ELISA as described by Teesalu et al. [43]. For assessment of IgG and IgM anti-tTG antibodies, a modified assay with purified human fibronectin was used [43].

All antibodies of interest, i.e. antibodies to deamidated gliadin, bovine β-lactoglobulin, bovine α-casein and tTG, were possible to evaluate in 33 sera.

Statistical Analysis

Statistical calculations were performed using the Statistica 8.0 software (Stat Soft) and the GraphPad Prism 5.03 software. The results obtained for different study groups were presented as means ± SE and compared using the t test for independent samples and the Mann-Whitney test. Correlation between the results of TJP1 gene expression and the level of antibodies was analysed by Spearman's rank correlation test. Age adjustment for mean antibody levels was performed using multiple regression analysis. Differences were considered statistically significant at $p < 0.05$.

Results

The lowest level of TJP1 mRNA expression was found in patients with CD and coexisting T1D, which was significantly different from the corresponding level in the control group (437.9 ± 52.5 vs. 713.2 ± 98.4 ; $p = 0.01$). The highest expression of FOXP3 mRNA was seen in pa-

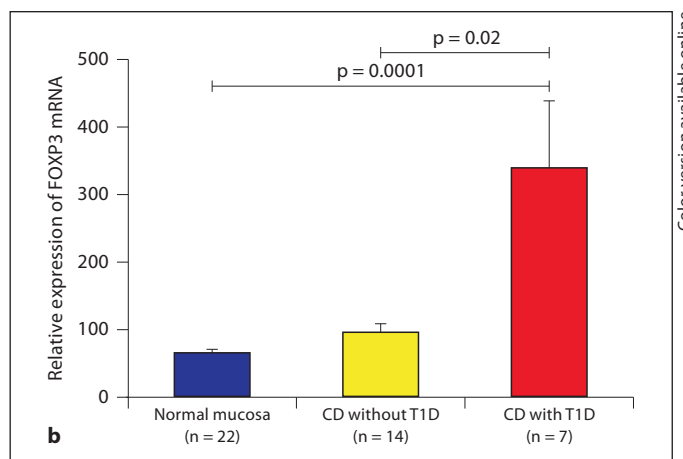
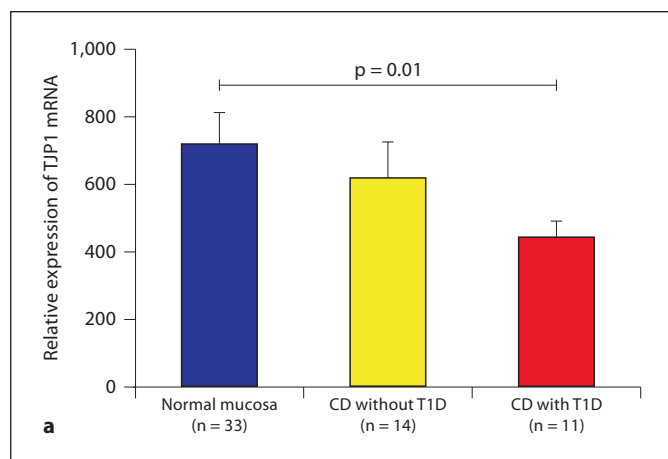


Fig. 1. Comparative expression of TJP1 (a) and FOXP3 mRNA (b) in small bowel biopsy specimens of the study groups. **a** The transcription level of the TJP1 (ZO-1) gene was evaluated using SYBR Green RT-PCR for human TJP1 (Hs.510833). As the housekeeping gene, β -actin (Hs.520640) was used. **b** The transcription level of

the FOXP3 gene (Hs00203958_m1) was evaluated using the TaqMan Gene Expression assay, and as the housekeeping gene, ribosomal 18s was used. The results are expressed in relative units based on the calculation of $2^{-\Delta\Delta C_t}$. Each bar represents the mean $\pm$ SE.

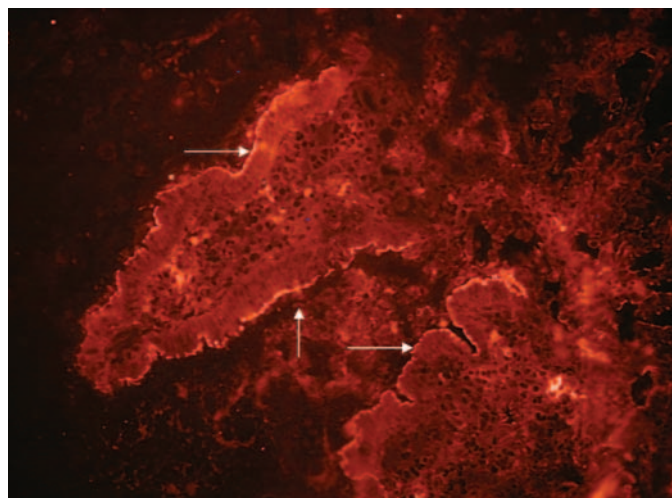


Fig. 2. Expression of TJP1 at the apical part of the epithelial cell layer (arrows) in normal intestinal mucosa (crenellated, without disruptions), with the staining intensity scored as 5 ($\times 20$ objective, $\times 10$ eyepiece). An immunofluorescence method was used, with antibodies to ZO-1 at a dilution of 1:100 (2.5 μ g/ml) and the secondary antibody sheep anti-rabbit IgG (whole molecule) Fab' fragment conjugated with Cy3 at a dilution of 1:100.

