Structural and Functional Changes in the Tight Junctions of Asymptomatic and Serology-negative First-degree Relatives of Patients With Celiac Disease

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Background: Ten to 15% of first-degree relatives (FDRs) of celiac disease (CeD) patients develop CeD. Although intestinal barrier functions (intestinal permeability) are abnormal in the subset of serology-negative FDRs, what leads to the abnormal barrier function is not known.

Goals: To study the ultrastructure and functions of tight junctions in serology-negative FDRs of CeD patients.

Study: The intestinal permeability was measured in 97 asymptomatic and anti-tissue transglutaminase antibody (anti-tTG Ab)-negative FDRs (using the lactulose mannitol ratio) and in 75 controls. The ultrastructure of tight junctions using transmission electron microscopy, and the expression of key tight junction proteins (claudin-2, claudin-3, occludin, JAM-A, and ZO-1) and zonulin using real-time PCR and immunohistochemistry were assessed in anti-tTG Ab-negative, HLA-DQ2/-DQ8-positive FDRs having normal villi and in disease controls. In addition, the serum zonulin level was measured in 172 anti-tTG Ab-negative FDRs and 198 controls.

Results: The intestinal permeability was significantly increased in FDRs than in controls. Ultrastructural abnormalities such as dilatation of the tight junction ($P = 0.004$) and loss of the pentalaminar structure ($P = 0.001$) were more common in FDRs than in disease controls. There was significant underexpression of tight junction proteins ZO-1 ($P = 0.040$) and occludin ($P = 0.041$) in FDRs. There was no significant difference in the serum zonulin level between FDRs and controls ($P = 0.154$).

Conclusions: Even asymptomatic, anti-tTG-Ab-negative FDRs with a normal villous histology have both ultrastructural and functional abnormalities in tight junctions. These findings are indirect evidence of the presence of tight junction abnormalities before the onset of the disease and may have therapeutic implications.

Key Words: intestinal permeability, paracellular permeability, tight junctions, celiac disease, zonulin

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In addition to genetic susceptibility and exposure to environmental triggers, intestinal barrier dysfunction has been implicated in the pathogenesis of autoimmune diseases including celiac disease (CeD).[1] CeD represents a unique autoimmune disease in which environmental factors (gluten) and genetic susceptibility loci, HLA-DQ2 and HLA-DQ8, are well known.[2,3] Gluten peptides are exposed to the immune system after entering through either the transcellular or the paracellular route.[4] Entry through the paracellular route is primarily maintained by tight junctions, which is a major physical barrier present at the apical-most part of the paracellular route.[5] Tight junctions are composed of multiprotein complexes including both cytoplasmic (ZO-1, ZO-2, and ZO-3) and transmembrane proteins (claudin, junctional adhesion molecule, and occludin).[6] The interaction between these tight junction proteins is an important event for the regulation and the maintenance of tight junction structure and paracellular permeability.[7]

The functional status of the paracellular route is assessed by the intestinal permeability, which is abnormal not only in patients with CeD and Crohn's disease, but also in other autoimmune diseases such as type I diabetes.[8–10] Furthermore, even first-degree relatives (FDRs) of patients with CeD, Crohn's disease, and type I diabetes have an abnormal intestinal permeability.[8–12] Some of these family members having an abnormal intestinal permeability later have developed full-blown diseases.[10,13,14] Although it is understandable that abnormal intestinal permeability in patients with CeD is secondary to the disease itself, it is unclear whether the abnormality in the intestinal permeability in them is a primary event or an effect of the disease. Preliminary evidences suggest that paracellular permeability is abnormal even before the onset of autoimmune diseases such as CeD or Crohn's disease.[8,14] Furthermore, the intestinal permeability has been reported to be abnormal even in asymptomatic family members of CeD.[8,11] Van Elburg et al[8] detected an increase in the intestinal permeability in FDRs with normal villous among 7 of 10 FDRs. Similarly, Vogelsang et al[11] also reported an increase in the intestinal permeability in 31% of the FDRs, 23% of whom had a negative celiac serology.

Zonulin is regarded as a modulator of intestinal permeability, and a zonulin-dependent increase in the intestinal

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✠T.K.D. is now deceased and the copyright is signed by the next of kin on his behalf.
This research was conceptualized, designed, and supervised by G.K.M. and V.A. The study design, participant recruitment, sample collection, performance of experiments, and acquisition of data were performed by A.M. S.P. supervised the HPLC work and the interpretation of data. T.K.D. supervised the acquisition of TEM data. S.D.G. supervised the pathology and IHC work. V.S. performed the biostatistical analysis. Data interpretation and drafting of the manuscript was performed by A.M., G.K.M., V.A., S.D.G., T.K.D., and V.S.
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permeability has been known to play an important role in the pathogenesis of type 1 diabetes in Biobreeding diabetes-prone rats.[15,16] Furthermore, an abnormal intestinal permeability and a higher zonulin concentration has been reported in 70% of type 1 diabetic patients and their relatives.[13] Higher serum levels of zonulin were also found in type 1 diabetes patients' sera collected before the diagnosis of the disease.[13]

There is limited information on the initial event in the pathogenesis of CeD, especially regarding what happens to tight junctions. At present, there are no suitable animal models for CeD wherein one can study the initial events of antigen entry and the status of tight junctions before the onset of the disease. Ten to 15% of FDRs develop CeD, and an increase in paracellular permeability is present in 20% to 30% of FDRs.[8,11,17–20] Although not an ideal, family members of CeD may be considered as a pre-CeD model. Whereas it is known that some of the serology-negative FDRs have an abnormal intestinal permeability, information on the tight junction ultrastructure and the expression of relevant tight junction proteins in serology-negative FDRs is not well known.

