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ORIGINAL ARTICLE

A Comparison of Antibody Testing, Permeability Testing, and Zonulin Levels with Small-Bowel Biopsy in Celiac Disease Patients on a Gluten-Free Diet

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Abstract Active celiac disease is associated with positive endomysial (EMA) and tissue transglutaminase (TTG) antibodies, elevated zonulin levels, and increased intestinal permeability. There is little known about what happens to these immunologic and structural abnormalities in patients on a gluten-free diet and their correlation with small-bowel biopsy changes. Adult patients previously diagnosed with celiac disease and on a gluten-free diet for greater than 1 year were considered for the study. All patients underwent the following: measurement of EMA and TTG antibodies, serum zonulin levels, intestinal permeability (IP) testing with lactulose/mannitol ratios, food diary analysis for gluten ingestion and small- bowel biopsy. A total of 21 patients on a gluten-free diet for a mean of 9.7 years completed the study. There were ten patients who had normalization of intestinal biopsies, IP and TTG, and EM antibodies. Six patients had Marsh type 2 or 3 lesions and all had either abnormal IP (5/6) or TTG antibody (4/6). In patients with Marsh type 3 lesions, there was a correlation between IP and zonulin levels. A subgroup of patients with celiac disease on a gluten-free diet has complete normalization of intestinal biopsies, intestinal permeability defects, and antibody levels. Patients with Marsh type 3 lesions have abnormal TTG antibodies and intestinal permeability with zonulin levels that correlate with IP. These abnormalities may be due to continued gluten ingestion. Further study is needed to determine the clinical utility of TTG antibodies and IP testing in following patients with celiac disease.

Keywords Celiac disease · Intestinal permeability · Gluten-free diet · Intestinal biopsy

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Introduction

Celiac disease is an autoimmune disease characterized by villous destruction and crypt hypertrophy in response to exposure to dietary gluten [1]. The pathogenesis of celiac disease involves changes in permeability that allow gluten peptides access to the small intestinal lamina propria and activate an intramucosal immune cascade [2]. Zonulin is a protein released from the lamina propria that alters intestinal permeability and is up-regulated in active celiac disease [3]. Intestinal permeability can be measured non-invasively using lactulose/mannitol ratios [4]. Previous studies have demonstrated increased intestinal permeability changes in patients with active celiac disease [5, 6]. In response to a gluten-free diet, these permeability changes decrease and, in some patients normalize completely [5, 6]. The relationship between histologic normalization and permeability change has not been well studied. Tissue transglutaminase antibodies (TTG) and endomysial antibodies (EMA) normalize in many patients on a gluten-free diet [7]. In patients who are on a gluten-free diet, the correlation of antibody levels with permeability, histology, and zonulin levels is unclear.

The purpose of this study is to explore the relationships between zonulin levels, intestinal permeability, celiac antibodies, and histology in celiac patients on a gluten-free diet.

Methods

Study Population

Adults were considered for the study if the diagnosis of celiac disease was based on a consistent small-bowel biopsy and a clinical response to a gluten-free diet. Individuals were also considered if the clinical presentation was consistent with celiac disease, serology for celiac disease was positive (positive IgA endomysial antibody) and there was a clinical response to a gluten-free diet. All patients considered for the study were on a gluten-free diet for over 1 year.

Exclusion Criteria

Exclusions included active peptic ulcer disease or chronic renal insufficiency (creatinine > 250 mmol/l). Participants in the study avoided the following prior to permeability testing: alcohol for 24 h, chewing gum containing mannitol for 72 h, and NSAIDS for 1 week.

Study Protocol

All patients were asked to complete the following study protocol (1) intestinal permeability testing, (2) antibody testing with EMA and TTG, (3) detailed dietary assessment, (4) serum zonulin measurements, and (5) small-bowel intestinal biopsy. The study protocol was approved by the University of Manitoba Research Ethics Board. All participants gave informed consent for participation in the study.