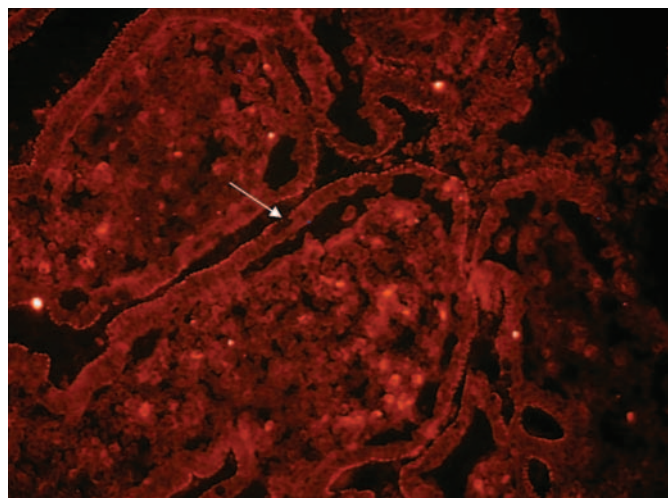


Fig. 3. Weak staining of TJP1 on the epithelial surface of the intestinal villae with disrupted patches (arrow) from a patient with CD and coexisting T1D, scored as 2 ($\times 20$ objective, $\times 10$ eyepiece). An immunofluorescence method was used, with antibodies to ZO-1 at a dilution of 1:100 (2.5 μ g/ml) and the secondary antibody sheep anti-rabbit IgG (whole molecule) Fab' fragment conjugated with Cy3 at a dilution of 1:100.

tients with CD and T1D (335.3 ± 102.6), which was different from FOXP3 mRNA expression in patients with CD alone (92.6 ± 15.8 ; $p = 0.02$) and in persons with normal mucosa (61.1 ± 10.1 ; $p = 0.0001$). Comparative expression of TJP1 and FOXP3 mRNA in small bowel bi-

opsy specimens for the different study groups is presented in figure 1a and b. Significant negative correlation was found between the expression of the TJP1 gene and the values of FOXP3 gene expression in the biopsy specimens of the studied patients ($r = -0.42$; $p = 0.003$; $n = 46$).

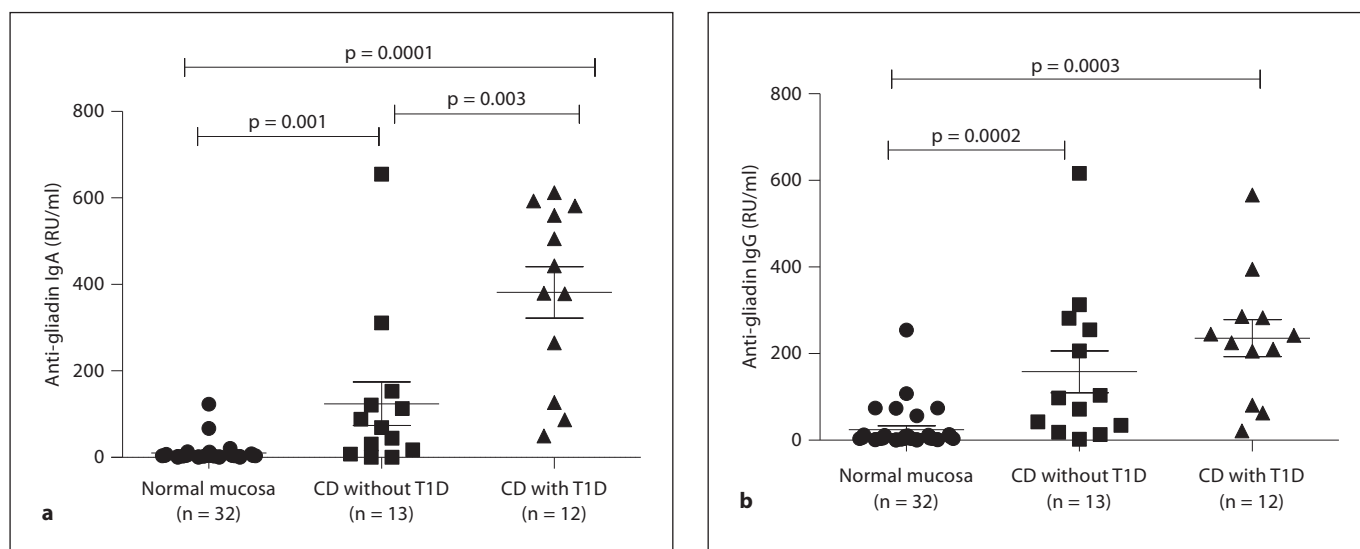


Fig. 4. Values of IgA (a) and IgG (b) antibodies to gliadin (GAF-3X) in the various study groups, detected by ELISA using the Euroimmun kit. Mean values are marked with horizontal lines $\pm$ SE. RU = Relative units.

