Original Article

Zonulin Upregulation Is Associated With Increased Gut Permeability in Subjects With Type 1 Diabetes and Their Relatives

Anna Sapone,[1,2] Laura de Magistris,[2] Michelle Pietzak,[3] Maria G. Clemente,[1] Amit Tripathi,[1] Francesco Cucca,[4] Rosanna Lampis,[4] Deborah Kryszak,[1] Maria Cartenì,[2] Maddalena Generoso,[2] Dario Iafusco,[2] Francesco Prisco,[2] Francesca Laghi,[2] Gabriele Riegler,[2] Romano Carratu,[2] Debra Counts,[5] and Alessio Fasano[1]

Zonulin, a protein that modulates intestinal permeability, is upregulated in several autoimmune diseases and is involved in the pathogenesis of autoimmune diabetes in the BB/Wor animal model of the disease. To verify the association between serum zonulin levels and in vivo intestinal permeability in patients with type 1 diabetes, both parameters were investigated in different stages of the autoimmune process. Forty-two percent (141 of 339) of the patients had abnormal serum zonulin levels, as compared with age-matched control subjects. The increased zonulin levels correlated with increased intestinal permeability in vivo and changes in claudin-1, claudin-2, and myosin IXB genes expression, while no changes were detected in ZO1 and occludin genes expression. When tested in serum samples collected during the pre–type 1 diabetes phase, elevated serum zonulin was detected in 70% of subjects and preceded by 3.5 ± 0.9 years the onset of the disease in those patients who went on to develop type 1 diabetes. Combined, these results suggest that zonulin upregulation is associated with increased intestinal permeability in a subgroup of type 1 diabetic patients. Zonulin upregulation seems to precede the onset of the disease, providing a possible link between increased intestinal permeability, environmental exposure to non–self antigens, and the development of autoimmunity in genetically susceptible individuals. *Diabetes* 55:1443–1449, 2006

From the [1]Mucosal Biology Research Center, University of Maryland School of Medicine, Baltimore, Maryland; the [2]Department Magrassi-Lanzara, Gastroenterology Unit, II University of Naples, Naples, Italy; the [3]University of Southern California Keck School of Medicine and the Division of Gastroenterology and Nutrition, Children's Hospital Los Angeles, Los Angeles, California; the [4]Department of Biomedical Science and Biotechnology, University of Cagliari, Cagliari, Italy; and the [5]Division of Pediatric Endocrinology, Department of Pediatrics, University of Maryland School of Medicine, Baltimore, Maryland.

Address correspondence and reprint requests to Alessio Fasano, MD, Mucosal Biology Research Center, University of Maryland School of Medicine, 20 Penn St., Room 345, Baltimore, MD 21201. E-mail: afasano@mbrc.umaryland.edu.

Received for publication 8 December 2005 and accepted in revised form 13 February 2006.

ELISA, enzyme-linked immunosorbent assay; GALT, gut-associated lymphoid tissue; ICA, islet cell antibody; IA-2, insulinoma-associated protein 2; LA/MA, lactulose/mannitol; MAdCAM-1, mucosal vascular addressin cell adhesion molecule 1; TBS-T, Tris-buffered saline 0.05% Tween 20; TJ, tight junction.

DOI: 10.2337/db05-1593

The driving antigen(s) of the autoimmune destruction of pancreatic β-cells in type 1 diabetes remains unclear. The interplay between environmental factors and specific susceptibility genes likely drives the immune response responsible for the onset of the disease (1–3). The observation that <10% of subjects with increased genetic susceptibility progress to clinical disease suggests a strong environmental component in the pre-diabetic process. Type 1 diabetes presents the same pathogenesis challenges of other autoimmune diseases, particularly as they pertain to identifying which driving antigen(s) is involved and how nonself antigens gain access to the immune system. While past work has been mainly focused on understanding the immunological consequences of the interplay between predisposing genes and antigens driving the autoimmune response, little is known about how these antigens reach the immune system. The gastrointestinal tract is likely the main system through which nonself antigens gain access. This theory is supported by studies outlining the central role of the gut-associated lymphoid tissue (GALT) in the pathogenesis of type 1 diabetes (4,5) and by clinical evidence suggesting that individuals affected by various autoimmune diseases, including type 1 diabetes, have an aberrant increase in intestinal permeability (6–11). Increased permeability precedes the onset of autoimmune diabetes in BioBreeding diabetic prone (BBDP) rats (12,13) and has been described in humans with type 1 diabetes (6,9,14–16).

We have recently reported a novel protein, zonulin, that modulates intestinal permeability by disassembling the intercellular tight junctions (TJs) (17,18). This protein is most likely involved in the innate immunity of the gut (19) and, when upregulated, appears to play a key role in the pathogenesis of autoimmune diseases, such as celiac disease (18,20). In the BBDP rat, we reported that a zonulin-dependent increase in intestinal permeability preceded the onset of type 1 diabetes by 2–3 weeks (13). Oral administration of the zonulin inhibitor AT-1001 to BBDP rats blocked autoantibody formation and zonulin-mediated intestinal permeability increase, reducing the incidence of diabetes (13). These studies suggested that the zonulin-dependent loss of intestinal barrier function is one

TABLE 1
Primers used for intestinal TJ quantitative genes expression by real-time PCR

Gene	Sense	Antisense
ZO-1	CAAGATAGTTTGGCAGCAAGAGATG	ATCAGGGACATTCAATAGCGTAGC
Occludin	AATTCTTCACTTCTAACAAATGGACCTC	CACATCACAATAATGAGCATAGACAGG
Claudin 1	TGGTGGTTGGCATCCTCCTG	AATTCGTACCTGGCATTGACTGG
Claudin 2	GGCGGTAGCAGGTGGAGTC	CTTGGTAGGCATCGTAGTAGTTGG
Myosin IXB	AGGGTACAGCGCCAAGTACA	CTCCTTCAGGAAGACCTTGG

of the initial steps in the pathogenesis of type 1 diabetes in the BBDP animal model of the disease.