Intestinal Permeability Measurements

After an overnight fast, participants ingested a carbohydrate solution consisting of 5 g lactulose and 2 g mannitol and a hyperosmotic agent, 100 g sucrose in 300 ml water. This protocol for measuring intestinal permeability has been validated in patients with celiac disease by other investigators [8]. All urine was collected for the following 5 h in a container with 5 ml of 10% thymol in methanol as preservative. Participants were permitted to drink water throughout the study, and could eat lightly after the first 2 h, avoiding foods containing sucrose. At conclusion of the test, the volume of urine was measured and an aliquot of sample was removed and stored at −2°C. Analysis of lactulose and mannitol was performed by HPLC with pulsed amperometric detection. The fractional excretion was calculated as follows:

$$\text{mg lactulose excreted} = \text{mg/l lactulose} \times \text{l urine}$$

$$\text{Fractional excretion of lactulose} = \text{mg lactulose excreted}/\text{mg lactulose consumed}$$

Results are expressed as a ratio of the fractional excretion of lactulose to the fractional excretion of mannitol (L/M ratio).

Antibody Analysis

On each visit, 5 ml of blood was drawn and the serum tested for IgA endomysial antibodies. For this testing, serum samples were diluted and incubated with human umbilical cord substrate for 30 min. They were then washed, incubated with fluorescein-conjugated guinea pig anti-human IgA for an additional 30 min, washed again, and examined by fluorescence microscopy. Sera were considered positive if fluorescence was seen at dilutions of 1:5 or greater.

TTG antibodies were measured using an ELISA test kit (EUROIMMUN, Germany). The test kit contains microtiter strips each with eight break-off reagent wells coated with human tissue transglutaminase. In the first reaction step, diluted patient samples are incubated in the wells for 30 min at room temperature. In the case of positive samples, specific IgA antibodies will bind to the antigens. To detect the bound antibodies, a second incubation of 30 min is carried out using a peroxidase-labeled anti-human IgA (rabbit). Then, a final incubation of 15 min is carried out with a chromagen/substrate solution (TMB/hydrogen peroxide) which gives a color reaction where the intensity of the color compares with the concentration of the antibody. A stop reagent of 0.5 M sulphuric acid is used to stop the reaction allowing the optical density of the wells to be read at a wavelength of 450 nm with a reference wavelength of between 620 and 650 nm. The upper limit of the normal range recommended by EUROIMMUN is 20 relative units (RU)/ml. All antibody testing was performed by a single technologist experienced in these assays.

Dietary Assessment

Prior to the permeability test, participants kept a 3-day food diary, incorporating one weekend day and two weekdays for analysis. A random 24-h dietary recall was also conducted by phone by a study investigator prior to each test. This method has been used by other investigators for determining compliance in patients with celiac disease [9].

Each participant's food record was analyzed, blind to test results, by a clinical dietitian experienced in celiac disease, and assigned to one of the following three categories; (N) no gluten ingested—analysis revealed no gluten detectable in diet: (T) trace amounts of gluten ingested—analysis demonstrated ingestion of one or more gluten-containing additives, e.g., malt in cereal; (G) gluten ingested—analysis revealed significant gluten ingestion, e.g., more than just as food additive ingestion.

For analyzing food records, patients were asked to give brands of the foods they consumed since some brands are gluten-free, while others may contain gluten (e.g., certain brands of ice cream may contain gluten). Food labels were examined and product manufacturers contacted to determine if there was gluten in any of the foods studied. In cases where the type of food consumed was unclear, the patient was contacted by phone for further clarification.