However, we expect serology-positive FDRs to have some form of ultrastructural and functional abnormalities or even light microscopic abnormalities; we therefore decided to include in this study only those FDRs who were asymptomatic, had normal hemoglobin, had negative celiac serology, and also had no villous abnormalities (Modified Marsh grade 0). This subgroup, we presume, represents the most healthy subset of FDRs. Therefore, to understand the initiating event in the pathogenesis of CeD, we studied the structure and functions of tight junctions in asymptomatic and serology-negative FDRs of patients with CeD.

PATIENTS AND METHODS

Screening of Participants

FDRs

We invited FDRs of CeD patients, following in the Celiac Disease Clinic at our center between May 2010 and September 2014, for screening for CeD. FDRs who agreed to participate were screened for CeD using commercially available IgA anti-tissue transglutaminase antibody (IgA anti-tTG Ab) ELISA kits (AESKU Diagnostik, Wendelsheim, Germany) (cut-off: 18 ng/mL). FDRs who were reported to be negative for IgA anti-tTG Ab and were asymptomatic or had mild symptoms and met the following exclusion criteria were included in the study after obtaining their informed consent: FDRs with a history of use of nonsteroidal anti-inflammatory drugs in the past 2 weeks, recent gastroenteritis, having used antibiotics or probiotics, chronic alcohol consumers, those having any systemic disease, or those who had undergone gastrointestinal surgery were excluded. Pregnant women and lactating mothers were also excluded. A 3 mL blood sample was collected for serum zonulin along with anti-tTG Ab testing. They were also requested to provide 5-hour urine samples for the measurement of intestinal permeability and to undergo upper gastrointestinal endoscopy to provide duodenal biopsies to study tight junction parameters.

Screening and Recruitment of Controls

In the present study, we recruited 2 types of controls: that is, healthy controls and disease controls. This study included both invasive (endoscopic examination for obtaining mucosal biopsies for tight junction structural studies) and noninvasive investigations (urine collection for tight junction functional study and serological tests). Endoscopic examination could not be performed in healthy participants because of ethical reasons; hence, we also recruited disease controls as described below.

Healthy Participants

Apparently healthy individuals who did not have any recent gastrointestinal infection or any autoimmune diseases and any of the above-mentioned exclusion criteria were included in the study. All of them also underwent screening for CeD using anti-tTG Ab. They were requested to provide blood samples for the measurement of serum zonulin along with anti-tTG Ab and 5-hour urine samples for the estimation of lactulose and mannitol ratio (LMR).

Disease Controls

Patients with gastroesophageal reflux disease, hepatitis B virus, and hepatitis C virus carriers undergoing endoscopic examination for their routine examination were invited to participate in the study if they met all the inclusion and exclusion criteria described above. They were also screened for CeD using anti-tTG Ab, and only anti-tTG Ab-negative participants were included in the study. Duodenal biopsies from the second part of the duodenum were collected from participants who had normal upper gastrointestinal endoscopy findings. Furthermore, only those biopsies were finally included in the study that had no histologic abnormality on light microscopy (villous abnormality Marsh grade 0). Biopsies were graded according to the modified Marsh criteria.[21]

The study was approved by the Ethics Committee of the All India Institute of Medical Sciences, and informed consent was obtained from each participants.

Genotyping for HLA-DQ2 and HLA-DQ8

The genotyping for HLA-DQ2 and HLA-DQ8 was performed using the reverse sequence-specific oligonucleotides method (One Lambda Inc., Thermo Fisher Scientific, Walthem, MA). Readings were taken through the Luminex X-Map technology (Luminex; Life Technologies Inc.).

Duodenal Biopsies

Multiple duodenal biopsies from both FDRs and disease controls were collected from the second part of the duodenum using spike biopsy forceps during upper gastroduodenoscopic examination. Four mucosal biopsies were collected in 10% neutral buffered formalin for light microscopic and immunohistochemistry (IHC) examination, 4-bites in RNAlater solution for RNA extraction and 2-bites in Karnovsky's solution for electron microscopic examination.

Whereas 42 FDRs and 39 controls underwent UGI endoscopic examination, duodenal biopsies for histopathology, IHC, transmission electron microscopy (TEM), and qPCR were collected from 39 FDRs and 36 controls once they had normal endoscopic findings. Three participants in each group were excluded because of mucosal lesions in the stomach or the duodenum. Biopsies were graded as per the Modified Marsh criteria. Data of 18 FDRs who were HLA-DQ2/HLA-DQ8 positive and had no villous abnormality on light microscopic examination

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and 24 controls were finally included for detailed evaluation of tight junctions.

Assessment of the Intestinal Permeability Using LMR

Collection of urine for the assessment of intestinal permeability was performed as described.[22,23] Standard concentrations of lactulose, mannitol, and cellobiose (internal control) (Sigma-Aldrich Laborchemikalien, Germany) were estimated using the Agilent 1200 (Agilent Technologies, CA) HPLC machine and a carbohydrate analysis column (250 × 4.6 mm) of 5 μm particle size. The chromatographic separation was carried out at a flow rate of 0.75 mL/min using HPLC-grade water (35%) as mobile phase A and acetonitrile (65%) as mobile phase B. Solvents were filtered through a 0.22-μm syringe filter and sonicated to remove gas before use. The column temperature was kept at 50°C. The run time for each sample was set at 12 minutes. The analytes were detected using an evaporating light-scattering device (ELSD). Analytes were detected by keeping the temperature of ELSD at 60°C and the flow of N_2 gas at a rate of 1.5 L/minute. Resolution of mannitol and lactulose peaks was found at 6.0 and 6.7 minutes and cellobiose at 7.8 minutes, respectively. The percentage excretion of lactulose and mannitol was compared between FDRs and controls. Validation of tests was carried out by constructing a calibration curve, and inter-assay and intra-assay variations were measured for each sugar by running them at 3 different concentration on each day. The LMR was assessed in 97 serology-negative FDRs, 54 healthy controls, and 21 disease controls (Fig. 1).