Combined, these observations provided the rationale to study the zonulin expression in type 1 diabetic patients and to establish the correlation between zonulin upregulation and intestinal permeability. In this article, we demonstrate that patients with type 1 diabetes and their relatives have elevated serum zonulin levels that correlate with increased intestinal permeability associated with altered genetic expression of intestinal TJ proteins. We also established that zonulin upregulation was detected during the pre-diabetic stage and precedes the onset of type 1 diabetes.

RESEARCH DESIGN AND METHODS

Prospective studies. Consent was obtained from all enrolled subjects after the nature of the investigation was explained and in accordance with the approved protocol from the institute review board at the University of Maryland and the University of Naples. A total of 339 type 1 diabetic patients (mean age 13.0 ± 6.8 years, mean age at diagnosis of diabetes 7.7 ± 4.4 years, 48% females), 89 first-degree relatives (mean age 41.5 ± 8.9 years, 58% females), and 97 sex- and age-matched normal control subjects (25.0 ± 10.5 years, 54% females) were tested for their serum zonulin levels. A random subgroup of these type 1 diabetic subjects (n = 36), along with their relatives (n = 56) and healthy control subjects (n = 43), also performed in vivo intestinal permeability using the lactulose/mannitol (LA/MA) double sugar test (see below). The diabetic patients had positive islet cell antibodies (ICAs), anti-GAD, and/or tyrosine phosphatase (insulinoma-associated protein 2 [IA-2]) autoantibodies at time of diagnosis (for specific methods on determination of autoantibodies, see below). Celiac disease was excluded by seronegative tests for anti-gliadin antibodies IgA and IgG, anti-endomysium antibodies IgA, and anti-human transglutaminase IgA antibodies (Eurospital, Trieste, Italy). None of the patients and/or their relatives who were enrolled in the study showed major GI symptoms and/or diseases.

Retrospective studies. To determine the temporal relationship between increased serum zonulin levels and the onset of type 1 diabetes, samples from a serum bank of patients at risk for the disease (positive family history of type 1 diabetes and HLA typing compatible with type 1 diabetes) were screened for their zonulin levels in the following three groups of subjects: *1*) type 1 diabetic patients whose serum sample was obtained after the diagnosis of the disease (n = 8), *2*) subjects at risk for the disease and with positive GAD and/or IA-2 autoantibodies and whose samples were collected before the onset of the disease (potential type 1 diabetes) (n = 10), and *3*) at-risk subjects who did not develop autoimmunity (n = 15).

In vivo intestinal permeability. In vivo permeability was determined by means of the LA/MA test, as we have previously described (21). Briefly, after overnight fast, patients drank a solution of lactulose and mannitol mixed in water (5 g lactulose and 2 g mannitol in 150 ml water). This was immediately followed by a 5-h urine collection. The detection and measurement of the two sugar probes in the urine was performed by high-performance anion exchange chromatography coupled with pulsed amperometric detection. This technique allows direct quantification of nonderivative carbohydrates by using a Dyonex DX-500 with a gradient pump module GP40 and sample loop of 50 μl. A CarboPac PA-100 guard column and elution with a NaOH-NaAc gradient was also used.

Serum zonulin measurement by sandwich enzyme-linked immunosorbent assay. Zonulin sandwich enzyme-linked immunosorbent assay (ELISA) was performed as we have previously described (19) with minor modifications. Briefly, plastic microtiter plates (Costar, Cambridge, MA) were coated with rabbit zonulin cross-reacting anti-Zonula occludens toxin (Zot) derivative ΔG IgG (13) antibodies (10 μg/ml in 0.1 mol/l sodium carbonate buffer, pH 9.0). After overnight incubation at 4°C, plates were washed four times in Tris-buffered saline 0.05% Tween 20 (TBS-T) and blocked by incubation for 1 h at 37°C with TBS-T. After four TBS-T washes, five ΔG serial standards (50, 25, 12.5, 6.2, 3.1, and 0 ng/ml) and patient sera samples (1:101 dilution in TBS-T) were added and incubated overnight at 4°C. After four washes with Tris-buffered saline 0.2% Tween 20 buffer, plates were incubated with biotinylated anti-Zot IgG antibodies for 4 h at 4°C. A color reaction was developed by using a commercial kit (ELISA amplification kit; Invitrogen). The absorbance at 495 nm was measured with a microplate auto-reader (Molecular Devices Thermomax Microplate Reader). To define the intra- and interassay precision of the ELISA sandwich method, the coefficient of variation (CV) was calculated using double replicates from two samples with different concentrations of zonulin, on three consecutive days. The interassay test of the ELISA sandwich method produced a CV of 9.8%. The CV of the intra-assay test was 4.2% at day 1, 3.3% at day 2, and 2.9% at day 3. Zonulin was expressed as ng/mg total protein (as measured by the Bradford method) detected in the tested samples.

Analysis of ICA, GAD, and IA-2 antibodies. The presence of ICAs was determined by indirect immunofluorescence. GAD and IA-2 antibodies were measured by protein A/G radiobinding assays using [^{35}S]methionine-labeled in vitro–translated recombinant human GAD65 and IA-2, respectively (22,23). Samples with antibody titers above the discriminatory range of the assays were titrated until they fell within this part of the standard curve, and the units were multiplied by the appropriate dilution factor. The positive thresholds in each assay were set at the 99th percentile of control subjects. These assays had sensitivities and specificities of 80 and 94% (GAD) and 58 and 100% (IA-2) in the First DASP Assay Proficiency Evaluation (24).