Zonulin Levels

This assay was performed as has been described [10] with minor modifications. Briefly, plastic microtiter plates (Costar, Cambridge, MA, USA) were coated with rabbit zonulin cross-reacting anti-Zot IgG antibodies (10 mg/ml in 0.1 M sodium carbonate buffer, pH 9.0). After overnight incubation at 4°C, the plates are washed 4× in TBS-0.05% Tween 20 (TBS-T), and blocked by incubation for 1 h at 37°C with TBS-T. After 4 TBS-T washes, 5 Zot serial standards (50, 25, 12.5, 6.2, 3.1 and 0 ng/ml) and patient sera (1:101 dilution in TBS-T) were added and incubated overnight at 4°C. After four washes with TBS-0.2% Tween 20 buffer, the 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, USA). The absorbance at 495 nm was measured with a microplate autoreader (Molecular Devices Thermomax Microplate Reader, USA). To define the intra-and inter-assay 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. Zonulin was expressed as ng/mg total protein (as measured by the Bradford method) detected in the tested samples.

Small-Bowel Biopsy

All biopsies were performed by one individual (DRD). Patients were sedated with midazolam and a gastroscopy was performed. Al least four small-bowel biopsies were taken from the descending duodenum and fixed in formalin. They were stained with hematoxylin and eosin stain. All biopsies were read in a blinded fashion by one individual (Dr. A Fasano) according to the modified Marsh criteria [11].

Statistical Analysis

Descriptive statistics were used to analyze the data. The biopsy groups were compared using Student's *t*-test. A *P*-value of <0.05 was accepted as statistically significant.

Results

A total of 22 patients (19 female and three male) completed the study. The mean age was 50.5 years and the mean time on a gluten-free diet was 9.7 years (range 1.3–50 years). One patient was subsequently excluded from further analysis because of an uninterpretable biopsy result (poorly oriented specimen).

Patients were divided into three groups according to their biopsy results. Group 1 had normal biopsies or biopsies that had intestinal epithelial cell lymphocytosis only (Marsh 0 and 1). Group 2 demonstrated crypt hyperplasia consistent with Marsh type 2 lesions and group 3 had histologic characteristics of a Marsh 3 lesion (evidence of villous destruction). All patients stated that they were asymptomatic with respect of celiac disease. It is possible that symptoms were under-reported due to study bias.

Table 1 demonstrates the demographics of the different groups. There were no differences between the groups and 91% of the patients were biopsy-proven celiac disease.

Table 2 summarizes the abnormalities of permeability testing, antibody testing, and zonulin-level testing. There were ten patients who had normal biopsies, negative EMA, and TTG antibodies and a normal intestinal permeability as measured by the lactulose/mannitol ratio. There were six patients who had Marsh type 3 lesions. Only two of these six patients had identifiable transgression on food diary analysis and these two individuals were minimally compliant with a gluten-free diet. These patients had abnormal EMA, TTG, and intestinal permeability. The other four patients had no detectable gluten in their diets but 3/4 had abnormal permeability and 2/4 had an elevated TTG antibody. All patients with Marsh type 3 lesions had either an elevated TTG or intestinal permeability. The three patients with Marsh type 2 lesions did not have abnormal dietary transgressions and only one had abnormal intestinal permeability and none had positive TTG antibodies.

Figure 1 compares the TTG and permeability results of all patients according to small-bowel biopsy. In group 3

Table 1 Patient demographics

	Mean age	Sex	Time on GFD (year)	Diagnosis biopsy	Diagnosis serology
Marsh 0/1	54.9	2M/10F	13.4	10	2
Marsh 2	58.8	3F	4.4	3	0
Marsh 3	53.5	1M/5F	6.0	6	0

Table 2 Comparison of permeability, antibody testing, zonulin level and diet according to biopsy result

Biopsy	*N*	Diet[a]	L/M[b]	EMA[c]	TTG[d]	Zonulin[e]
Marsh 0/1	12	4	2	0	0	9
Marsh 2	3	0	1	0	1	2
Marsh 3	6	2	5	2	4	6