TEM

A mucosal biopsy collected in Karnovsky's solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer) was used to make blocks for TEM. Blocks were prepared using graded acetone dehydration and polymerization in araldite CYC212. Ultrathin sections were cut using a Reichert-Jung (Leica UC6; Leica, MA) ultramicrotome. Only those EM blocks were finally included in the study that did not have artifacts and had preserved morphologic features. TEM viewing was performed using a Gatan CCD camera (Olympus Soft Imaging Solution, Munster, Germany) attached with an electron microscope. The ImageJ software was used for measuring the diameter of the tight junction and the microvillous length. The tight junction pentalaminar structure is formed by the fusion of a membrane of the adjacent cell forming a structure composed of 2 outer lighter bands and a middle denser band (Fig. 2C). The pentalaminar structure of the tight junction was considered normal or maintained when all the 3 bands (2 outer lighter bands and the middle denser band) were present (Fig. 2C). The diameter of the tight junctions, the length, and the gap between microvillous were measured at 3 or more sites, and finally the mean value was reported.

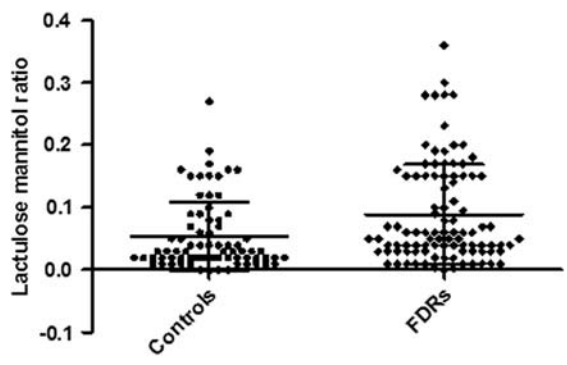

FIGURE 1. A scatter plot showing the LMR in FDRs and controls (data have been shown as mean ± SD). A significantly high LMR was observed in FDRs compared with controls ($P = 0.002$). FDRs indicates first-degree relatives; LMR, lactulose mannitol ratio.

Expression of Tight Junction Proteins in Duodenal Biopsies Using Real-time PCR

The total RNA was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany). The quality and the quantity of extracted RNA were checked using Bioanalyzer 2100 (Agilent Technology, Waldenbronn, Germany). Reverse transcription reaction (cDNA synthesis) was carried out using 2 μg of total RNA and the Maxima first-strand cDNA synthesis kit (Thermo Fisher Scientific Inc.). Real-time PCR was performed using Mx3005P thermocycler (Stratagene; Agilent Technologies) using the Maxima probe qPCR master mix (Thermo Fisher Scientific). Primers and probes of claudin-2 and claudin-3, occludin, JAM-A, ZO-1, and zonulin were synthesized using the Beacon Designer software (PREMIER Biosoft), and primers and probes were procured from Sigma-Aldrich Laborchemikalien (Table 1). GAPDH was used for the normalization of gene expression and the calculation of the fold difference in the FDR group with respect to the control group. Analysis of fold differences was carried out using the REST 2009 software.[24]

Expression of Tight Junction Proteins in Duodenal Biopsies Using IHC

Thin 5-μm sections of paraffin-embedded blocks were cut, and tissue sections were mounted on slides coated with amino-propyl-tri-ethoxy-silane. Sections were dewaxed using xylene and then incubated in graded acetone. The endogenous peroxidase activity was blocked by 3% hydrogen peroxide. Heat-induced antigen retrieval was performed using citrate buffer (pH 6.0) and EDTA buffer (pH 8 and 9). The sections were then stained with primary antibodies and incubated overnight at 4°C. Anti-human primary Ab for claudin-2 and claudin-3 (1:20, 1:40; Zymed Laboratories Inc., San Francisco, CA), occludin (1:200; Abcam, Cambridge, UK), JAM-A (1:200; Zymed Laboratories Inc.), ZO-1 (1:50; Abcam), and zonulin (1:200; Immundiagnostik AG, Bensheim, Germany) were used. The universal secondary Ab (CRF anti-polyvalent HRP polymer kit; Scytek Laboratories, Logan, UT) was used. The antigen-antibody reaction was finally visualized with peroxidase-substrate reaction using 3,3′-Diaminobenzidine as the chromogen. Each stain was evaluated using a semi-quantitative scoring system for both the intensity and the percentage of positive epithelial cells. When at least 5% of the epithelial cells displayed distinct immunostaining, they were considered to be positive. IHC expression was graded according to the stain distribution and the intensity separately both in the villous and the crypt epithelium. Finally, the total score was obtained by adding the score of the percentage distribution of the protein in cells and the intensity, individually for villous and crypts. A minimum of 500 cells per slide were counted.