RNA extraction, cDNA synthesis, and real-time quantitative PCR. Quantification of intestinal TJ protein genes expression was performed by real-time PCR as we have previously described (25) with minor modifications. Briefly, small intestinal biopsies obtained from type 1 diabetic subjects (n = 5), type 1 diabetic relatives (n = 3), and healthy control subjects (n = 5) were used to extract total RNA using Trizol reagent (Invitrogen, Grand Island, NY) per the manufacture's instructions. RNA samples (2 μg) were reverse transcribed to c-DNA using the First Strand cDNA Synthase Kit (MBI Fermentas, Hanover, MD) with random hexamer primer. Real-time quantitative PCR was performed with the Applied Biosystems 7500 Fast Real-Time PCR System. Primer sequences were designed using Beacon Designer 4.0 (Premier Biosoft International) and synthesized by the Biopolymer Laboratories of the University of Maryland. The primer sequences for the TJ protein genes studied are listed in Table 1. PCR was performed in a 25-μl volume using SYBR Green PCR Master Mix (Applied Biosystems, Warrington, U.K.). Amplification conditions were as follows: 50°C for 2 min and 95°C for 10 min, followed by 50 cycles at 95 C° for 15 s and 60 C° for 1 min. Results were normalized by the expression of the housekeeping gene S18 and expressed as fold changes compared with gene expression in healthy control subjects.

Statistical methods. All data are expressed as means ± SE. Sample size estimates were calculated for patients with type 1 diabetes and were based on comparisons between subjects with elevated zonulin levels and those without elevated zonulin levels. The cutoff for elevated zonulin was defined as >2 SD above mean levels in age-matched healthy control subjects. Sample size was determined to detect a mean serum zonulin level change of 0.4 ng/mg protein among the three groups of patients (type 1 diabetic patients, their relatives, and healthy control subjects), using a significance level of 5%, a power of 85%, and one-way ANOVA for data analysis.

For the subgroup of subjects in which intestinal permeability was performed, groups were compared on proportions with increased intestinal permeability (>2 SD above the mean of normal control subjects [21]), assumed to be ~5% in subjects without elevated zonulin. Calculations based on χ^2 tests indicate that for a total sample size of 35 type 1 diabetic subjects, we would be able to detect differences in proportions with increased intestinal permeability of 23% with 80% power, α = 0.05. That is, if 28% or more of patients with elevated serum zonulin had increased intestinal permeability, compared with 5% of those without elevated zonulin, we would obtain a power

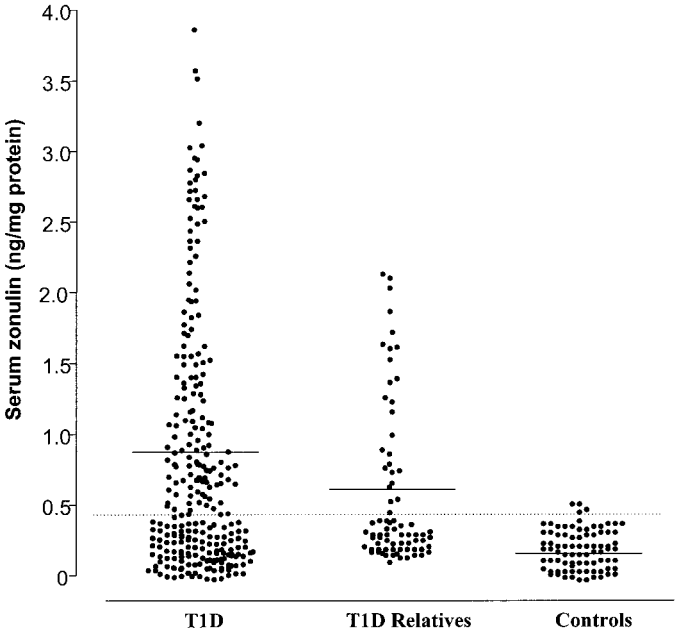

FIG. 1. Serum zonulin levels in type 1 diabetic subjects (T1D; n = 339), their first-degree relatives (n = 89), and age- and sex-matched control subjects (n = 97). Type 1 diabetic patients showed higher serum zonulin levels than their relatives (P = 0.011) and control subjects ($P = 2.13E^{-9}$)

of 80% or more to detect this difference. Nonparametric methods were used for statistical analysis of data, since both serum zonulin and LA/MA variables were not normally distributed. The correlation between serum zonulin and LA/MA was analyzed by Spearman's coefficient. The comparisons among the three groups of subjects studied (type 1 diabetic patients, their relatives, and healthy control subjects) were performed by the Kruskal-Wallis test. Because this test has an asymptotic efficiency equal to 96% relative to the one-way parametric ANOVA, the number of patients recruited to perform both tests guaranteed a level of study power not <80%.

RESULTS

Serum zonulin levels in subjects with type 1 diabetes, their first-degree relatives, and healthy control subjects. Type 1 diabetic patients showed statistically higher serum zonulin levels (0.83 ± 0.05 ng/mg protein) than both their relatives (0.62 ± 0.07 ng/mg protein) and control subjects (0.21 ± 0.02 ng/mg protein) (Fig. 1). Forty-two percent (141 of 339) of type 1 diabetic patients and 29% of their first-degree relatives (26 of 89) had serum zonulin levels that were 2 SD above the mean zonulin levels detected in age-matched healthy control subjects. Only 4% (4 of 97) of control subjects had zonulin levels 2 SD above the mean ($P < 0.01$). Serum zonulin was higher in type 1 diabetic patients than in their relatives ($P = 0.01$). Serum zonulin levels did not correlate with age or sex of any of the subjects studied. Furthermore, in type 1 diabetic patients, serum zonulin levels did not correlate with age of diagnosis of type 1 diabetes, its duration, daily insulin dose, HbA_{1c}, or serum glucose levels (data not shown).