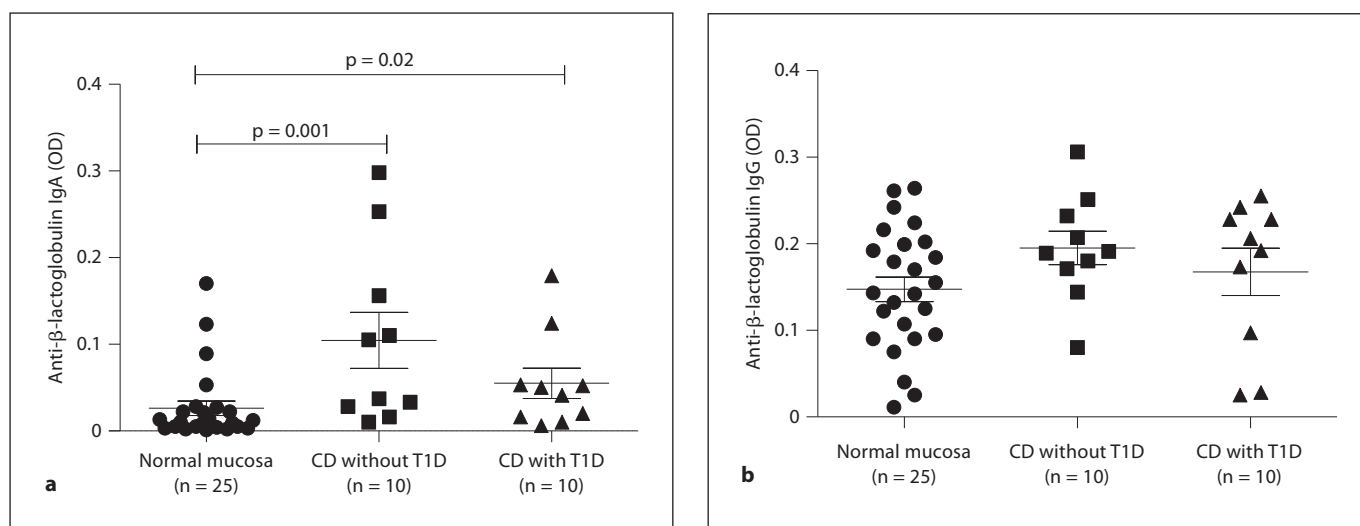


Fig. 5. Values of IgA (a) and IgG (b) antibodies to β -lactoglobulin in the various study groups, expressed as optical density (OD) (ELISA, serum diluted 1:20). Mean values are marked with horizontal lines $\pm$ SE.

The pattern of TJP1 expression in normal intestinal mucosa (fig. 2) appeared crenellated and more clearly distinguished compared with that in the intestinal mucosa of patients with villous atrophy (fig. 3). There was no disruption of the fluorescence pattern in the normal mucosa, while in patients with atrophy, TJP1 reactivity appeared as a disrupted pattern. The immunofluorescence intensity varied considerably within both study groups;

however, the mean staining intensity score did not differ significantly between the group of patients with CD and T1D (2.42 ± 0.36 ; $n = 7$) and the control group with normal mucosa (2.42 ± 0.61 ; $n = 7$; $p = 0.5$).

As expected, the levels of IgA and IgG antibodies to deamidated gliadin were significantly higher in CD patients (without T1D as well as with T1D) than in patients with normal gut mucosa (fig. 4a, b). In the group of pa-

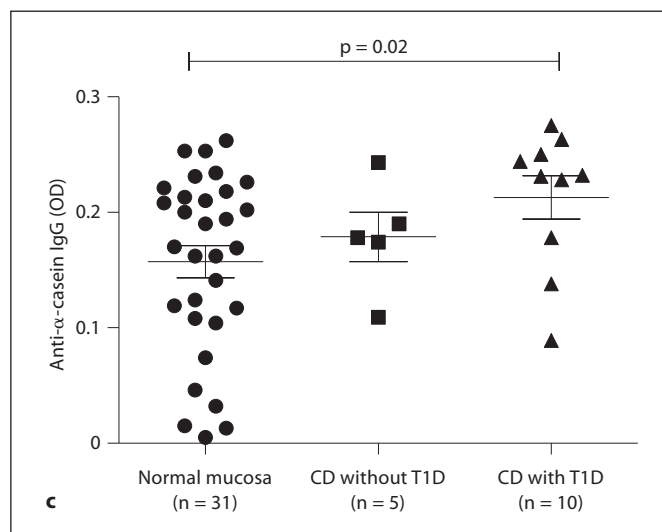
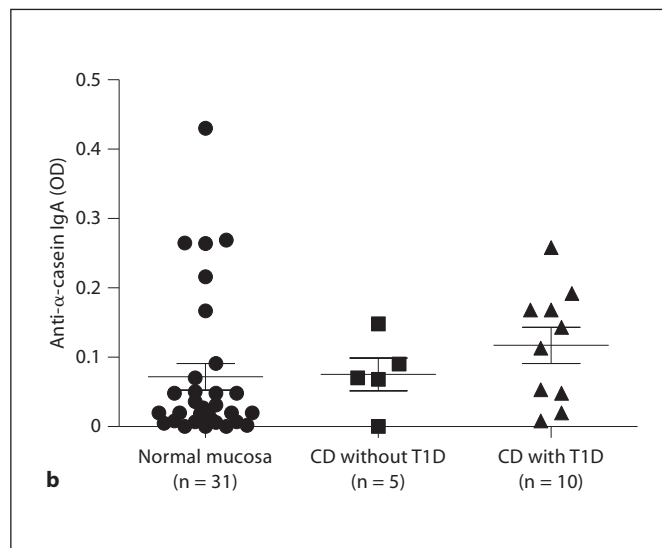
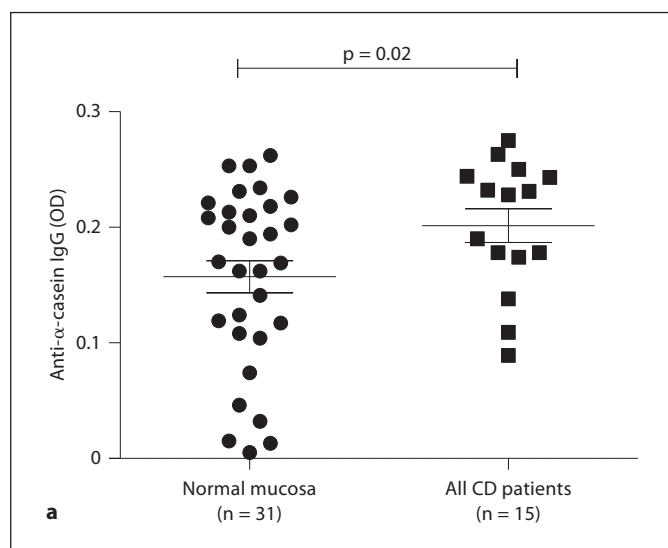