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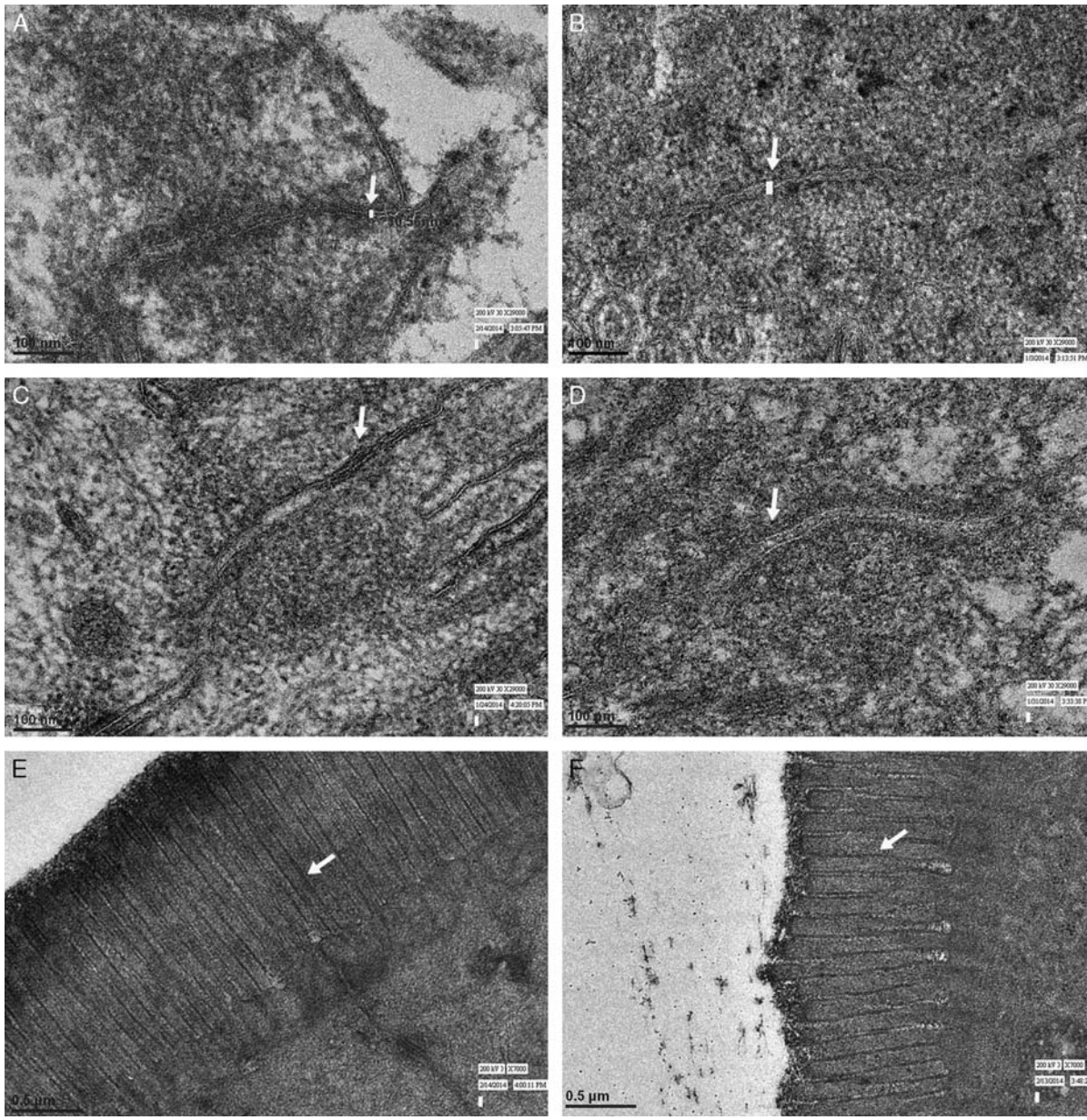

FIGURE 2. Transmission electron microphotograph. A, A representative electron micrograph of a disease control showing the measurement of the tight junction diameter (10.5 nm) (shown by an arrow). B, A representative electron microphotograph showing the measurement of FDRs' tight junction diameter (22 nm) (shown by an arrow). C, Distinct bands of the pentalaminar structure of tight junctions in a disease control (shown by an arrow). D, The distorted pentalaminar structure of tight junctions of an FDR could be seen as the loss of strands of the tight junction (shown by an arrow). E, A representative electron micrograph showing microvilli of a disease control (shown by an arrow). F, A representative electron micrograph of microvilli (shown by an arrow) of an FDR showing an increase in the distance between adjacent microvilli. FDRs indicates first-degree relatives.

Estimation of Serum Zonulin

Serum zonulin concentrations were measured using the zonulin ELISA Kit (Immundiagnostik AG). The assay was conducted according to the manufacturer's manual. Serum samples were blinded before running the tests, and tests were carried out in duplicates for 10% samples. Readings were taken using the TECAN ELISA reader (TECAN Group Limited, Maennedorf, Switzerland) at 450 nm against the reference of 620 nm. Mean intra-assay and inter-assay coefficients of variance were 1.8% and 4.10%, respectively. Data were analyzed using 4 PL algorithms using GraphPad Prism (GraphPad Software Inc., CA).

Statistical Analysis

Quantitative variables were summarized as mean ± SD. Qualitative variables were summarized as proportions (%). Intestinal permeability parameters and quantitative parameters of electron microscopy were summarized as mean ± SD and median (IQR). These parameters were compared between anti-tTG-negative FDRs and controls using Student

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TABLE 1. Primer and Probe Sequences Used in the Quantification of Tight Junction Proteins

Protein	Genbank No.	Sequences	Base Pair
Claudin-2	NM_001171092.1	F 5′-TGC TCA TCC CAG AGA AAT CG-3′ R-5′-GCT GTA GGA ATT GAA CTC ACT C-3′ P-5′-TAC GAT GCC TAC CAA GCC CAA CCT C-3′	122
Caludin-3	NM_001306.3	F 5′-CCA CGC GAG AAG AAG TAC AC-3′ R-5′-GTA GTC CTT GCG GTC GTA GC-3′ P-5′-AAG GTC GTC TAC TCC GCG CCG C-3′	102
Occludin	NM_002538.2	F-5′-TAT AAA TCC ACG CCG GTT CCT-3′ R-5′-ACG AGG CTG CCT GAA GTC AT-3′ P-5′-AAG TGG TTC AGG AGC TTC CAT TAA CTT CGC-3′	80
JAM-A	NM_016946.4	F-5-GAG GCC ACT TTG ACA GAA CA-3′ R-5′-CTCA CAC CAG GAA TGA CGA G-3′ P-5′-CAG CCA GCC TAG TGC CCG AA-3′	114
ZO-1	NM_003257.3	F-CAA GAT AGT TTG GCA GCA AGA GAT G-3′ R-ATC AGG GAC ATT CAA TAG CGT AGC-3′ P-5′-TAT TGA AGA TAA ATG GTA CTG TGA CAG-3′	160
Zonulin	NM_001126102.	F'-5′-CAA GAT TTC AGC CTG GAA GAG G-3′ R-5′-TTA TTG ATT GAC TCA GCA ATG CAG-3′ P-5′-CGG GAG TGG ACA GGA GTG GAT GCG-3′	114

JAM-A indicates junctional adhesion molecule; ZO-1, zonula occludens.

t test/Rank sum test. Qualitative parameters of electron microscopy were compared using Fisher exact test.