[a] Number of patients whose diet records demonstrated abnormal results

[b] Number of patients with L/M ratio of >0.03

[c] Number of patients with EMA titer of >1:8

[d] Number of patients with TTG > 20 RU/ml

[e] Number of patients with zonulin level of >0.6 mg/ml

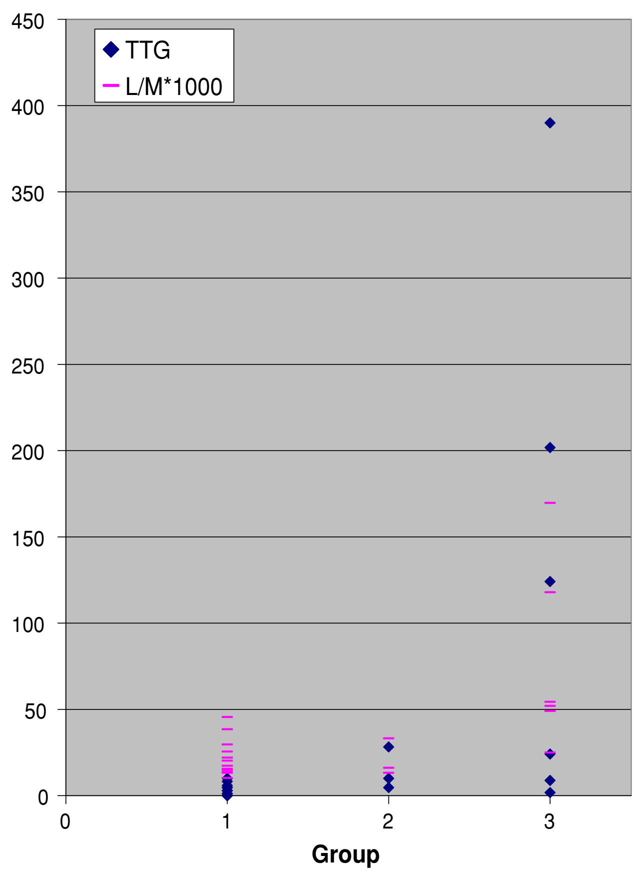

Fig. 1 A comparison of TTG and L/M according to small-bowel biopsy. A TTG of >20 is considered positive and a L/M × 1,000 > 30 is considered abnormal

patients, 4/6 patients had abnormal TTG antibodies while 5/6 had abnormal intestinal permeability. The sensitivity and specificity for TTG in predicting an abnormal biopsy was 56 and 100% while for permeability testing was 67 and 83%.

Serum zonulin did not correlate well with histology and remained elevated in the majority of patients, confirming previous data of a constitutive up-regulation of zonulin expression in celiac disease patients [12]. Figure 2 demonstrates the correlation between serum zonulin level and intestinal permeability as measured by lactulose/mannitol ratio in patients who had Marsh 3 lesions on intestinal biopsies. While serum zonulin did not normalize in many patients, in those who had a destructive intestinal histology, there was a significant relationship between permeability and serum zonulin levels.