Correlation between serum zonulin and intestinal permeability in type 1 diabetic subjects and their relatives. To establish whether serum zonulin levels correlated with intestinal permeability, LA/MA urine ratio was determined in both a subset of type 1 diabetic subjects with documented zonulin upregulation (n = 36) and their relatives (n = 56). Intestinal permeability was higher in both the type 1 diabetic subjects (LA/MA 0.037 ± 0.003) and their relatives (LA/MA 0.025 ± 0.01) than in healthy control subjects (n = 43) (LA/MA 0.017 ± 0.0018; $P <$ 0.0001 vs. type 1 diabetic patients and $P < 0.02$ vs. their relatives) (Fig. 2*A*). The increase in intestinal permeability detected in both type 1 diabetic patients and their relatives was related to an increased lactulose detection in urine, without any significant change in mannitol detection (Table 2). Since lactulose is a marker of the paracellular pathway, while mannitol is a marker of transcellular pathway, these data suggest that the observed changes in LA/MA reflect an increase permeability of the paracellular pathway. The increased intestinal permeability was paralleled by increased serum zonulin levels, and both parameters were significantly higher in type 1 diabetic patients than in their relatives (Fig. 2*B*). There was a direct correlation between LA/MA and serum zonulin (R = 0.36, P = 0.0004) (Fig. 2*C*).

Serum zonulin as an early marker for type 1 diabetes. Based on our previous results obtained in the BBDP animal model of autoimmune diabetes (13), we formulated the hypothesis that a zonulin-dependent increase in intestinal permeability precedes the onset of type 1 diabetes in human as well. To test this hypothesis, serum zonulin levels were determined in three groups of patients: subjects with established type 1 diabetes (group 1, n = 8), at-risk subjects with elevated auto-antibodies but not established disease at the moment of the serum sampling (group 2, n = 10), and at-risk subjects who did not develop autoimmunity (group 3, n = 15) (Table 3). Four of eight of type 1 diabetic patients whose blood was drawn after the diagnosis of the disease (group 1) showed elevated zonulin levels (Table 3). The percentage of zonulin-positive patients in group 1 was similar to that detected in the prospective studies (see Fig. 1 and relative text). Seven of 10 potential type 1 diabetic subjects (group 2) showed elevated zonulin. Four of these subjects (three of which showed elevated serum zonulin levels) went on to develop type 1 diabetes 3.5 ± 0.9 years after the serum sampling (Table 3). Finally, 3 of 15 at-risk subjects who did not develop type 1 diabetes (group 3) had elevated serum zonulin levels without a parallel increase in autoantibodies (Table 3). The percentage of zonulin-positive subjects in group 3 was similar to that found among first-degree relatives enrolled in our prospective studies (see Fig. 1 and relative text).

Gene expression of intestinal TJ proteins. TJ integral membrane proteins occludin, claudin-1, and claudin-2 and scaffold proteins ZO-1 and myosin IXB are considered key elements on the TJ complex, and their expression and localization dictate TJ competency (10). To establish whether the intestinal permeability and serum zonulin changes observed in patients with type 1 diabetes and their relatives were associated with modification in TJ gene proteins expression, intestinal tissues obtained from type 1 diabetic patients (n = 5), their relatives (n = 3), and normal control subjects (n = 5) were immediately processed for mRNA extraction following their collection during an upped gastrointestinal endoscopy. RNA levels of the five proteins were quantified by real-time PCR. No changes in occludin or ZO-1 gene expression were observed (Fig. 3). Expression of claudin-1 and myosin IXB, a scaffold protein recently described to be possibly involved in the increased intestinal permeability in celiac disease (26), were increased twofold, while the expression of claudin-2 was decreased twofold (Fig. 3). Despite suggestive of a possible biological importance in dictating the