Results of LMR obtained from 54 healthy controls were used to determine the upper limit of LMR at the one tailed 90% tolerance interval, assuming that in the normal population, 10% of the individuals will have an abnormal intestinal permeability. The cut-off value of LMR corresponding to the 90th percentile was 0.15; hence, LMR > 0.15 was considered as abnormal.

The relative gene expression by qPCR was calculated between FDRs and controls using the relative expression software tools (REST) 2009 software.[24] Data were represented as the mean factor of the expression level, the range of SE, and the 95% confidence interval. Statistical analysis was carried out using the IBM statistic 19.0 software version. The distribution and the intensity of tight junction protein expression in villous and crypts were categorized and were compared between FDRs and controls using Fisher exact test. Log-transformed values were used for the analysis of variables with a higher SD. All tests were 2-tailed. A *P*-value of <0.05 was considered as statistically significant. All analyses were carried out using Stata version 12.1 (College Station, TX).

TABLE 2. Characteristics of Participants Included in the Study

Test	N	Age (Mean ± SD)	M:F
Lactulose and mannitol ratio			
FDRs	97	31.2 ± 12.1	58:39
Controls	75	27.7 ± 6.26	69:6
Transmission electron microscopy			
FDRs	10	33.0 ± 1.4	6:4
Controls	12	30.9 ± 8.6	11:1
Expression pattern of tight junction and zonulin proteins			
FDRs	18	34,0 ± 8.9	8:10
Controls	24	30.7 ± 8.7	20:4
Serum zonulin			
FDRs	172	31.2 ± 12.1	105:67
Controls	198	26.8 ± 4.7	164:34

FDRs indicate first-degree relatives.

RESULTS

Characteristics of the Participants Included in This Study

Details of the participants included in this study have been shown in Table 2.

HLA-DQ2/HLA-DQ8 Haplotyping

Among 18 FDRs whose duodenal biopsies were included in this study for detailed evaluation of the ultrastructure and the expression of tight junction proteins, 17 were heterozygous HLA-DQ2 and 1 FDR was carrying a complex heterozygous HLA-DQ2 and HLA-DQ8. HLA haplotyping could be performed in 20 of 24 disease controls, who underwent detailed evaluation for the tight junction protein structure and expression. Only 13 of them were carrying either HLA-DQ2 or HLA-DQ8 (11 heterozygous HLA-DQ2 and 2 heterozygous HLA-DQ8).

Functional Analysis of Tight Junctions by the LMR in FDRs and Controls

The LMR was measured in 54 healthy controls, 21 disease controls, and 97 FDRs. The mean value of the percentage excretion of lactulose was 0.32 ± 0.36 and 0.68 ± 1.05 in healthy controls and disease controls, respectively. The mean value of the percentage excretion of mannitol was 8.17 ± 8.6 and 11.6 ± 15.02 in healthy controls and disease controls, respectively. Overall, the mean value of LMR was 0.04 ± 0.04 in healthy controls and 0.06 ± 0.07 in disease controls. When compared between total control and FDRs, the mean value of the percentage excretion of lactulose was significantly higher in FDRs in comparison with controls (0.76 ± 0.92 vs. 0.42 ± 0.65, $P < 0.001$) (Table 3, Fig. 1). There, however, was no significant difference in the percentage excretion of mannitol in FDRs compared with controls (9.1 ± 10.8 vs. 11.2 ± 12.8, $P = 0.12$) (Table 3, Fig. 1). Overall, LMR was significantly higher in FDRs compared with controls (0.08 ± 0.07 vs. 0.05 ± 0.05, $P = 0.002$) (Table 3). Using the cut-off value of LMR at 0.15 (as determined by the 90th percentile of LMR in healthy controls), a significantly

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TABLE 3. Results of the Measurement of Intestinal Permeability Using the Percentage Excretion of the Lactulose and Mannitol Ratio

Subjects	N	Excretion of Lactulose (%)	Excretion of Mannitol (%)	Lactulose Mannitol Ratio
Controls	75	0.42 ± 0.65	9.1 ± 10.8	0.05 ± 0.05
FDRs	97	0.76 ± 0.92	11.2 ± 12.81	0.08 ± 0.07
P		< 0.001	0.123	0.002

FDRs indicate first-degree relatives.

higher number of serology-negative FDRs had an abnormal intestinal permeability compared with controls [28 (29%) vs. 10 (13%), ($P = 0.04$)].

Comparison of Ultrastructural Changes in Tight Junctions and Microvilli in HLA DQ2+/− DQ8-positive FDRs and Disease Controls (Both Serology Negative and Having No Villous Abnormalities) Using TEM

Serology-negative FDRs having a normal villous architecture on light microscopy had a significantly higher mean diameter of tight junctions in comparison with that in controls (23.0 ± 7.6 vs. 14.09 ± 5.12 nm, $P = 0.004$) (Figs. 2A, B). Seven of 10 FDRs also showed destruction of the pentalaminar structure of tight junctions, whereas all controls showed a normal pentalaminar structure (Figs. 2C, D). Whereas the mean distance between microvilli was higher in FDRs compared with controls (24.3 ± 9.1 vs. 16.7 ± 6.8 nm, $P = 0.03$), there was no significant difference in the microvillous length between FDRs and controls (Table 4; Figs. 2E, F).