Fig. 6. a Values of IgG antibodies to α -casein for persons with normal mucosa and for all studied CD patients, expressed as optical density (OD) (ELISA, sera diluted 1:20). Mean values are marked with horizontal lines $\pm$ SE. **b, c** Values of IgA (**b**) and IgG (**c**) antibodies to α -casein in the various study groups, expressed as optical density (OD) (ELISA, sera diluted 1:20). Mean values are marked with horizontal lines $\pm$ SE.

tients with CD and coexisting T1D, the level of IgA antibodies to deamidated gliadin was significantly higher than that in CD patients without T1D ($p = 0.003$; fig. 4a). Age adjustment (using multiple regression analysis) showed that the levels of IgA and IgG antibodies to gliadin were not dependent on age ($\beta = 0.15$, $p = 0.35$, and $\beta = 0.048$, $p = 0.76$, respectively). A significant negative correlation between expression of the TJP1 gene and the level of IgA to gliadin ($r = -0.24$; $p = 0.03$; $n = 57$) was established by analysing the data of the whole studied group.

The level of IgA but not IgG antibodies to bovine β -lactoglobulin was significantly elevated in CD patients compared with the group of patients with normal mu-

cosa (fig. 5a, b), while a significantly higher level of IgG-type antibodies to bovine α -casein was found in patients with CD (including those with associated T1D) compared with patients with normal mucosa ($p = 0.02$; fig. 6a–c). The level of IgA as well as that of IgG and IgM antibodies to tTG was significantly higher in CD patients than in patients with normal small bowel mucosa ($p = 0.009$, 0.0005 and 0.004 , respectively; fig. 7a–c). The highest level of IgA antibodies to tTG was demonstrated in CD patients with coexisting T1D, i.e. 81.2 ± 13.9 arbitrary units (AU) versus 30.0 ± 12.9 AU in CD patients without T1D ($p = 0.01$) and 3.2 ± 0.4 AU in the control group ($p = 0.009$; fig. 7a). Age adjustment (using multiple regression analysis) showed that the value of IgA anti-

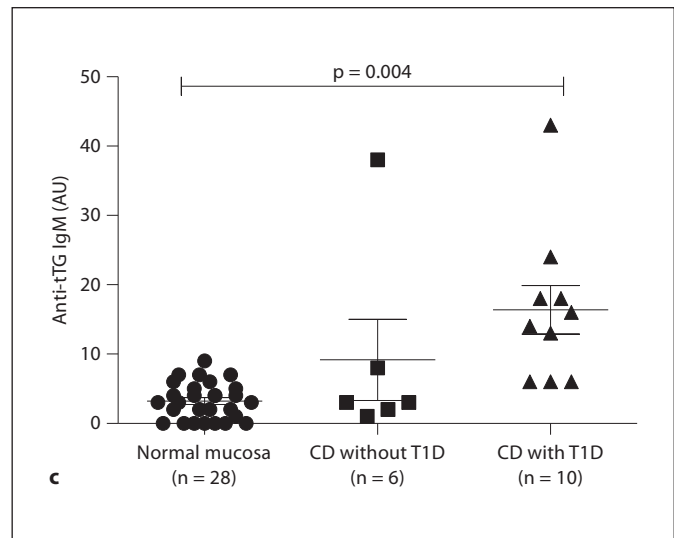
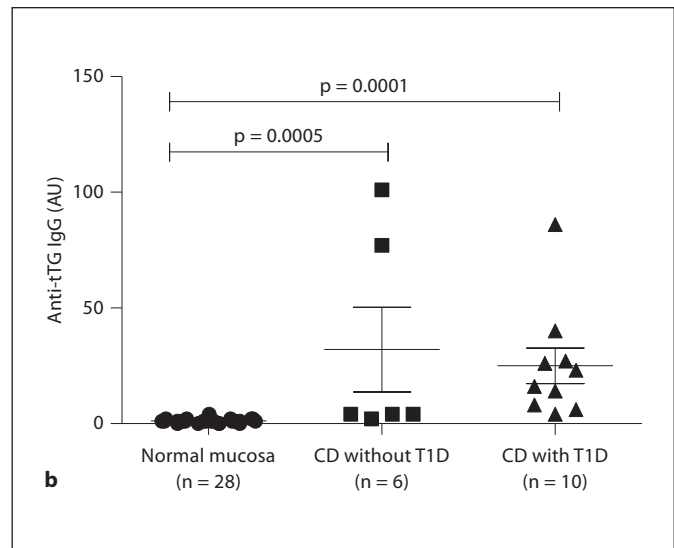
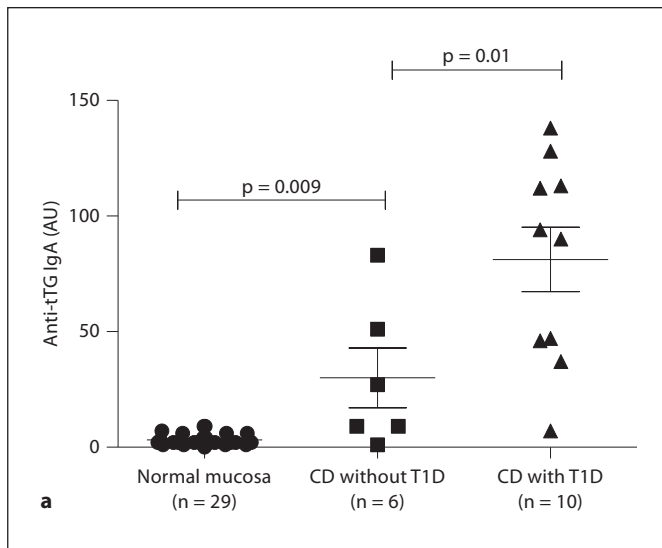