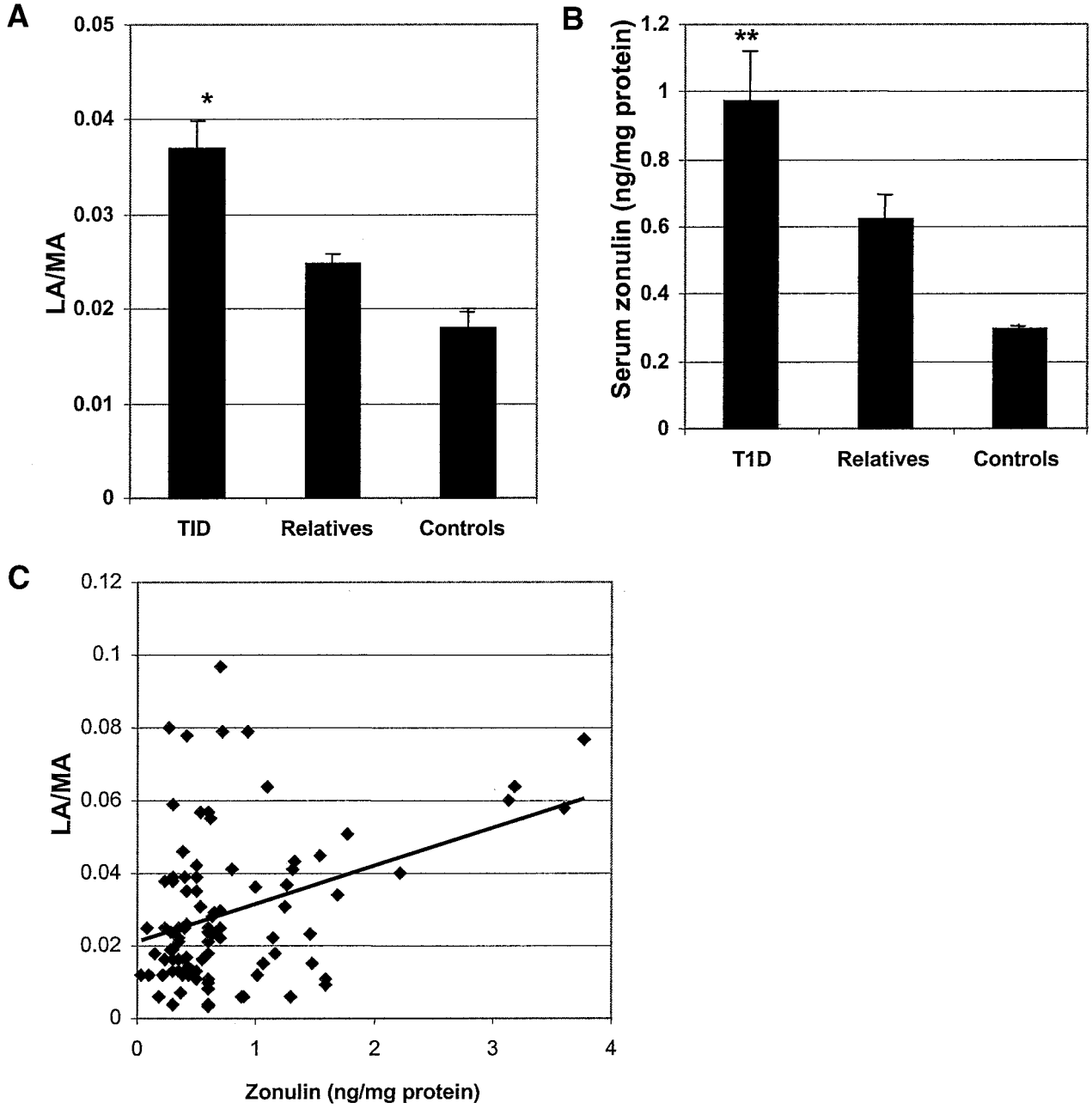

FIG. 2. Intestinal permeability (*A*), serum zonulin levels (*B*), and their correlation (*C*) in type 1 diabetic patients ($n = 36$), their relatives ($n = 56$), and healthy control subjects ($n = 43$). Both parameters were significantly higher in type 1 diabetic subjects than in their relatives. (*$P = 0.04$ and **$P = 0.02$) and control subjects (*$P < 0.01$ and **$P < 0.0001$). LA/MA and serum zonulin showed a significant coefficient of correlation (multiple $R = 0.36$; intercept $P = 1.71\text{E}^{-10}$; x variable 1 $P = 0.0004$).

TABLE 2
Percentage of lactulose and mannitol detected in the urine of type 1 diabetic patients, their relatives, and healthy control subjects

Group (n)	Lactulose (%)	Mannitol (%)
Type 1 diabetic patients (36)	0.79 ± 0.11*	21.27 ± 2.22
Relatives of type 1 diabetic patients (56)	0.63 ± 0.14*	24.77 ± 3.20
Control subjects (43)	0.48 ± 0.12	23.25 ± 3.36

*$P < 0.05$ vs. control subjects

observed effects on intestinal TJ, these changes did not reach statistical significance due to the limited number of cases studied. Conversely, the expression of all five genes studied was unchanged in relatives of type 1 diabetes (data not shown).

DISCUSSION

Several lines of evidence suggest that the GALT plays a key role in the development of type 1 diabetes (5). Lymphocytes are known to circulate between the GALT, lymph nodes, and other tissues. The migration of lymphocytes to the pancreas appears to be mediated through mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) and α4β7 integrin, a gut-specific homing

TABLE 3
Serum zonulin as an early marker for type 1 diabetes in humans

Subject number	Age (years)	Age onset (years)	Age at blood drawing	GAD-Ab*	IA-2-Ab*	Zonulin* (ng/mg protein)
Group 1: Type 1 diabetic subjects whose blood was drawn after the onset of the disease						
1	18	6	9	**62.62**	**209.76**	**7.1**
2	23	11	16	**55.2**	0.45	0.2
3	23	9	13	0.97	**16.58**	0.5
4	25	11	15	**75.38**	1.76	**1.3**
5	17	6	7	**3.89**	0.33	**1.8**
6	23	12	14	N.D.	N.D.	0.3
7	11	3	4	N.D.	N.D.	0.4
8	17	5	8	**90.57**	**87.3**	**0.7**
Group 2: Subjects at risk for type 1 diabetes and positive autoantibodies (potential type 1 diabetes)						
1	23	19	14	**16.88**	1.92	**1.0**
2	14	6	5	**95.12**	**198.81**	**2.3**
3	20	15	10	**52.19**	**161.77**	0.5
4	14	10	7	**13.23**	**3.88**	**1.1**
5	27	NA	20	**85.15**	**11.45**	0.2
6	25	NA	16	**4.79**	0.4	0.1
7	23	NA	13	**4.76**	**150.64**	**0.9**
8	28	NA	19	**31.71**	**181.5**	**0.9**
9	19	NA	10	**84.28**	**8.57**	**1.4**
10	25	NA	16	**29.46**	2.90	**1.1**
Group 3: Subjects at risk for type 1 diabetes who did not develop the disease						
1	51	NA	42	0.39	0.57	0.3
2	24	NA	15	0.57	0.44	0.4
3	50	NA	40	0.28	0.27	0.5
4	33	NA	24	0.59	0.43	**0.8**
5	41	NA	32	0.89	0.53	**1.3**
6	52	NA	43	0.42	0.38	0.2
7	42	NA	33	0.39	0.41	0.1
8	43	NA	33	0.37	0.39	0.4
9	43	NA	33	0.45	0.36	0.3
10	58	NA	48	0.57	0.33	0.6
11	55	NA	45	0.36	0.41	**0.7**
12	35	NA	28	N.D.	N.D.	0.2
13	37	NA	30	N.D.	N.D.	0.4
14	62	NA	55	0.47	0.55	0.2
15	47	NA	38	0.34	0.37	0.5

*Abnormal values are identified in bold.