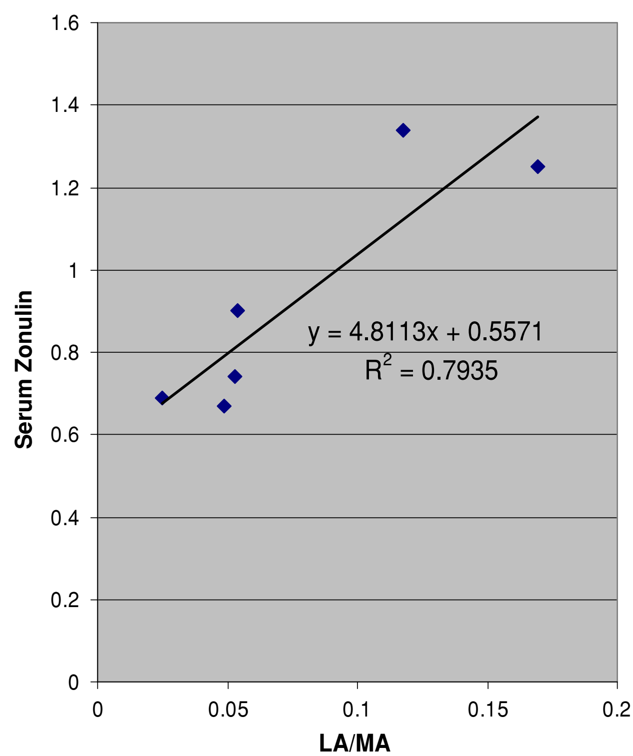

Fig. 2 Correlation of serum zonulin levels and intestinal permeability in patients with Marsh type 3 intestinal histology

Discussion

In this study we found that biopsies normalized in ∼50% of celiac patients on a gluten-free diet and that these patients also had negative celiac antibodies and normal intestinal permeability (10/21 patients). This study supports the theory that adherence to a gluten-free diet results in histologic normalization as well as resolution of immunologic abnormalities and intestinal permeability changes in a subgroup of patients.

In other patients, biopsies may remain abnormal despite resolution of symptoms and apparent compliance to a gluten-free diet. In the individuals with a Marsh 2 or 3 lesions, 7/9 of them had abnormal IP, abnormal TTG, or both. By 3-day food record, only 2/9 were noncompliant with a gluten-free diet according to food-record analysis. This persistent abnormality may be due to low levels of gluten ingestion or to an underlying immunologic up-regulation that persists in some celiac patients. We would hypothesize the former for the following reasons. Dietary analysis in this study was based on a 3-day food record done the week prior to the study. While this gives some information as to the potential gluten content of an individual's diet, a global assessment by a dietitian expert utilizing a clinical interview and food ingredient quiz may be more accurate in determining adherence to a gluten-free diet [13]. Therefore, it is probable that gluten ingestion was underestimated in this study. The most common reason for persistently abnormal symptoms or biopsies is continued ingestion of gluten, which is due to small amounts of gluten [14]. Previous studies have demonstrated that there is a high incidence of gluten ingestion in patients who have been diagnosed with celiac disease and treated with a gluten-free diet [14–16]. The range of noncompliance is from 40 to 60% depending on the level of gluten that is being ingested. The reasons for this include inadvertent gluten ingestion secondary to trace ingestion [17] and ambiguous food labeling, [18] as well as difficulty adapting to the restriction imposed by a gluten-free diet. Many patients have minimal symptoms with gluten ingestion and this compounds the difficulty of maintaining a gluten-free diet [19]. In our study, the 3-day food record may have underestimated the potential for dietary transgressions, which can be inadvertent or intentional. In this study, we did not re-examine compliance in patients who had abnormal studies.

Positive antibodies and abnormal histology have also been found in patients with refractory celiac disease (RCD) particularly RCD I [20]. The patients in this study did not meet the criteria for refractory celiac disease.

Exposure to gluten results in increased zonulin release, which ultimately binds to an apical receptor, and, through a complex series of intracellular events, opens the paracellular space [4]. This results in increased intestinal permeability. The correlation of serum zonulin levels with permeability abnormalities in patients with Marsh 3 mucosal lesions supports this hypothesis. A similar relationship was also demonstrated in patients with type 1 diabetes without significant intestinal mucosa damage [21]. In celiac disease patients with destructive histology, there is a linear correlation between zonulin release and mucosal permeability change. In patients with normal histology or Marsh 1 lesions, zonulin levels may remain elevated and do not correlate with intestinal permeability. While increased intestinal permeability appears to be important in the development of celiac disease, this study demonstrates that permeability can normalize if there is no exposure to gluten. Zonulin levels do not seem to be clinically helpful in following patients with celiac disease.