Fig. 7. Values of IgA (a), IgG (b) and IgM (c) antibodies to tTG (human recombinant) in the various study groups. A modified ELISA with purified human fibronectin was performed as described by Teesalu et al. [43]. Mean values are marked with horizontal lines $\pm$ SE.

bodies to tTG did not depend on age ($\beta = 0.06$; $p = 0.7$) but did depend on the study group ($\beta = 0.5$; $p = 0.003$). In CD patients (including those with T1D), a higher level of IgA antibodies to tTG correlated with lower expression of the TJP1 gene in biopsy specimens ($r = -0.84$; $p = 0.03$; $n = 6$).

The expression of FOXP3 mRNA correlated highly with the level of anti-gliadin IgA antibodies ($r = 0.3$; $p = 0.02$; $n = 43$), anti-gliadin IgG antibodies ($r = 0.37$; $p = 0.01$; $n = 41$) and anti-tTG IgA antibodies ($r = 0.52$; $p = 0.004$; $n = 27$) for the whole study group. The level of anti-tTG IgA in CD patients with coexisting T1D correlated with higher expression of FOXP3 mRNA ($r = 0.90$; $p = 0.04$; $n = 5$).

Discussion

The results of the present study revealed a significant decline in TJP1 gene expression in CD patients, particularly in those with associated T1D, suggesting that these persons may suffer considerable loss of epithelial tight junctions, leading to an increase in intestinal permeability. Our data support the notion of impaired intestinal integrity and permeability in patients with CD and T1D [13, 17, 44]. The results of our study are in concordance with the data of Drago et al. [45], who showed significantly lower expression of ZO-1 (TJP1) and occludin genes in CD patients compared to non-CD controls. Also, Pizzuti et al. [42] demonstrated a significant decrease in

the mRNA level as well as the protein expression of ZO-1 in the small bowel mucosa of untreated CD patients compared with normal mucosa. However, we failed to find a clear concordance between the results for the expression of TJP1 mRNA and the expression of this protein in the small bowel mucosa of our patients with the use of a semi-quantitative histochemical approach to protein detection. We suppose that the TJP1 (ZO-1) staining pattern is quite difficult to interpret from biopsy sections, as it seems to be patchy compared with mRNA expression from the entire biopsy in quantitative analysis. According to a recent extensive study of Ravelli et al. [46], some variability in histological lesions can be found in newly diagnosed CD, as was the case with our study group, even within the same duodenal biopsy specimen.

In our study, CD patients with lower expression of TJP1 had higher levels of anti-gliadin as well as anti-tTG IgA antibodies, which might reflect greater intestinal permeability to gluten in these persons [22, 26]. The link between enhanced gut permeability to gliadin and production of anti-gliadin IgA antibodies was already reported by Bonamico et al. [47] and was confirmed by other authors [20]. An interesting finding of our study was the particularly high level of anti-gliadin IgA antibodies in CD patients with coexisting T1D, who also displayed the lowest expression of TJP1. This is consistent with the concept of the leaking gut syndrome and its role in the pathogenesis of T1D [13]. In addition, our study showed that the small bowel mucosa of CD patients with associated T1D may have significant peculiarities in terms of its basic immunological function.

Increased levels of antibodies reactive with dietary proteins such as cow's milk proteins have been well studied in patients with CD and T1D [12, 48–50]. Rodriguez-Juan et al. [49] found that the level of antibodies to bovine serum albumin was significantly higher in CD patients and in those T1D patients who had antibodies to reticulin, endomysium or gliadin compared with controls. These authors suggested that antibodies to bovine serum albumin appear in T1D patients with compromised epithelial permeability and with a predisposition to autoimmunity. The results of our study showed that IgA and IgG antibodies to bovine milk proteins were mostly elevated in CD patients, also in those with coexisting T1D.

Another important finding of our study was that lower expression of TJP1 in patients with CD and coexisting T1D was associated with pronounced FOXP3 mRNA expression and, consequently, higher Treg expression in the intestinal mucosa [37]. This is in line with the data of Tiittanen et al. [31], who also found higher expression of

FOXP3 mRNA and an increased number of FOXP3-stained CD25+ cells in the lamina propria of the intestinal mucosa of children with active CD alone or with CD and coexisting T1D, compared with children with normal mucosa. Moreover, as we already showed in our previous study [37], patients with CD, especially those with coexisting T1D, had a higher number of FOXP3-positive cells in their intestinal mucosa than persons with normal mucosa. The results of our double-staining study show that the majority of FOXP3+ cells were CD4+ and CD25+ [37], which confirms that the studied patients with small bowel villous atrophy had an increased number of Treg in their proximal jejunum. These findings are consistent with recent data about the activation of immunoregulatory mechanisms after loss of the intestinal barrier by induction of Treg generation to prevent inappropriate inflammatory responses [3, 35].

