

Gender Differences in Serotonin Signaling in Patients with Diarrhea-predominant Irritable Bowel Syndrome

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Abstract

Objective Gender differences, including differences in the prevalence, subtypes and the effectiveness of treatment, are generally recognized in irritable bowel syndrome (IBS). Although serotonin type 3 receptor (5-HT₃R) antagonists appear to be more effective in women with diarrhea predominant IBS (IBS-D) than they are in men, the mechanisms underlying these effects remain unclear. The aim of the present was to investigate the gender differences in 5-HT signaling.

Methods The subjects were selected from outpatients with IBS-D and healthy controls. Their rectal mucosal *S100A*, tryptophan hydroxylase (*TPH*) and 5-HT transporter (5-HTT, SERT, *SLC6A4*) mRNA expression levels were measured. Clinical symptoms were evaluated using the gastrointestinal symptom rating scale (GSRS) and the self-rating depression scale (SDS).

Results The study population of 100 subjects included 47 IBS-D patients and 53 age- and gender-matched healthy controls. The *S100A9* (5.20 vs. 1.90, $p=0.001$) and *SLC6A4* (2.00 vs. 1.00, $p=0.019$) mRNA levels in the rectal mucosa of women with IBS-D were significantly higher than those in men. Among the healthy controls, the *S100A10* expression levels in men were higher than those in women (1.33 vs. 0.82, $p=0.005$). The *S100A8* and *S100A10* expression levels in women with IBS-D were positively correlated with their diarrhea scores ($r=0.55$ and 0.58 , $p<0.05$).

Conclusion 5-HT signaling might be a major contributor to the symptoms of IBS in men, and the differences may be associated with the effectiveness of 5-HT₃R antagonists.

Key words: irritable bowel syndrome, serotonin, serotonin transporter, tryptophan hydroxylase, *S100A10*

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Introduction

Irritable bowel syndrome (IBS) is a functional gastrointestinal (GI) disorder that is commonly seen in gastroenterological practice. A meta-analysis reported that the prevalence of IBS is 10.9% (1). There are several well-known gender differences in IBS and the prevalence of IBS in women is reported to be higher than that in men (14% vs. 8.9%) (1). A meta-analysis to investigate the gender differences in IBS symptoms revealed that women were more likely report to constipation and abdominal pain, while men tended to report diarrhea (2). Diarrhea-predominant IBS

(IBS-D) was more frequently confirmed in men, whereas constipation-predominant IBS (IBS-C) was more frequent in women (3). Studies from western countries showed that alosetron, a 5-hydroxytryptamine (serotonin, 5-HT) type 3 receptor (5-HT₃R) antagonist, seems to be more effective in women (4, 5). The prescription of another 5-HT₃ antagonist, ramosetron, which was developed in Japan, has been limited to men with IBS-D, because the clinical efficacy has only been confirmed in men. However, in a later randomized, placebo-controlled study that included female patients, a half-dose of ramosetron (2.5 μ g per day) reduced the symptoms of IBS and improved the patients' quality of life (QOL) (6). Among Japanese IBS-D patients, women appear

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to require lower doses of 5-HT₃R antagonists than men (6, 7).

5-HT is crucial neurotransmitter that affects both the central nervous system (CNS) and the gut; its signaling plays a pathophysiological role in IBS, especially in IBS-D (8). It has been suggested that the release of 5-HT contributes to the development of abdominal pain and regulates GI motility and secretion (9, 10). The synthesis of 5-HT is regulated by the rate-limiting enzyme tryptophan hydroxylase (TPH). Two isoforms of TPH exist: TPH1 is dominantly expressed in the peripheral organs, especially the gut, and TPH2 is primarily expressed in the CNS and the peripheral serotonergic neurons (11). 5-HT transporters (5-HTT, SERT, *SLC6A4*), which are located in the synaptic cleft, play an important role in regulating serotonergic signaling (12). 5-HTT was detected on the mucosal surface, especially on the epithelial cells of the crypt and the myenteric neurons using immunostaining (12, 13). It was previously reported that the *TPH1* and *SLC6A4* mRNA levels are decreased in the rectal mucosa of IBS patients and that they are associated with diarrhea and constipation symptoms (14).