Relative Gene Expression of Tight Junction Proteins in Serology-negative HLA-DQ2/HLA-DQ8-positive FDRs Having a Normal Villous Architecture and Controls Using qPCR

There was an underexpression of occludin by a mean factor of 0.630 ($P = 0.04$) in FDRs in comparison with controls. Similarly, an underexpression of cytoplasmic protein ZO-1 was observed in FDRs by a mean factor of 0.630 ($P = 0.041$) in comparison with that in the controls. There, however, was no significant difference in the transcriptional expression of other tight junction proteins such as claudin-2, claudin-3, JAM-A, and zonulin (Table 5).

Semiquantitative Expression of Tight Junction Proteins in Serology-negative HLA-DQ2/HLA-DQ8-positive FDRs Having a Normal Villous Architecture and Controls Using IHC

There was significant underexpression of cytoplasmic protein ZO-1 in the villi of FDRs in comparison with that in controls ($P = 0.02$) (Table 6; Figs. 3A, B). There, however, was no significant difference in the expression of ZO-1 in crypts in FDRs and controls ($P = 0.59$).

Although there was a trend of underexpression of the transmembrane protein occludin in the villi of FDRs in comparison with controls, the difference in the expression was not statistically significant ($P = 0.34$). There was no significant difference in the expression of other tight junction proteins claudin-2, claudin-3, and JAM-A, and zonulin in the villi and the crypts of both FDRs and controls.

Comparison Between Serum Levels of Zonulin in FDRs and Controls

The level of serum zonulin was estimated in 172 serology-negative FDRs and 198 controls (serology negative) using the commercially available ELISA kit. Mean values of serum zonulin in FDRs and controls were 47.5 ± 23.0 and 51.2 ± 26.12 ng/mL, respectively, and there was no statistically significant difference between the 2 groups ($P = 0.15$) (Fig. 4).

DISCUSSION

We observed that even asymptomatic and anti-tTG Ab-negative FDRs had evidences of both structural and functional changes in the small-intestinal tight junctions. Specifically, they had dilatation of the tight junctions, loss of the pentalaminar structure of tight junctions, and an increase in the intermicrovillous distance on TEM examination. The intestinal permeability was abnormal in FDR, as evidenced by a higher LMR in one third of them. There was underexpression of ZO-1 and occludin, which regulate the tight junction structure and functions. No significant alteration, however, was observed in them in the serum levels of zonulin.

Observations of 2 previous studies and the present study suggest that a higher number of even asymptomatic and serology-negative FDRs have an abnormal intestinal permeability in comparison with controls. Not only FDRs of patients with CeD and IBD, but 25% to 35% FDRs of patients with type 1 diabetes also have an increase in the intestinal permeability.[8,10,13,25] It is quite interesting and intriguing to observe the abnormal intestinal permeability in FDRs of type 1 diabetes, where the primary pathology is in the β-cells of pancreas and not in the intestine. Furthermore,

TABLE 4. Results of Transmission Electron Microscopy in HLA-DQ2/HLA-DQ8-positive FDRs and Controls (Both Serology Negative and Having Normal Villous Architecture)

Subjects	N	Tight Junction Diameter (Mean ± SD) (nm)	Tight Junction Structure Maintained	Microvillous Length (Mean ± SD) (nm)	Intermicrovillous Distance (Mean ± SD) (nm)
Controls	12	14.09 ± 5.12	12/12	1203 ± 238.7	16.7 ± 6.86
FDRs	10	23.08 ± 7.63	2/10	1138 ± 296.37	24.35 ± 9.17
P		0.04	0.001	0.57	0.03

FDRs indicate first-degree relatives.

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TABLE 5. Relative Gene Expression of Tight Junction Proteins in HLA-DQ2/HLA-DQ8-positive First-degree Relatives and Controls (Both Serology Negative and Having Normal Villous Architecture Using Immunohistochemistry) Using qPCR

Gene	Relative Gene Expression	SE	95% CI	*P*
Membranous tight junction proteins				
Claudin-2	0.566	0.148-2.137	0.027-5.214	0.078
Claudin-3	0.724	0.250-2.099	0.028-5.432	0.256
Occludin	0.630	0.333-1.378	0.036-3.317	**0.041**
JAM-A	0.677	0.255-1.806	0.027-4.510	0.172
Cytoplasmic proteins				
ZO-1	0.610	0.271-1.548	0.038-3.095	**0.040**
Zonulin	0.813	0.394-2.085	0.048-4.252	0.399

Bold values represent significant *P* values.
CI indicates confidence interval; JAM-A, junctional adhesion molecule; ZO-1, zonula occludens-1.

an increase in the intestinal permeability was observed 2 weeks before the onset of diabetes in Biobreeding diabetic-prone rats, which develop diabetes spontaneously at approximately 75 days of their age.[16]

The observations of a case report, as described below, also add to the evidence to support the intestinal permeability being an early event in the pathogenesis and preceding the onset of the disease.[14] A 13-year-old girl, a family member of a patient with Crohn's disease, had an increase in intestinal permeability while she was asymptomatic and had no microscopic or macroscopic lesions of Crohn's disease. She became symptomatic at the age of 21 years and was detected to have ileocolonic Crohn's disease.[14]

Tight junctions, present at the apical-most part of tight junctions, play a crucial role in allowing entry through the paracellular route. Abnormal tight junctions with a wider gap between intercellular junctions allow the entry of larger molecules and increase the intercellular permeability. While serology-positive FDRs of CeD patients are expected to have ultrastructural and light microscopic abnormalities, we in this study observed dilated and disrupted tight junctions in 7 of 10 asymptomatic and anti-tTG Ab-negative, HLA-DQ2/HLA-DQ8-positive FDRs. We also observed a loss of the pentalaminar structure of tight junctions in them. In our opinion, such an observation, albeit in relatively small numbers, is the strongest evidence of the abnormal structure of tight junctions in them

Ultrastructural changes of tight junctions have been described in patients with CeD and Crohn's disease, which could be secondary to the disease process itself. Observations of ultrastructural changes in tight junctions in serology-negative FDRs, as shown in this study, and in other autoimmune diseases where the intestine is not even the primary site of diseases, such as in type 1 diabetes, Hashimoto thyroiditis, and dermatitis herpetiformis, are indirect