In a study by Kivling et al. [51], children with both T1D and CD showed higher FOXP3 mRNA expression in peripheral blood mononuclear cells than children with T1D alone. The authors suggest that these results could be a sign of increased Treg-associated activity in children with two autoimmune diseases. In the same study, Kivling et al. [51] found that gluten was able to induce FOXP3 mRNA expression in children with CD but not in controls. We found that expression of intestinal FOXP3 mRNA correlated significantly with the levels of anti-gliadin IgA and IgG antibodies, as well as with anti-tTG IgA antibodies. This situation can be interpreted as a compensatory increase in Treg towards down-regulation of the immune response to foreign and self-antigens to maintain immunological tolerance. Enhanced immune activation in the small intestine in paediatric patients with T1D was demonstrated in a study by Westerholm-Ormio et al. [52], and elevated levels of anti-tTG IgA antibodies in the small intestine of children with T1D were demonstrated by Maglio et al. [53]. Most probably, both the inflammatory and regulatory arms of the immune response are activated after loss of intestinal barrier function, while the balance between them determines the severity of immune pathology in the gut.

Recent studies [3, 36] of the association between increased intestinal epithelial tight junction permeability, accumulation of dendritic cells in the lamina propria and their role in Treg differentiation under conditioning by epithelial cells could cast some light on existing immunological mechanisms in the intestinal mucosa. It is hypothesized that interaction between the dendritic cells and antigenic material in the intestinal lumen is regulated by tight junction permeability and has an important role in maintaining immune homeostasis [3]. From this

point of view, further study of the dendritic cells as integrators of mucosal homeostasis by intestinal barrier dysfunction would be highly important.

The issue deserving special attention in further research would be determination of the mechanisms that lead to the most prominent changes in TJP1 and FOXP3 expression in the small bowel mucosa of patients with CD and coexisting T1D. At present, one can only speculate that the integrity of the small bowel mucosa in CD patients with T1D is substantially impaired due to the simultaneous influence of different factors, among which microorganisms may play a specific role. Tight junction proteins, including TJP1, are a well-known target for different infectious agents [4], and the small intestinal mucosa, harboring viruses like enteroviruses, might serve as one of the candidates [54].

Conclusion

Lower expression of the TJP1 gene in patients with coexisting CD and T1D indicates failed mucosal integrity and increased small bowel permeability. In the present study, lower TJP1 expression in CD patients was significantly associated with a higher level of anti-tTG IgA. A simultaneous increase in FOXP3 expression might contribute to maintaining intestinal immunological tolerance in CD patients with T1D, which can be interpreted as a compensatory increase in these cells in order to

achieve down-regulation of the immune response to foreign and self-antigens.

Our study shows that small bowel mucosal integrity and immune homeostasis might be significantly changed in CD associated with T1D. This is reflected by higher levels of IgA antibodies to different food antigens as well as by elevated expression of FOXP3 mRNA and Treg in the small bowel mucosa of patients with CD and coexisting T1D.

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References

- Stevenson BR, Siliciano JD, Mooseker MS, Goodenough DA: Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J Cell Biol* 1986;103:755–766.
- Fasano A, Shea-Donohue T: Mechanisms of disease: the role of intestinal barrier function in the pathogenesis of gastrointestinal autoimmune diseases. *Nat Clin Pract Gastroenterol Hepatol* 2005;9:416–422.
- Turner JR: Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol* 2009;9:799–809.
- Guttman JA, Finlay BB: Tight junctions as targets of infectious agents. *Biochim Biophys Acta* 2009;1788:832–841.
- Green PH, Jabri B: Celiac disease. *Lancet* 2003;362:383–391.
- Ciccocioppo R, Di Sabatino A, Corazza GR: The immune recognition of gluten in coeliac disease. *Clin Exp Immunol* 2005;140:408–416.
- Hopper AD, Hadjivassiliou M, Butt S, Sanders DS: Adult coeliac disease. *BMJ* 2007;335:558–562.
- Myléus A, Ivarsson A, Webb C, Danielsson L, Hernell O, Högborg L, Karlsson E, Lagerqvist C, Norström F, Rosén A, Sandström O, Stenhammar L, Steinlund H, Wall S, Carlsson A: Celiac disease revealed in 3% of Swedish 12-year-olds born during an epidemic. *J Pediatr Gastroenterol Nutr* 2009;49:170–176.
- Ress K, Harro M, Maaroos H-I, Harro J, Uibo R, Uibo O: High prevalence of celiac disease: need for increasing awareness among physicians. *Dig Liver Dis* 2007;39:136–139.
- Schumann M, Richter JF, Wedell I, Moos V, Zimmermann-Kordman M, Schneider T, Daum S, Zeitz M, Fromm M, Schulzke JD: Mechanisms of epithelial translocation of the alpha (2)-gliadin-33mer in coeliac sprue. *Gut* 2008;57:747–754.
- Åkerblom HK, Knip M: Putative environmental factors in type 1 diabetes. *Diabetes Metab Rev* 1998;14:31–67.
- Luopajarvi K, Savilahti E, Virtanen SM, Ilonen J, Knip M, Åkerblom HK, Vaarala O: Enhanced levels of cow's milk antibodies in infancy in children who develop type 1 diabetes later in childhood. *Pediatr Diabetes* 2008;9:434–441.
- Vaarala O, Atkinson M, Neu J: The 'perfect storm' for type 1 diabetes. The complex interplay between intestinal microbiota, gut permeability, and mucosal immunity. *Diabetes* 2008;10:2555–2562.
- Holmes GKT: Screening for coeliac disease in type 1 diabetes. *Arch Dis Child* 2002;87:495–499.
- Rewers M, Liu E, Simmons J, Redondo MJ, Hoffenberg EJ: Celiac disease associated with type 1 diabetes mellitus. *Endocrinol Metab Clin North Am* 2004;33:197–214.
- Uibo O, Heilman K, Rägo T, Shor R, Paal M, Metsküla K, Tillmann V, Uibo R: Symptomless coeliac disease in type I diabetes: 12-year experience in Estonia. *Pediatr Int* 2010;52:230–233.