S100 proteins consist of calcium-binding proteins and are involved in the regulation of the cytoplasmic calcium concentration, signal transduction and various intracellular and extracellular functions (15, 16). Inflammatory and epithelial cells express *S100A8* (also known as calgranulin A; myeloid-related protein 8, MRP8) and *S100A9* (calgranulin B; MRP14). *S100A8* and *S100A9* are abundantly expressed throughout the lamina propria and the epithelial cells in the inflamed mucosa of patients with inflammatory bowel disease (IBD), but not in the non-inflamed mucosa (17, 18). Thus, these proteins and calprotectin (the hetero-complex formed by the non-covalent association of *S100A8* and *S100A9*) are thought to be associated with gut inflammation and the pathogenesis of IBD (19-22). The fecal calprotectin levels are significantly increased in patients with IBD and are correlated with the clinical disease activity (23); this can be used to reliably differentiate between patients with IBD and IBS (24, 25). However, few papers have investigated the association between the mucosal expression of *S100A8* or *S100A9* and IBS (26). *S100A10* (also known as p11) transports members of the voltage-gated sodium and potassium channel families and enhances the functional expression of acid-sensing ion channels (ASICs) (27), which play a key role in the activation and sensitization of the visceral nociceptors (28). *S100A10* interacts and co-localizes with 5-HT_{1B} receptors (29), and its alteration is associated with depression (30). Previous studies evaluating the expression levels of *S100A10* and *SLC6A4* in the rectal and sigmoid mucosa of IBS patients and healthy controls confirmed the significant overexpression of *S100A10* in the rectal mucosa of IBS-D patients (26, 31). However, no studies have so far investigated gender differences in the mucosal expression, and the specific role of 5-HT signaling in the pathophysiology and the gender differences of IBS remain unclear. Thus, to identify whether the expression levels of *TPH1*, *S100A8*,

S100A9, *S100A10* and *SLC6A4* differ between the genders, we measured the mRNA levels in biopsy specimens taken from the rectal mucosa of IBS-D patients and healthy controls.

Materials and Methods

We performed a case control study of IBS-D patients and age- and sex matched healthy controls. This study was approved by Kawasaki Medical School Ethical Committee. Written informed consent was obtained from each of the subjects.

Subjects

Patients with IBS-D were diagnosed with IBS according to the Rome III criteria (32); IBS-D was subtyped if >25% of their stools were loose (mushy) or watery. Subjects with other subtypes of IBS, previous surgery of the GI tract and self-reported organic GI disorders (malignancy, inflammatory bowel disease, peptic ulcer, gall bladder disorder, pancreatitis or liver disease) were excluded.

The enrolled healthy controls were individuals who underwent a routine checkup and patients undergoing colon cancer screening who were positive for fecal occult blood, but otherwise demonstrated no meaningful findings.

Biopsy sample

Experienced GI endoscopists performed colonoscopies. Rectal specimens were taken using endoscopic forceps (FB240U Olympus, Tokyo, Japan), and they were frozen with liquid nitrogen and stored until use at -80°C.

RNA extraction and polymerase chain reaction

RNA extraction and a quantitative mRNA analysis of *S100A8*, *S100A9*, *S100A10*, *TPH1* and *SLC6A4* were performed using a reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR), as previously reported (33).

Questionnaire

The GI symptom rating scale (GSRS) was performed to evaluate the clinical GI symptoms. The test consists of 15 questions on a scale of 1 to 7, and can evaluate the following five major GI symptoms: abdominal pain, reflux, diarrhea, indigestion and constipation (34). The subjective depressive status was assessed using a self-rating depression scale (SDS) that was composed of 20 questions; depression was defined as a total score of >50 (35).

Analysis

Continuous and normally distributed values were expressed as the mean and standard deviation (SD). Categorical data or non-normally distributed continuous values were expressed as counts with the percentage or median and the interquartile range. Normal distribution and homoscedasticity were assessed using the Shapiro-Wilk test and the Levene test. Comparisons between two groups were tested

Table 1. Comparison of Demographic Data, Questionnaire Scores, and mRNA Expressions between the IBS-D Patients and the Healthy Controls.