TABLE 6. Expression of Tight Junction Proteins in HLA-DQ2/HLA-DQ8-positive FDRs and Controls (Both Serology Negative and Having Normal Villous Architecture Using Immunohistochemistry)

			Villous n (%)			Crypts n (%)		
Location	Proteins	Grades of Immunostaining	Controls	FDRs	*P*	Controls	FDRs	*P*
Transmembranous	Claudin-2		n = 24	n = 18		n = 24	n = 18	
		Negative (0)	2 (8.3)	0 (0)	0.44	2 (8.7)	1 (5.5)	0.80
		Focal (≤3)	2 (8.3)	2 (11.1)		4 (17.4)	2 (11.1)	
		Diffuse (> 3)	20 (83.3)	16 (88.8)		18 (73.9)	15 (83.3)	
	Claudin-3		n = 24	n = 18		n = 24	n = 18	
		Negative (0)	1 (4.3)	1 (5.5)	0.69	4 (17.4)	1 (5.5)	0.53
		Focal (≤3)	5 (21.7)	2 (11.1)		2 (8.7)	2 (11.1)	
		Diffuse (> 3)	18 (73.9)	15 (83.3)		18 (73.9)	15 (83.3)	
	Occludin		n = 24	n = 17		n = 24	n = 17	
		Negative (0)	6 (25)	7 (41.2)	0.34	9 (37.5)	6 (35.3)	0.73
		Focal (≤3)	3 (12.5)	4 (23.5)		12 (50)	10 (58.5)	
		Diffuse (> 3)	15 (62.5)	6 (35.3)		3 (12.5)	1 (5.9)	
	JAM-A		n = 20	n = 18		n = 20	n = 18	
		Negative (0)	2 (10)	1 (5.5)	0.75	6 (30)	2 (11.1)	0.15
		Focal (≤3)	3 (15)	5 (33.3)		7 (35)	12 (66.6)	
		Diffuse (> 3)	15 (75)	12 (66.6)		7 (35)	4 (22.2)	
Cytoplasmic	ZO-1		n = 24	n = 18		n = 24	n = 18	
		Negative (0)	2 (8.3)	8 (44.4)	**0.02**	6 (25)	7 (38.8)	0.59
		Focal (≤3)	15 (62.5)	8 (44.4)		17 (70.8)	10 (55.5)	
		Diffuse (> 3)	7 (29.2)	2 (11.1)		1 (4.2)	1 (5.5)	
	Zonulin		n = 13	n = 12		n = 13	n = 12	
		Negative (0)	0 (0)	0 (0)	0.99	0 (0)	0 (0)	0.99
		Focal (≤3)	11 (84.6)	10 (83.3)		11 (84.6)	10 (83.3)	
		Diffuse (> 3)	2 (15.4)	2 (16.6)		2 (15.4)	2 (16.6)	

FDRs indicate first-degree relatives; JAM-A, junctional adhesion molecule; ZO-1, zonula occludens-1.

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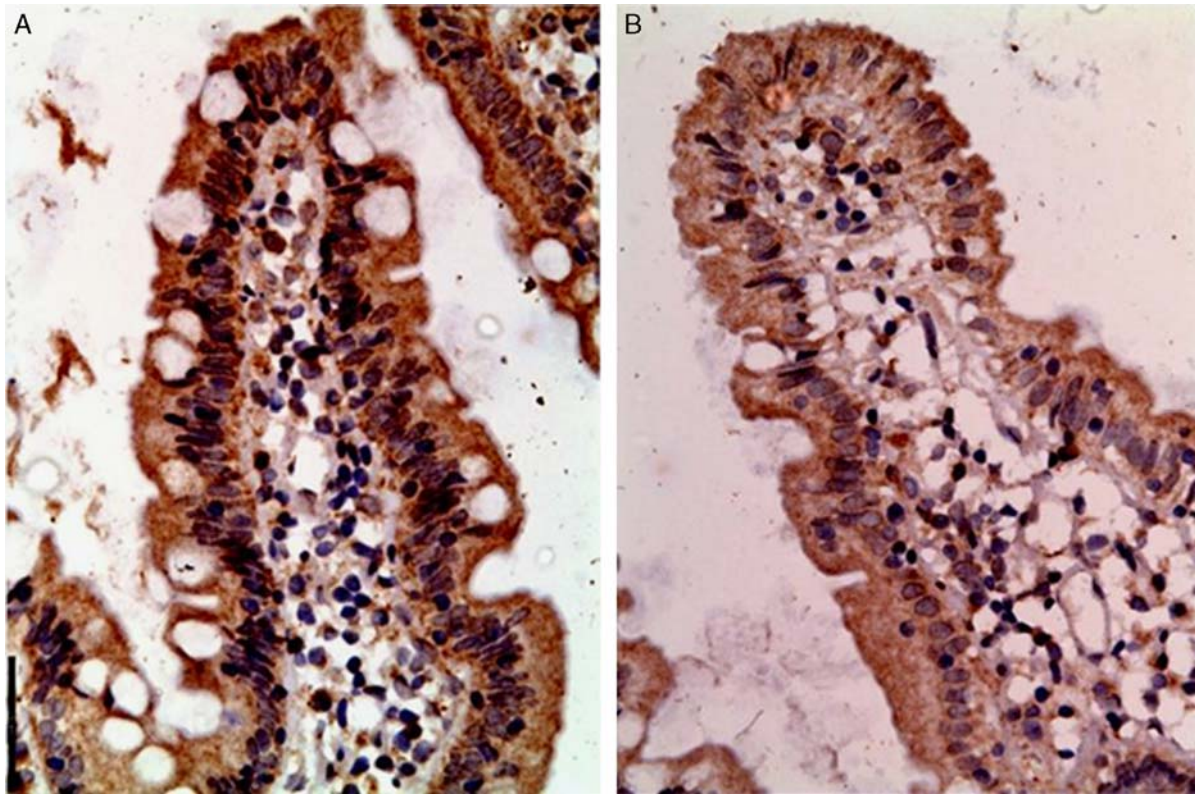