- 17 Visser J, Rozing J, Sapone A, Lammers K, Fasano A: Tight junctions, intestinal permeability, and autoimmunity. Celiac disease and type 1 diabetes paradigms. *Ann NY Acad Sci* 2009;1165:195–205.
- 18 Vaarala O: Leaking gut in type 1 diabetes. *Curr Opin Gastroenterol* 2008;24:701–706.
- 19 Groschwitz KR, Hogan SP: Intestinal barrier function: molecular and disease pathogenesis. *J Allergy Clin Immunol* 2009;124:3–20.
- 20 Vilela EG, de Abreu Ferrari Mde L, De Gama Torres HO, Martins FP, Goulart EM, Lima AS, da Cunha AS: Intestinal permeability and antigliadin antibody test for monitoring adult patients with celiac disease. *Dig Dis Sci* 2007;52:1304–1309.
- 21 Schulzke JD, Bentzel CJ, Schulzke I, Riecken EO, Fromm M: Epithelial tight junction in the jejunum of children with acute and treated celiac sprue. *Pediatr Res* 1998;43:435–441.
- 22 Fasano A, Not T, Wang W, Uzzau S, Berti I, Tommasini A, Goldblum SE: Zonulin, a newly discovered modulator of intestinal permeability, and its expression in celiac disease. *Lancet* 2000;355:1518–1519.
- 23 van Elburg RM, Uil JJ, Mulder CJJ, Heymans HAS: Intestinal permeability in patients with celiac disease and relatives of patients with celiac disease. *Gut* 1993;34:354–357.
- 24 Ciccocioppo R, Finamore A, Ara C, Di Sabatino A, Mengheri E, Corazza GR: Altered expression, localization, and phosphorylation of epithelial junctional proteins in celiac disease. *Am J Clin Pathol* 2006;125:502–511.
- 25 Zanoni G, Navone R, Lunardi C, Tridente G, Bason C, Sivori S, Beri R, Dolcino M, Valletta E, Corrocher R, Pucetti A: In celiac disease, a subset of autoantibodies against transglutaminase binds toll-like receptor 4 and induces activation of monocytes. *PLoS Med* 2006;9:1637–1652.
- 26 Duerksen DR, Wilhelm-Boyles C, Veitch R, Kryszak D, Parry DM: A comparison of antibody testing, permeability testing, and zonulin levels with small-bowel biopsy in celiac disease patients on a gluten-free diet. *Dig Dis Sci* 2010;55:1026–1031.
- 27 Watts T, Berti I, Sapone A, Gerarduzzi T, Not T, Zielke R, Fasano A: Role of the intestinal tight junction modulator zonulin in the pathogenesis of type 1 diabetes in BB diabetic-prone rats. *Proc Natl Acad Sci USA* 2005;102:2916–2921.
- 28 Bosi E, Molteni L, Radaelli MG, Folini L, Fermo L, Bazzigaluppi E, Piemonti L, Pastore MR, Paroni R: Increased intestinal permeability precedes clinical onset of type 1 diabetes. *Diabetologia* 2006;49:2824–2827.
- 29 Neu J, Reverte M, Mackey AD, Liboni K, Tuohacek-Tenace LM, Hatch M, Li N, Caicedo RA, Schatz DA, Atkinson M: Changes in intestinal morphology and permeability in the biobreeding rat before the onset of type 1 diabetes. *J Pediatr Gastroenterol Nutr* 2005;40:589–595.
- 30 Tritt M, Sgouroudis E, d’Hennezel E, Albanese A, Piccirillo CA: Functional waning of naturally occurring CD4⁺ regulatory T-cells contributes to the onset of autoimmune diabetes. *Diabetes* 2008;57:113–123.
- 31 Tiittanen M, Westerholm-Ormio M, Verkasalo M, Savilahti E, Vaarala O: Infiltration of forkhead box P3-expressing cells in small intestinal mucosa in coeliac disease but not in type 1 diabetes. *Clin Exp Immunol* 2008;152:498–507.
- 32 Barnes MJ, Powrie F: Regulatory T cells reinforce intestinal homeostasis. *Immunity* 2009;31:401–411.
- 33 Maynard CL, Weaver CT: Intestinal effector T cells in health and diseases. *Immunity* 2009;31:389–400.
- 34 Su L, Shen L, Clayburgh DR, Nalle SC, Sullivan EA, Meddings JB, Abraham C, Turner JR: Targeted epithelial tight junction dysfunction causes immune activation and contributes to development of experimental colitis. *Gastroenterology* 2009;136:551–563.
- 35 Boirivant M, Amendola A, Butera A, Sanchez M, Xu L, Marinaro M, Kitani A, Di Giacinto C, Strober W, Fuss IJ: A transient breach in the epithelial barrier leads to regulatory T-cell generation and resistance to experimental colitis. *Gastroenterology* 2008;5:1612–1623.
- 36 Iliev ID, Mileti E, Matteoli G, Chieppa M, Rescigno M: Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. *Mucosal Immunol* 2009;4:340–350.
- 37 Vorobjova T, Uibo O, Heilman K, Rāgo T, Honkanen J, Vaarala O, Tillmann V, Ojakivi I, Uibo R: Increased FOXP3 expression in small-bowel mucosa of children with celiac disease and type 1 diabetes mellitus. *Scand J Gastroenterol* 2009;44:422–430.