	Controls (n=53)	IBS-D (n=47)	p
Age mean (SD)	44.1 (16.0)	46.1 (18.3)	0.801 ^a
Gender men (%)	32 (60)	29 (62)	0.892 ^b
SDS scores median (IQR)	34 (31-41)	42.5 (35-51)	<0.001 ^c
GSRs scores median (IQR)			
Reflux	1 (1-1.5)	1.8 (1-3)	0.016 ^c
Abdominal pain	1 (1-1.7)	2 (1.3-3)	<0.001 ^c
Indigestion	1.3 (1-1.5)	2.1 (1.3-2.8)	<0.001 ^c
Diarrhea	1 (1-2)	5.2 (3.7-6)	<0.001 ^c
Constipation	1.3 (1-2)	2.3 (1.8-3)	<0.001 ^c
mRNA expression median (IQR)			
TPH1 $\times 10^{-3}$	5.02 (4.12-8.11)	6.19 (3.72-10.11)	0.532 ^c
<i>S100A8</i> $\times 10^{-5}$	5.50 (2.00-11.50)	3.00 (1.00-9.00)	0.216 ^c
<i>S100A9</i> $\times 10^{-4}$	2.05 (1.30-6.40)	2.80 (1.30-5.50)	0.599 ^c
<i>S100A10</i> $\times 10^{-1}$	1.06 (0.82-1.41)	1.23 (0.99-1.48)	0.148 ^c

IBS-D: diarrhea predominant irritable bowel syndrome, SDS: Self-rating Depression Scale, GSRs: gastrointestinal symptom rating scale

p values calculated using a: unpaired t-test, b: chi-squared test, c: Mann-Whitney U test

Table 2. Gender Differences of Age and Questionnaire Scores in IBS-D Patients.

	Men (n=29)	Women (n=18)	p
Age mean (SD)	46.4 (15.7)	45.6 (22.4)	0.548 ^a
SDS scores	44 (37-50)	42 (36-46)	0.481 ^c
GSRs scores			
Reflux	2 (1-3)	1.5 (1-2)	0.617 ^c
Abdominal pain	1.8 (1.3-2.8)	2.3 (1.5-4)	0.301 ^c
Indigestion	1.5 (1.3-2.1)	2.3 (1.3-3)	0.715 ^c
Diarrhea	5 (3.5-5.8)	5.3 (4-5.8)	0.622 ^c
Constipation	2.3 (1.3-3)	2 (1.8-2.5)	0.571 ^c

IBS-D: diarrhea predominant irritable bowel syndrome, SDS: Self-rating Depression Scale, GSRs: gastrointestinal symptom rating scale

p values calculated using a: unpaired t-test, c: Mann-Whitney U

by the unpaired *t*-test or the non-parametric Mann-Whitney U test (continuous values), and the chi-squared test (categorical values). Spearman's correlation coefficient was calculated to evaluate the correlation. Two-sided p values of <0.05 were considered to indicate statistical significance. All of the statistical analyses were performed using the SPSS software program (version 11 for Windows, SPSS Inc., Chicago, IL, USA).

Results

The study population included 47 IBS-D patients (29 men and 18 women) and 53 controls (32 men and 21 women). The GSRs and SDS scores of the IBS-D patients were significantly higher than those of the controls (Table 1). These scores did not differ to a significant extent between the genders in patients with IBS-D (Table 2).

mRNA expression

The *TPH1*, *S100A8*, *S100A9*, *S100A10* and *SLC6A4* mRNA expression levels in the IBS-D patients and controls did not differ to a statistically significant extent (Table 1). The expression levels of *S100A9* (5.20 vs. 1.90, $p=0.001$) and *SLC6A4* (2.00 vs. 1.00, $p=0.019$) in women with IBS-D were those in men with IBS-D (Fig. 1). The expression levels of *S100A10* in male controls were significantly higher than those in female controls (1.33 vs. 0.82, $p=0.005$) (Fig. 2) (Table 3).

The association between the mRNA expression levels and the clinical scores (Table 4)

There were some significant correlations between the mRNA expression and the GSRs scores in the IBS-D patients. The *S100A8*, *S100A9*, and *S100A10* expression levels were positively correlated with the diarrhea scores in the female patients ($r=0.55$, 0.44, and 0.58) (Fig. 3).