receptor for addressin (27) that is highly expressed in β-cell–reactive lymphocytes of type 1 diabetic patients. MAdCAM-1 is specifically implicated in the homing and recirculation of lymphocytes in the early phase of autoimmune diabetes in NOD mice (28). Inhibition of MAdCAM-1 before the onset of insulitis results in a reduced incidence of type 1 diabetes (28). Conversely, inhibition of α4 integrin blocks the spontaneous development of type 1 diabetes and passive transfer of diabetes from splenic lymphocytes of diabetic mice (29). An important factor for the intestinal immunological responsiveness is the major histocompatibility complex. HLA class I and class II genes are located in the major histoconpatibility complex on chromosome 6. These genes code for antigen-presenting cells (APCs) glycoprotein receptors, which bind peptides, and this HLA-peptide complex is recognized by certain T-cell receptors in the intestinal mucosa (8). Susceptibility to at least 50 diseases, including type 1 diabetes, has been associated with specific HLA class I or class II alleles (8). Certain HLA class II alleles account for 40% of the genetic susceptibility to type 1 diabetes in Caucasians; however, the majority of the genetically predisposed individuals do not develop type 1 diabetes. This supports some driving antigen(s) of the autoimmune destruction of β-cells that precedes type 1 diabetes. A common denominator of autoimmune diseases is the presence of several preexisting conditions leading to an autoimmune process. The first is a genetic susceptibility for the host immune system to recognize, and potentially misinterpret, an environmental antigen presented within the gastrointestinal tract. Second, the host must be exposed to the antigen. Finally, the antigen must be presented to the gastrointestinal mucosal immune system following its paracellular passage (normally prevented by the TJ competency) from the intestinal lumen to the gut submucosa. In all cases, increased permeability appears to precede disease and causes an abnormality in antigen delivery that triggers the multiorgan process leading to the autoimmune response (10).

We have recently reported the discovery of the protein zonulin that modulates the intestinal permeability by disassembling the intercellular tight junctions (17). Our preliminary data suggest that zonulin is a protease that activates its target receptor in a manner similar to other serine proteases (30). The zonulin system is likely involved in several functions, including the protection against microorganism colonization of the proximal intestine (innate immunity) (19). Given the complexity of both cell signaling events and intracellular structures involved in the zonulin system, it is not surprising that this pathway may be affected when the physiological state of epithelial cells is changed as occurs in many autoimmune diseases in which TJ dysfunction appears to be the primary defect (8). This hypothesis has been confirmed both in human autoimmune disease states, such as celiac disease (31), and in an animal model of autoimmune diabetes, in which the role of zonulin in the pathogenesis of the disease was directly demonstrated (13). These data provided the rationale for the studies objects of this report on the causal role of zonulin on intestinal barrier dysfunction typical of type 1 diabetic subjects. Our results showed that a large subgroup of type 1 diabetic patients has high serum zonulin levels that correlated with increased intestinal permeability. We also provided preliminary evidence suggesting that, like in the BBDP rat model of the disease, zonulin upregulation precedes the diagnosis of the disease in type 1 diabetic patients. Albeit preliminary, these data provide the rationale for prospective studies of at-risk populations to study the temporal relationship between zonulin upregulation, autoantibodies seroconversion, and the diagnosis of type 1 diabetes.

TJ is assumed to be a complex meshwork of several transmembrane and cytoplasmic proteins that show differential expression in varying tissues. So far, little is known about factors influencing genomic regulation of TJ protein expression. Our group (25) and other investigators (32) have recently reported that changes in intestinal permeability detected in celiac disease patients are secondary to reduction in TJ proteins ZO-1 and occludin gene expression. Conversely, our real-time PCR results of this study failed to show any changes in the gene expression of these proteins, while we detected a trend toward either increase (claudin-1 and myosin IXB) or decrease (claudin-2) gene expression of other TJ proteins.

Interestingly, first-degree relatives also showed in-

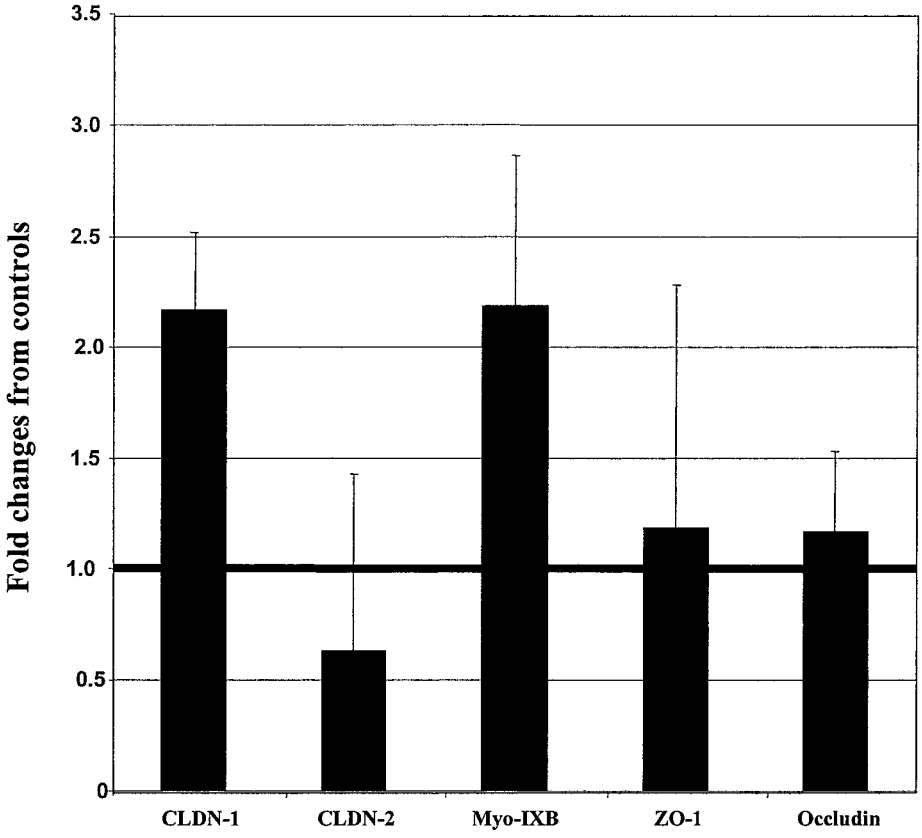

FIG. 3. Quantitative real-time PCR of intestinal TJ protein gene expression in type 1 diabetic patients. mRNA extracted from intestinal biopsies of type 1 diabetic patients (T1D; $n = 5$) was used to measure quantitative expression of the integral membrane TJ protein genes occludin, claudin-1 (CLDN-1), and claudin-2 (CLDN-2) and the scaffold proteins ZO-1 and myosin IXB (Myo-IXB). Results were normalized by the housekeeping gene S18 and expressed as fold changes from results obtained from healthy control subjects ($n = 5$) (thick line). No significant changes in gene expression were detected.