What are the clinical implications of this study? Although this was a small study, a significant number of individuals continue to have abnormal biopsies despite being on a gluten-free diet for a mean of 9.7 years and do not have any significant symptoms. The persistence of abnormal biopsies on a gluten-free diet has been demonstrated before [22] although the significance of this is uncertain. Intestinal permeability testing and/or TTG antibody testing were abnormal in the majority of patients with Marsh 2 and 3 lesions and all patients with type 3 lesions had either an abnormal permeability test or abnormal TTG. As mentioned above, the most likely explanation for these abnormalities is continued gluten exposure. Currently, many experts recommend that patients who have celiac disease should be followed long term to monitor for complications as well as compliance with a gluten-free diet [18, 23–25]. While detailed dietary analysis may be the most accurate way to detect continued gluten ingestion, this is labor-intensive, and is associated with significant interobserver variability [13]. Previous studies have emphasized the limitations of follow-up antibody testing of patients [7, 26]. It has also been suggested that permeability testing may offer important clinical information in the follow-up of patients with celiac disease [27]. The data from this study suggests that TTG antibodies and intestinal permeability may be useful non-invasive markers to monitor compliance. These markers may identify individuals who require more intensive dietary assessment. Further clinical research is needed to evaluate their role in this regard.

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References

1. Green PH, Cellier C. Celiac disease. *N Engl J Med*. 2007;357:1731–1743. doi:10.1056/NEJMra071600.
2. Sollid LM, Khosla C. Future therapeutic options for celiac disease. *Nat Clin Pract Gastroenterol Hepatol*. 2005;2:140–147. doi:10.1038/ncpgasthep0111.
3. Fasano A, Not T, Wang W, et al. Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. *Lancet*. 2000;355:1518–1519. doi:10.1016/S0140-6736(00)02169-3. (Letter).
4. Arrieta MC, Bistritz L, Meddings JB. Alterations in intestinal permeability. *Gut*. 2006;55:1512–1520. doi:10.1136/gut.2005.085373.
5. Smecuol E, Bai JC, Vazquez H, et al. Gastrointestinal permeability in celiac disease. *Gastroenterology*. 1997;112:1129–1136. doi:10.1016/S0016-5085(97)70123-9.
6. Duerksen DR, Wilhelm-Boyles C, Parry DM. Intestinal permeability in long-term follow-up of patients with celiac disease on a gluten-free diet. *Dig Dis Sci*. 2005;50:785–790. doi:10.1007/s10620-005-2574-0.
7. Vahedi K, Mascart F, Mary JY, et al. Reliability of antitransglutaminase antibodies as predictors of gluten-free diet compliance in adult celiac disease. *Am J Gastroenterol*. 2003;98:1079–1087. doi:10.1111/j.1572-0241.2003.07284.x.
8. Uil JJ, van Elburg RR, Janssens PMW, Mulder CJJ, Heymans HSA. Sensitivity of a hyperosmolar or low-osmolar test solution for sugar absorption in recognizing small intestinal mucosal damage in coeliac disease. *Dig Liver Dis*. 2000;32:195–200. doi:10.1016/S1590-8658(00)80820-8.
9. Mariani P, Viti MG, Montuori M, et al. The gluten-free diet: a nutritional risk factor for adolescents with celiac disease? *J Pediatr Gastroenterol Nutr*. 1998;27:519–523. doi:10.1097/00005176-199811000-00004.
10. El Asmar R, Panigrahi P, Bamford P, et al. Host-dependent zonulin secretion causes the impairment of the small intestine barrier function after bacterial exposure. *Gastroenterology*. 2002;123:1607–1615. doi:10.1053/gast.2002.36578.