Discussion

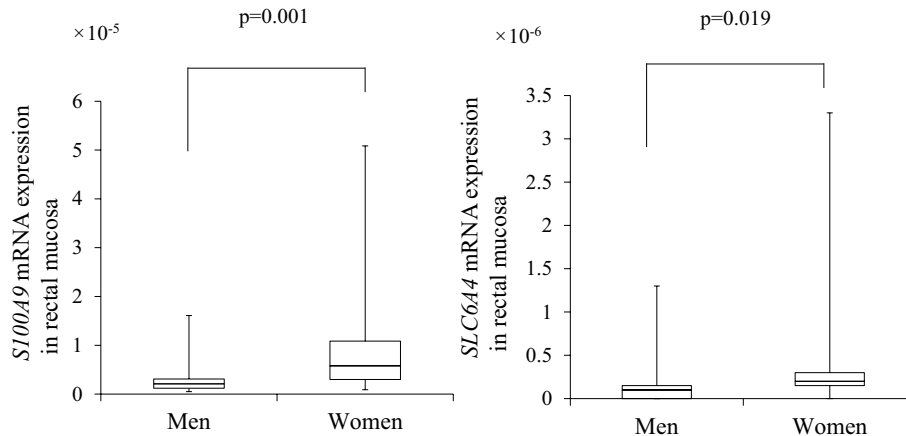
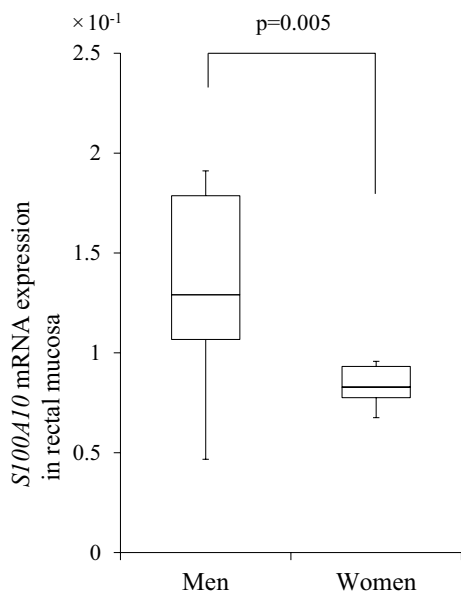
This is the first study to identify a gender difference in the expression levels of *S100A* and *SLC6A4* in the rectal mucosa of IBS-D patients and healthy controls. The median *S100A9* expression level in the female patients was significantly higher than that in the male patients. The association between gut micro-inflammation and the pathogenesis of IBS was previously reported (36). Shulman et al. reported that the gut permeability and an inflammatory status were associated with the severity of GI symptoms in IBS patients (37). Indeed, we demonstrated that *S100A8* was positively correlated with the diarrhea and indigestion scores of the IBS-D patients. This result suggested that micro-

Table 3. Gender Differences of mRNA Expressions in the IBS-D Patients and the Controls.

		IBS-D patients			Controls		
		Men (n=29)	Women (n=18)	p	Men (n=32)	Women (n=21)	p
TPH1	$\times 10^2$	5.89 (2.76-8.70)	6.00 (4.78-8.41)	0.062	6.68 (4.45-9.28)	4.79 (4.01-7.44)	0.596
<i>S100A8</i>	$\times 10^5$	2.00 (0.50-7.50)	5.00 (2.00-2.30)	0.198	5.00 (1.00-13.00)	7.50 (2.50-11.50)	0.487
<i>S100A9</i>	$\times 10^4$	1.90 (1.05-3.00)	5.20 (2.80-12.20)	0.001	1.50 (1.20-7.00)	2.35 (1.40-3.60)	0.818
<i>S100A10</i>	$\times 10^1$	1.22 (0.99-1.44)	1.26 (0.94-1.47)	0.948	1.33 (1.07-1.79)	0.82 (0.76-1.03)	0.005
<i>SLC6A4</i>	$\times 10^5$	1.00 (0-1.50)	2.00 (2.00-3.00)	0.019	-	-	-

IBS-D: diarrhea predominant irritable bowel syndrome

p values calculated using Mann-Whitney U test

**Figure 1.** The gender difference in the expression of *S100A9* and *SLC6A4* mRNA in the rectal mucosa of the IBS-D patients. Horizontal bar: median, Box: interquartile range, Vertical lines: the range of values. The p values were calculated using the non-parametric Mann-Whitney U test.**Figure 2.** The gender difference in the expression of *S100A10* in the rectal mucosa of the healthy controls. Horizontal bar: median, Box: interquartile range, Vertical lines: the range of values. The p values were calculated using the non-parametric Mann-Whitney U test.