FIGURE 3. Immunohistochemistry (IHC) of ZO-1 at ×400 magnification. A, A representative IHC picture of a control. B, A representative IHC picture of first-degree relatives; scale bar = 50 μm. full color online

evidences to support the view that these changes may be present before the onset of the disease.[26–28]

As the structure and the function of tight junctions is maintained by a series of tight junction proteins, we in this study observed a significant underexpression of ZO-1 and occludin, and no difference in the expression of other transmembrane (claudin-2, claudin-3, and JAM-A) and secretory cytoplasmic (zonulin) proteins at the transcriptional level in serology-negative FDRs compared with that in controls. At the level of localization of tight junction proteins in tissues by IHC, there was significant underexpression of ZO-1 only and not in the occludin. This difference in the expression of proteins could be due to differences in sensitivities of both the techniques. In addition, the extraction of RNA was performed from the whole mucosal biopsy (including all the contents), whereas IHC showed site-specific localization.

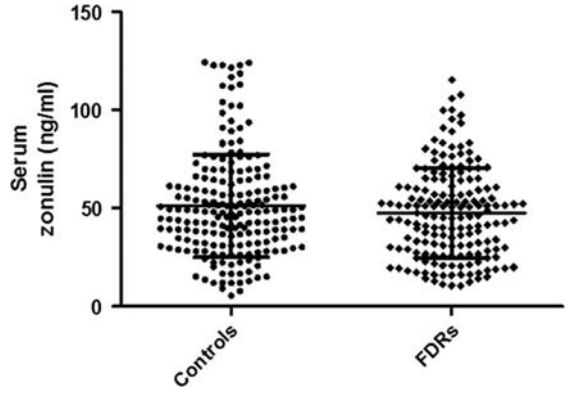

FIGURE 4 . A scatter plot showing the serum zonulin level in controls (n = 198) and FDRs of celiac disease patients (n = 172) and has been shown as mean ± SD. There was no significant difference in the serum zonulin level between controls and FDRs ($P = 0.15$). FDRs indicates first-degree relatives.

Underexpression of ZO-1 and occludin is likely to be the molecular basis of the abnormal structure and function of tight junctions in FDRs of patients with CeD. ZO-1 is the earliest molecule to localize in the nascent tight junction complex, and forms a connecting link between the cytoplasmic actin filament (skeleton) and transmembranous proteins.[29–31] Underexpression of ZO-1 with the concomitant loss of F-actin organization had been reported in CeD.[32] Occludin interacts with other tight junction proteins such as ZO-1 and F-actin and is responsible for the paracellular flux of macromolecules and bacterial antigens.[33] The underexpression of occludin and its localization into the cytoplasm has been shown to be associated with an increase in the intestinal permeability in the murine model of graft-versus-host disease.[34] Furthermore, underexpression and redistribution of ZO-1 and occludin has been reported in the small intestine of active CeD patients, resulting in an increase in intestinal permeability.[35]

Zonulin modulates the intestinal permeability by causing the disruption of interaction between the transmembrane protein occludin and the cytoplasmic anchoring protein ZO-1.[15] An increase in the intestinal permeability is zonulin dependent, and the administration of zonulin inhibitor FZI/0 into BioBreeding diabetes-prone rats

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prevents an increase in the intestinal permeability.[36] In the present study, we did not observe any significant difference in the level of serum zonulin between asymptomatic and anti-tTG Ab-negative FDRs and asymptomatic and anti-tTG Ab-negative controls. Sapone et al[13] showed a significantly higher serum zonulin level in type I diabetes patients (0.83 ± 0.05 ng/mg of protein) and their FDRs (0.62 ± 0.07 ng/mg of protein) in comparison with controls (0.21 ± 0.02 ng/mg of protein). Duerksen et al[37] recently reported a higher serum zonulin level in CeD patients ($n = 21$), which got normalized after they were put on a gluten-free diet. The literature on the levels of serum zonulin is still evolving, and there are not many studies on the level of serum zonulin.

The strength of this study is the recruitment of asymptomatic anti-tTG Ab-negative FDRs and the use of strict inclusion criteria to exclude individuals having microscopic villous abnormalities. There are many limitations in the study. Whereas we showed abnormalities in the structure and the function in serology-negative FDRs, we have not followed them longitudinally for the development of CeD in those with and without tight junction structural and functional abnormalities. It will be interesting to follow longitudinally participants without evidence and those having evidence of disruption in the tight junction structure and functions, for many years, to observe how many in each subset develop CeD autoimmunity, or CeD, or even other autoimmune disease(s). Although the ideal controls for the study of the tight junction structure and function would have been healthy individuals, they could not be included for ethical reasons, and thus, we chose the nearest best possible individuals, such as patients with achalasia, and HBV/HCV carriers, in whom intestinal tight junctions are expected to be normal. Data on the ultrastructure of tight junctions could not be obtained in all FDRs who agreed to undergo biopsies because of technical reasons such as proper fixation. Inclusion of more male participants in the control group is another limitation of this study.

What could be the implications of such a finding in context with the pathogenesis of CeD? We now know that even serology-negative FDRs have abnormalities in tight junction functions and structure, which may predispose them to CeD or other autoimmune diseases. Whereas the modulation of tight junctions is now being explored for therapeutic intervention in CeD, the scope of such an intervention can be widened to include FDRs also.

in conclusion, even asymptomatic, anti-tTG Ab-negative FDRs and individuals having no villous abnormalities had both ultrastructural and functional abnormalities of the tight junctions. Such changes in the paracellular permeability may be responsible for the entry of various food antigens in a subset of FDRs and may predispose them to develop CeD or other autoimmune diseases.

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