creased serum zonulin levels and altered intestinal permeability compared with control subjects, suggesting that in a subgroup of type 1 diabetic patients, a zonulin-associated loss in intestinal barrier function is a necessary but not sufficient condition to develop autoimmunity. Taken collectively, these results support our overall hypothesis that a subgroup of subjects with type 1 diabetes experiences a zonulin-dependent increase in intestinal permeability. The loss of intestinal barrier function secondary to zonulin upregulation and/or an inflammatory state of the intestinal mucosa (33) may cause a switch from tolerance to immunity to nonself antigens that continuously cross the intestinal mucosa. The aberrant passage of antigens underlies an increased risk for type 1 diabetes in those individuals in which other genetic determinants (both HLA and non-HLA associated) will cause an inappropriate processing/presentation by the GALT of nonself antigens that have crossed the intestinal barrier. Our hypothesis is further supported by a series of evidence generated both by us and other groups suggesting that the pathogenesis of autoimmune diseases involve a miscommunication between innate and adaptive immunity (19,34). Molecular mimicry or bystander effects alone may not entirely explain the complex events involved in the pathogenesis of autoimmune diseases. Rather, the continuous stimulation by nonself antigens appears necessary to perpetuate the process, as clearly demonstrated in celiac disease (20,25,30,31). This concept implies that the autoimmune response can be theoretically stopped and perhaps reversed if the interplay between autoimmune predisposing genes and trigger(s) is prevented or eliminated (35–39). The option to prevent this interaction by removing the driving antigen is only available for celiac disease, since this is the only autoimmune disorder in which the trigger (gluten) is known (40–42). For other autoimmune diseases, including type 1 diabetes, reestablishing the intestinal barrier function can represent a valid, innovative alternative. Therefore, understanding the interactions between the upregulation of zonulin, increased intestinal permeability, and the development of autoimmune diseases may lead to novel preventative and possibly therapeutic strategies for type 1 diabetes.

REFERENCES

1. Blanas E, Carbone FR, Allison J, Miller JF, Heath WR: Induction of autoimmune diabetes by oral administration of autoantigen. *Science* 274:1707–1709, 1996
2. Sabbah E, Savola K, Kulmala P, Vahasalo P, Ilonen J, Salmela PI, Knip M: Genetic, autoimmune, and clinical characteristics of childhood- and adult-onset type 1 diabetes. *Diabetes Care* 23:1326–1332, 2000
3. Persaud DR, BarrancoMendoza A: Bovine serum albumin and insulin-dependent diabetes mellitus: is cow's milk still a possible toxicological causative agent of diabetes? *Food Chem Toxicol* 42:707–714, 2004
4. Vaarala O: The gut immune system and type 1 diabetes. *Ann N Y Acad Sci* 958:39–46, 2002
5. DeMeo MT, Mutlu EA, Keshavarzian A, Tobin MC: Intestinal permeation and gastrointestinal disease. *J Clin Gastroenterol* 34:385–396, 2002
6. Carratù R, Secondulfo M, de Magistris L, Iafusco D, Urio A, Carbone MG, Pontoni G, Cartenì M, Prisco F: Altered intestinal permeability to mannitol in diabetes mellitus type1. *J Ped Gastroenterol Nutr* 28:264–269, 1999
7. De Magistris L, Secondulfo M, Sapone A, Carratù R, Iafusco D, Prisco F, Generoso M, Cartenì M, Mezzogiorno A, Esposito V: Infection with Giardia and intestinal permeability in humans. *Gastroenterology* 125:277–279, 2003
8. Fasano A: Pathological and therapeutical implications of macromolecule passage through the tight junction. In *Tight Junctions*. Cereijido M, Anderson JM, Eds. Boca Raton, FL, CRC Press, 2001, p. 697–722
9. Secondulfo M, Iafusco D, Carratù R, de Magistris L, Sapone A, Generoso M, Mezzogiorno A, Sasso FC, Carteni M, De Rosa M, Prisco F, Esposito V: Ultrastructural mucosal alterations and increased intestinal permeability in non-celiac, type I diabetic patients. *Dig Liver Dis* 36:35–45, 2004
10. Fasano A, Shea-Donohue T: Mechanism of disease: the role of intestinal