inflammation is associated with GI dysfunction. Furthermore, the emergence of IBS after a cured intestinal infection

is known as post-infectious IBS (PI-IBS) (38). Thabane et al identified that women were at greater risk of PI-IBS (39). However, the expression levels of *S100A8* and *S100A9* did not differ between our IBS-D patients and healthy controls, as previously reported (26, 31). Although gut inflammation was thought to be associated with the pathogenesis of IBS, its contribution might be small. In a previous meta-analysis, blood biochemical markers such as C-reactive protein (CRP) were not elevated in IBS patients and none of the biomarkers reliably distinguished between IBS and healthy controls (40). The inflammatory changes are so subtle that it might only be possible to confirm on a microscopic scale (41). Considering our results and our previous research, we assume in Japanese subjects, gut micro-inflammation might make a greater contribution to the pathogenesis of IBS in women than it does in men.

In addition to *S100A9*, we confirmed *SLC6A4* was significantly overexpressed in the rectal mucosa of female patients in comparison to male patients. A decrease in the expression of *SLC6A4* in the intestinal mucosa, resulting in lower *SLC6A4* activity was reported to be associated with the symptoms of diarrhea and constipation (13). Chen et al. (42) reported a high frequency of diarrhea in transgenic mice lacking the *SLC6A4* gene. In practice, the use of selective 5-HT reuptake inhibitors (SSRI) induces diarrhea as an adverse event (43). An SSRI, paroxetine, was also reported to

Table 4. Correlation between mRNA Expressions and Questionnaire Scores in the IBS-D Patients.

	Men					Women				
	<i>TPH1</i>	<i>S100A8</i>	<i>S100A9</i>	<i>S100A10</i>	<i>SLC6A4</i>	<i>TPH1</i>	<i>S100A8</i>	<i>S100A9</i>	<i>S100A10</i>	<i>SLC6A4</i>
SDS	-0.35	-0.13	-0.75	0.06	0.18	0.07	-0.51	-0.28	-0.12	0.31
GSRs scores										
Reflux	-0.89	0.03	0.09	0.23	0.25	0.13	-0.09	-0.09	0.19	0.25
Abdominal pain	0.22	0.35	-0.02	0.02	-0.24	-0.16	-0.15	-0.01	-0.06	0.38
Indigestion	-0.17	0.42*	0.26	0.33	0.26	-0.07	-0.22	-0.22	0.02	0.51
Diarrhea	-0.17	0.01	-0.22	0.02	0.18	0.23	0.55*	0.44	0.58*	0.42
Constipation	-0.13	0.3	0.16	0.12	-0.85	0.41	0.46	0.05	0.19	0.14

IBS-D: diarrhea predominant irritable bowel syndrome, SDS: Self-rating Depression Scale, GSRs: gastrointestinal symptom rating scale

* $p < 0.05$, p values by Spearman's correlation coefficient

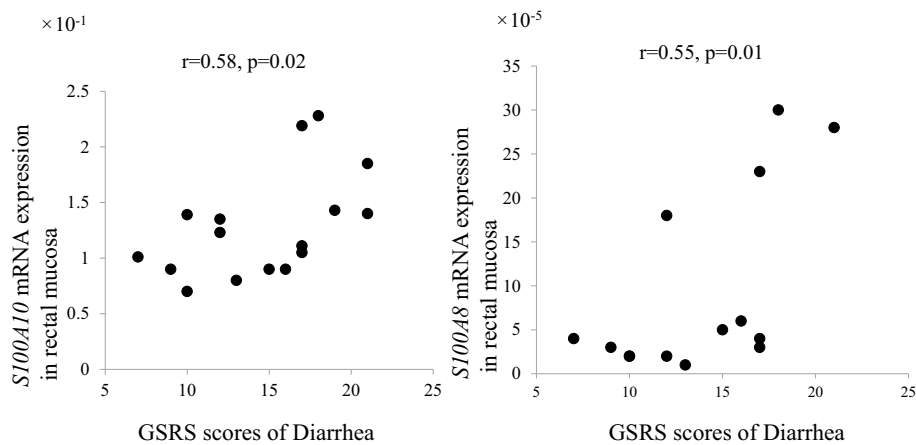


Figure 3. The correlation between the expression of *S100A10* or *S100A8* mRNA and the diarrhea scores of the gastrointestinal symptoms rating scale (GSRs). The p values were calculated using Spearman's correlation coefficient.

increase the jejuna propagation velocity of phase III probably through the enhancement of 5-HT signaling (44). Thus, 5-HTT is thought to be associated with colonic motility and its reduction might lead to the facilitation of 5-HT signaling and impaired motility. The present results regarding the gender difference in the expression of *SLC6A4* suggests that 5-HT signaling was dominant in male patients in comparison to female patients.