- 38 Walker-Smith J, Guandalini S, Schmitz J, Shmerling DH, Visakorpi JK: Revised criteria for diagnosis of celiac disease. Report of working group of European Society of Pediatric Gastroenterology and Nutrition. *Arch Dis Child* 1990;65:909–911.
- 39 Marsh MN: Gluten, major histocompatibility complex, and the small intestine: a molecular and immunobiologic approach to the spectrum of gluten sensitivity (‘celiac sprue’). *Gastroenterology* 1992;102:330–354.
- 40 Green PHR, Rostami K, Marsh MN: Diagnosis of coeliac disease. *Best Pract Res Clin Gastroenterol* 2005;19:389–400.
- 41 Anderson JM, Van Itallie CM, Peterson MD, Stevenson BR, Carew EA, Mooseker MS: ZO-1 mRNA and protein expression during tight junction assembly in Caco-2 cells. *J Cell Biol* 1989;109:1047–1056.
- 42 Pizzuti D, Bortolami M, Mazzon E, Buda A, Guariso G, D’Odorico A, Chiarelli S, D’Inca RD, De Iazzari F, Martinez D: Transcriptional downregulation of tight junction protein ZO-1 in active coeliac disease is reversed after a gluten-free diet. *Dig Liver Dis* 2004;36:337–341.
- 43 Teesalu K, Agardh D, Panarina M, Utt M, Uibo O, Uibo R: A modified ELISA for improved detection of IgA, IgG, and IgM anti-tissue transglutaminase antibodies in celiac disease. *Clin Chim Acta* 2009;403:37–41.
- 44 Sapone A, de Magistris L, Pietzak M, Clemente MG, Tripathi A, Cucca F, Lampis R, Kryszak D, Carteni M, Generoso M, Iafusco D, Prisco F, Laghi F, Riegler G, Carratu R, Counts D, Fasano A: Zonulin upregulation is associated with increased gut permeability in subjects with type 1 diabetes and their relatives. *Diabetes* 2006;55:1443–1449.
- 45 Drago S, El Asmar R, Di Pierro M, Clemente MG, Tripathi A, Sapone A, Thakar M, Iacono G, Carroccio A, D’Agate C, Not T, Zampini L, Catassi C, Fasano A: Gliadin, zonulin and gut permeability: effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. *Scand J Gastroenterol* 2006;41:408–419.
- 46 Ravelli A, Villanacci V, Monfredini C, Martinazzi S, Grassi V, Manenti S: How patchy is villous atrophy?: distribution pattern of histological lesions in the duodenum of children with celiac disease. *Am J Gastroenterol* 2010;105:2103–2110.
- 47 Bonamico M, Ballati G, Mariani P, Latini M, Triglione P, Rana I, Porro E, Mesturino MA, Criscione S: Screening for coeliac disease: the meaning of low titers of anti-gliadin antibodies (AGA) in non-coeliac children. *Eur J Epidemiol* 1997;13:55–59.
- 48 Savilahti ES, Saukkonen TT, Virtala ET, Tuomilehto J, Åkerblom HK: Increased levels of cow’s milk and β -lactoglobulin antibodies in young children with newly diagnosed IDDM. The Childhood Diabetes in Finland Study Group. *Diabetes Care* 1993;16:984–989.
- 49 Rodriguez-Juan C, Sala-Silveira L, Pérez-Blas M, Valeri AP, Aguilera N, López-Santalla M, Fuentes A, Martín-Villa JM: Increased levels of bovine serum albumin antibodies in patients with type 1 diabetes and celiac disease-related antibodies. *J Pediatr Gastroenterol Nutr* 2003;37:132–135.
- 50 Monetini L, Cavallo MG, Manfrini S, Stefanini L, Picarelli A, Di Tola M, Petrone A, Bianchi M, La Presa M, Di Giulio C, Baroni MG, Thorpe R, Walker BK, Pozzilli P, IM-DIAB Group: Antibodies to bovine beta-casein in diabetes and other autoimmune diseases. *Horm Metab Res* 2002;34:455–459.
- 51 Kivling A, Nilsson L, Fält-Magnusson K, Sölvander S, Johanson C, Faresjö M: Diverse FOXP3 expression in children with type 1 diabetes and celiac disease. *Ann NY Acad Sci* 2008;1150:273–277.
- 52 Westerholm-Ormio M, Vaarala O, Pihkala P, Ilonen J, Savilahti E: Immunologic activity in the small intestinal mucosa of pediatric patients with type 1 diabetes. *Diabetes* 2003;52:2287–2295.
- 53 Maglio M, Florian F, Vecchiet M, Auricchio R, Paparo F, Spadaro R, Zanzi D, Rapacciuolo L, Franzese A, Sblattero D, Marzari R, Troncone R: Majority of children with type 1 diabetes produce and deposit anti-tissue transglutaminase antibodies in the small intestine. *Diabetes* 2009;58:1578–1584.
- 54 Salminen KK, Vuorinen T, Oikarinen S, Helminen M, Simell S, Knip M, Ilonen J, Simell O, Hyöty H: Isolation of enterovirus strains from children with preclinical type 1 diabetes. *Diabet Med* 2004;21:156–164.