barrier function in the pathogenesis of gastrointestinal autoimmune diseases. *Nat Clin Prac Gastro Hepatol* 2:416–422, 2005
11. Yacyshyn B, Meddings J, Sadowski D, Bowen-Yacyshyn MB: Multiple sclerosis patients have peripheral CD45RO+ B cells and increased intestinal permeability. *Dig Dis Sci* 41:2493–2498, 1996
12. Meddings JB, Jarand J, Urbanski SJ, Hardin J, Gall DG: Increased gastrointestinal permeability is an early lesion in the spontaneously diabetic BB rat. *Am J Physiol* 276:G951–G957, 1999
13. Watts T, Berti I, Sapone A, Gerarduzzi T, Not T, Zielke R, Fasano A: Role of intestinal tight junction modulator zonulin in the pathogenesis of type1 diabetes in BB diabetic prone rats. *Proc Natl Acad Sci U S A* 102:2916–2921, 2005
14. Ellenberg M: Non neurologic manifestations of diabetic neuropathy. *Mt Sinai J Med* 47:561–567, 1980
15. Kuitunen M, Saukkonen T, Ilonen J, Akerblom HK, Savilahti E: Intestinal permeability to mannitol and lactulose in children with type 1 diabetes with the HLA-DQB1*02 allele. *Autoimmunity* 35:365–368, 2002
16. Damci T, Nuhoglu I, Devranoglu G, Osar Z, Demir M, Ilkova H: Increased intestinal permeability as cause of fluctuating postprandial blood glucose levels in type 1 diabetic patients. *Eur J Clin Invest* 33:397–401, 2003
17. 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 coeliac disease. *Lancet* 355:1518–1519, 2000
18. Wang W, Uzzau S, Goldblum SE, Fasano A: Human zonulin, a potential modulator of intestinal tight junctions. *J Cell Sci* 113:4425–4440, 2001
19. El Azmar R, Panigrahi P, Bamford P, Berti I, Not T, Coppa GV, Catassi C, Fasano A: Host-dependent activation of the zonulin system is involved in the impairment of the gut barrier function following bacteria colonization. *Gastroenterology* 123:1607–1615, 2002
20. Clemente MG, De Virgiliis S, Macatagney R, Congia M, Fasano A: New insights on celiac disease pathogenesis: gliadin-induced zonulin release, actin polymeration, and early increased gut permeaility. *Gut* 52:218–223, 2003
21. Generoso M, De Rosa M, De Rosa R, de Magistris L, Secondulfo M, Fiandra R, Carratù R, Cartenì M: Cellobiose and lactulose coupled with mannitol and determined using ion-exchange chromatography with pulsed amperometric detection are reliable probes for investigation of intestinal permeability. *J Chromatogr* 783:349–357, 2003
22. Ziegler AG, Hummel M, Schenker M, Bonifacio E: Autoantibody appearance and risk for development of childhood diabetes in offspring of parents with type 1 diabetes: the 2-year analysis of the German BABYDIAB Study. *Diabetes* 48:460–468, 1999
23. Naserke HE, Bonifacio E, Ziegler AG: Immunoglobulin G insulin autoantibodies in BABYDIAB offspring appear postnatally: sensitive early detection using a protein A/G-based radiobinding assay. *J Clin Endocrinol Metab* 84:1239–1243, 1999
24. Bingley PJ, Bonifacio E, Mueller PW: Diabetes antibody standardization program: first assay proficiency evaluation. *Diabetes* 52:1128–1136, 2003
25. Drago S, El Asmar R, Di Pierro M, Clemente MG, 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*. In press
26. Monsuur AJ, Bakker PI, Alizadeh BZ, Zhernakova A, Bevova MR, Strengman E, Franke L, Slot RV, Belzen MJ, Lavrijsen IC, Diosdado B, Daly MJ, Mulder CJ, Mearin ML, Meijer JW, Meijer GA, Oort E, Wapenaar MC, Koeleman BP, Wijmenga C: Myosin IXB variant increases the risk of celiac disease and points toward a primary intestinal barrier defect. *Nat Genet* 37:1341–1344, 2005
27. Paronen J, Klemetti P, Kantele JM, Savilahti E, Perheentupa J, Akerblom HK, Vaarala O: Glutamate decarboxylase-reactive peripheral blood lymphocytes from patients with IDDM express gut-specific homing reeptor alpha4beta-integrin. *Diabetes* 46:583–588, 1997
28. Hanninen A, Jaakkola I, Jalkanen S: Mucosal addressin is required for the development of diabetes in nonobese diabetic mice. *J Immunol* 160:6018–6025, 1998
29. Yang XD, Michie SA, Roland T, Karin N, Steinman L, McDevitt HO: A predominant role of integrin alpha 4 in the spontaneous development of autoimmune diabetes in nonobese diabetic mice. *Proc Natl Acad Sci U S A* 91:12604–12608, 1994
30. Fasano A, Clemente MG, DeVirgilis S, Musu M, Usai P, Porqueddu P, Cicotto L, Massidda C: Intestinal Zot/zonulin receptor is upregulated in active celiac disease and co-localizes with protease-activated receptor (Par)-2. *J Pediat Gastroenterol Nutr* 39:S57, 2004
31. Fasano A: Celiac disease: how to handle a clinical chameleon. *N Engl J Med* 348: 2568–2570, 2003
32. Pizzuti D, Bortolami M, Mazzon E, Buda A, Guariso G, D'Odorico A, Chiarelli S, D'Inca R, De Lazzari F, Martines D: Transcriptional downregulation of tight junction protein ZO-1 in active coeliac disease is reversed after a gluten-free diet. *Dig Liver Dis* 36:337–341, 2004
33. 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* 52:2287–2295, 2003
34. Londei M, Maiuri L: Gliadin as stimulator adaptive and innate immune responses in celiac disease. *J Pediatr Gastroenterol Nutr* 39:Suppl. 3:S729, 2004
35. Ziegler AG, Schmid S, Huber D, Hummel M, Bonifacio E: Early infant feeding and risk of developing type 1 diabetes-associated autoantibodies. *JAMA* 290:1721–1728, 2003
36. Funda DP, Kaas A, Bocl T, Tlaskalova-Hogenova H, Buschard K: Gluten-free diet prevents diabetes in NOD mice. *Diabetes Metab Res Rev* 15: 323–327, 1999
37. Norris JM, Barriga K, Klingensmith G, Hoffman M, Eisenbarth GS, Erlich HA, Rewers M: Timing of initial cereal exposure in infancy and risk of islet autoimmunity. *JAMA* 290:1713–1720, 2003
38. Saukkonen T, Savilahti E, Vaarala O, Virtala ET, Tuomilehto J, Akerblom HK: Children with newly diagnosed IDDM have increased levels of antibodies to bovine serum albumin but not to ovalbumin. *Diabetes Care* 17:970–976, 1994
39. Kimpimaki T, Erkkola M, Korhonen S, Kupila A, Virtanen SM, Ilonen J, Simell O, Knip M: Short-term exclusive breastfeeding predisposes young children with increased genetic risk of type I diabetes to progressive beta-cell autoimmunity. *Diabetologia* 44:63–69, 2001
40. Kohout P: Small bowel permeability in diagnosis of celiac disease and monitoring of compliance of a gluten-free diet (gut permeability in celiac disease). *Acta Medica (Hradec Kralove)* 44:101–104, 2001
41. Farrell RJ, Kelly CP: Celiac sprue. *N Engl J Med* 346:180–188, 2002
42. Dickey W, Hughes DF, McMillan SA: Disappearance of endomysial antibodies in treated celiac disease does not indicate histological recovery. *Am J Gastroenterol* 95, 712–714, 2000