Women complain of diarrhea symptoms less frequently than men (45, 46). On a molecular scale, the 5-HT concentration in platelets of women was confirmed to be lower than that in men (47). Interestingly, the median *S100A10* expression level in the rectum of healthy men was significantly higher than that in healthy women. Moreover, the expression of *S100A10* was correlated with diarrhea symptoms in our female patients. This result might indicate that men are generally more susceptible to 5-HT than women; however, there was no gender difference in the rectal expression levels of *S100A10* in IBS-D patients. This trend suggests that *S100A10* may be an important factor, not only in the onset of IBS, but also in the severity of IBS-D. *S100A10* is known to recruit the surface expression of 5HT type 4 receptor (5-HT₄R), leading its increase of concentration at the synapse thereby facilitating 5-HT₄R signaling (48, 49). In clinical

practice, 5-HT₄R agonists are used to promote propulsive motility in the treatment of constipation (50). Hence, *S100A10* seems to be associated with the pathogenesis of IBS-D through hypersensitivity and the enhancement of GI motility; the overexpression of *S100A10* has been confirmed in the rectal mucosa of IBS-D patients (26, 31).

Thus, the basic 5-HT signaling in men with IBS might receive greater stimulation due to the overexpression of *S100A10* and the low expression of *SLC6A4*. This hypothesis is compatible with the fact that women appear to require lower doses of 5-HT₃R antagonists than men (4, 6). In our recent pilot study of biomarkers for predicting the effectiveness of ramosetron, the increased expression of *S100A10* and *TPH1* mRNA as well as *TPH1* promoter and intronic SNPs appeared to predict higher 5-HT signaling, which is correlated with both diarrhea symptoms and the effectiveness of ramosetron in IBS-D patients (51). Moreover, the fact that 5-HT₄ agonists are more effective in women in comparison to men supports our hypothesis (52).

The present study was associated with some limitations. Firstly, the small number of cases might have induced a selection bias. A large-scale multicenter study is needed to minimize the influence of variation in the characteristics of the patients. Previous studies have reported a significant dif-

ference in the number of immune cells in the sigmoid colon and ileum of IBS patients in comparison to healthy controls (53, 54). However, other studies evaluating the expression of *S100A* and *SLC6A4* in the rectal and sigmoid mucosa of IBS patients and healthy controls indicated that the significant overexpression of *S100A10* was only confirmed in the rectal mucosa of IBS-D patients (26, 31). Based on our previous study (26), we selected the rectum as a biopsy site in the present study. The evaluation of the right-side colon or performing biopsies at multiple sites may lead to different results. Another limitation is that our investigation was not based on microarray profiling and thus other genes may be significantly related to micro-inflammation and 5-HT signaling. Although we discussed the possible association between micro-inflammation and the pathogenesis of IBS, we did not confirm whether our subjects had a history of infectious colitis. This important factor should be identified in our next study. In addition, various other factors that influence the symptoms of IBS, such as ovarian hormones (55), interpersonal relationships (56), the autonomic nervous system and brain processing (include stress vulnerability) (57), might induce gender differences. However, the associations between these factors and the pathogenesis of IBS remain unclear. In particular, the effect of the menstrual cycle on the symptoms of IBS is inadequately investigated in systemic reviews, and retrospective studies may overestimate the effect of the menstrual cycle on the symptoms of IBS and are less accurate than prospective assessments (2). Well-designed large-scale studies will be necessary to reveal the factors contributing to the clinical symptoms in IBS-D patients.

In conclusion, we identified gender differences in the expression of *S100A* and *SLC6A4* in the rectal mucosa of IBS patients and healthy controls. Enhanced 5-HT signaling seems to make a greater contribution to the IBS symptoms of men, and the differences may be associated with the effectiveness of 5-HT₃R antagonists. These gender differences may lead to a further understanding of the pathogenesis of IBS.

The authors state that they have no Conflict of Interest (COI).

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