11. Ravelli A, Bolognini S, Gambarotti M, Villanacci V. Variability of histologic lesions in relation to biopsy site in gluten-sensitive enteropathy. *Am J Gastroenterol*. 2005;100:177–185. doi:10.1111/j.1572-0241.2005.40669.x.
12. Drago S, El Asmar R, Di Pierro M, et al. Gliadin, zonulin and gut permeability: effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. *Scand J Gastroenterol*. 2006;41:408–419. doi:10.1080/00365520500235334.
13. Leffler DA, Edwards George JB, Dennis M, Cook EF, Schuppan D, Kelly CP. A prospective comparative study of five measures of gluten-free diet adherence in adults with coeliac disease. *Aliment Pharmacol Ther*. 2007;26:1227–1235.
14. Ciacci C, Cirillo M, Cavallaro R, Mazzacca G. Long-term follow-up of celiac adults on gluten-free diet: prevalence and correlates of intestinal damage. *Digestion*. 2002;66:178–185. doi:10.1159/000066757.
15. Fabiani E, Taccari LM, Ratsch IM, Di-Giuseppe S, Coppa GV, Catassi C. Compliance with gluten-free diet in adolescents with screening-detected celiac disease: a 5-year follow-up study. *J Pediatr*. 2000;136:841–843.
16. Bardella MT, Molteni N, Prampolini L, et al. Need for follow up in coeliac disease. *Arch Dis Child*. 1994;70:211–213. doi:10.1136/adc.70.3.211.
17. Faulkner-Hogg KB, Selby WS, Loblay RH. Dietary analysis in symptomatic patients with coeliac disease on a gluten-free diet: the role of trace amounts of gluten and non-gluten food intolerances. *Scand J Gastroenterol*. 1999;34:784–789. doi:10.1080/003655299750025714.
18. Pietzak MM. Follow-up of patients with celiac disease: achieving compliance with treatment. *Gastroenterology*. 2005;128:S135–S141. doi:10.1053/j.gastro.2005.02.025.
19. Brandimarte G, Tursi A, Giorgetti GM. Changing trends in clinical form of celiac disease. Which is now the main form of celiac disease in clinical practice? *Minerva Gastroenterol Dietol*. 2002;48:121–130.
20. Rubio-Tapia A, Kelly DG, Lahr BD, Dogan A, Wu TT, Murray JA. Clinical staging and survival in refractory celiac disease: a single center experience. *Gastroenterology*. 2009;136:99–107. doi:10.1053/j.gastro.2008.10.013.
21. Sapone A, de Magistris L, Pietzak M, et al. Zonulin upregulation is associated with increased gut permeability in subjects with type 1 diabetes and their relatives. *Diabetes*. 2006;55:1443–1449. doi:10.2337/db05-1593.
22. Lee SK, Lo W, Memeo L, Rotterdam H, Green PH. Duodenal histology in patients with celiac disease after treatment with a gluten-free diet. *Gastrointest Endosc*. 2003;57:187–191. doi:10.1067/mge.2003.54.
23. Ciclitira PJ, Johnson MW, Dewar DH, Ellis HJ. The pathogenesis of coeliac disease. *Mol Aspects Med*. 2005;26:421–458. doi:10.1016/j.mam.2005.05.001.
24. Hill ID, Dirks MH, Liptak GS, et al. Guideline for the diagnosis and treatment of celiac disease in children: recommendations of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition. *J Pediatr Gastroenterol Nutr*. 2005;40:1–19. doi:10.1097/00005176-200501000-00001.
25. Rostom A, Murray JA, Kagnoff MF. American Gastroenterological Association (AGA) Institute technical review on the diagnosis and management of celiac disease. *Gastroenterology*. 2006;131:1981–2002. doi:10.1053/j.gastro.2006.10.004.
26. Dickey W, Hughes DF, McMillan SA. Disappearance of endomysial antibodies in treated celiac disease does not indicate histological recovery. *Am J Gastroenterol*. 2000;95:712–714. doi:10.1111/j.1572-0241.2000.01838.x.
27. Vilela EG, Abreu Ferrari ML, Gama Torres HO, et al. Intestinal permeability and antigliadin antibody test for monitoring adult patients with celiac disease. *Dig Dis Sci*. 2007;52:1304–1309. doi:10.1007/s10620-006-9511-8.