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Platelet Serotonin Transporter in Patients With Diarrhea-Predominant Irritable Bowel Syndrome Both Before and After Treatment With Alosetron

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OBJECTIVES: Serotonin reuptake is mediated by a transporter protein (SERT), and its dysfunctions can alter serotonergic transmission. The present study examines the binding profile of platelet SERT in healthy volunteers as well as in patients with diarrhea-predominant irritable bowel syndrome (D-IBS), both before and after treatment with the 5-HT_3 receptor antagonist alosetron.

METHODS: Binding of [^{3}H]paroxetine to SERT was assayed in platelet membranes collected from D-IBS patients (12 women, age 21–73 yr) and healthy volunteers (12 women, age 24–68 yr). Both maximal binding capacity (B_{max}) and dissociation constant (K_d) were estimated. In D-IBS patients, binding parameters and symptom severity score were evaluated at baseline and after treatment with alosetron (1 mg *b.i.d.* for 8 wk).

RESULTS: At baseline, B_{max} and K_d values of [^{3}H]paroxetine binding were respectively lower and higher in D-IBS patients than in healthy volunteers (B_{max}: 518.7 ± 155.9 *vs* 1151.9 ± 187.4 fmol/mg, $p < 0.001$; K_d: 0.19 ± 0.05 *vs* 0.06 ± 0.02 nmol/L, $p < 0.001$). Symptom severity score in D-IBS patients (50.9 ± 18.8) was negatively correlated with B_{max} ($r = -0.964$; $p < 0.001$) but not K_d values ($r = -0.164$; $p = 0.609$). After treatment with alosetron, symptom severity score decreased significantly (14.4 ± 3.7; $p < 0.001$), whereas B_{max} (522.7 ± 39.7 fmol/mg) and K_d values (0.17 ± 0.07 nmol/L) did not change.

CONCLUSIONS: The present results indicate that SERT expressed on platelet membranes of D-IBS patients is characterized by low density and binding affinity and suggest a possible correlation between the reduced capacity of serotonin reuptake and the severity of D-IBS symptoms. (Am J Gastroenterol 2003;98:2705–2711. © 2003 by Am. Coll. of Gastroenterology)

INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional disorder characterized by abdominal discomfort, pain and changes in bowel habits (constipation or diarrhea), with a significant impact on the quality of life and relevant health care costs (1). This digestive disorder has an incidence of 15–20% among adults in developed countries and occurs with higher frequency among women (2). Diarrhea-predominant IBS (D-IBS) is often associated with accelerated small bowel or colonic transit and with rectal hypersensitivity (3).

At present, the mechanisms underlying the pathophysiology of IBS have not been clarified (1). In recent years, however, there has been a growing interest in a possible involvement of serotonin (5-HT) in this syndrome. Indeed, strong evidence has been provided that 5-HT, acting as a mediator in the digestive tract, participates in mucosal sensory transduction processes, plays a modulating role in visceral sensation, and regulates both peristaltic and secretory reflexes (4, 5).

As a mediator 5-HT exerts its actions by means of interaction with distinct receptors, which are differentiated on the basis of structures, molecular mechanisms, and pharmacological profiles (6). Most of 5-HT receptor subtypes are expressed throughout the gut and regulate a variety of digestive functions. On this basis, drugs acting on 5-HT receptors are currently being used or investigated for the clinical management of different digestive disorders, including nausea, gastroesophageal reflux, noncardiac chest pain, functional dyspepsia, and IBS (7). Among drugs targeting 5-HT receptors, alosetron is a selective 5-HT_3 receptor antagonist, which can inhibit 5-HT-mediated excitation of extrinsic sensory nerves and may slow colonic transit in patients with D-IBS (8, 9). Accordingly, clinical studies have demonstrated the efficacy of alosetron in promoting the relief of abdominal discomfort and pain, as well as in

decreasing stool frequency and improving consistency and urgency in these patients (10, 11).

Analogous to other transmitters, the endogenous activity of 5-HT is controlled by a specific 5-HT transporter (SERT), which mediates the intracellular reuptake of 5-HT and can be specifically blocked by selective 5-HT (serotonin) reuptake inhibitors (SSRIs), such as paroxetine and fluoxetine (12). SERT is widely expressed in intestinal epithelial cells, central or peripheral serotonergic neurons, and platelets, where it shares common molecular and physiological features (12, 13). There is also evidence suggesting that changes in the expression or pharmacological profile of SERT can be associated with dysfunctions of central serotonergic transmission in different disorders, including depression and migraine (14, 15).

As far as the digestive tract is concerned, although the role of 5-HT in IBS is being extensively investigated (16, 17), very few studies have examined a possible involvement of SERT in the pathophysiology of this syndrome (18, 19). In the present study, both the density and binding affinity of SERT were determined in platelets collected from D-IBS patients, for the following reasons: 1) to perform a comparison with the pharmacologic profile of platelet SERT in healthy volunteers and 2) to examine putative correlations of SERT characteristics with age, severity of symptoms, or alosetron treatment.

MATERIALS AND METHODS

Study Population

Twelve consecutive female patients who were aged 21–73 yr (median 37.5 yr) and were affected by D-IBS were included in the present study. Enrolled patients met the Rome II criteria for IBS (20). Organic diseases were ruled out in all patients by routine physical examination, blood chemistry, hematology, urine analysis, and total colonoscopy. Subjects under treatment with drugs known to interact with central or peripheral serotonergic pathways were excluded. Enrolled patients were required to discontinue drug treatments for IBS at least 14 days before entering the trial. For comparison, 12 healthy volunteers matched for sex and age (age range 24–68 yr, median 37 yr), were also included in the study. The investigation was approved by the local University Hospital Ethics Committee and was carried out in accordance with the Helsinki Declaration revised in Edinburgh in 2000. Written informed consent was obtained from all subjects before entry into the trial.

Study Design

The pharmacologic characterization of platelet SERT was carried out in patients with D-IBS as well as in healthy volunteers. In the former group, platelets were collected both at baseline and at the end of a treatment course with alosetron, given at the dose of 1 mg *b.i.d. p.o.* for 8 wk. The severity of IBS symptoms was evaluated at baseline and at end of alosetron treatment by a specific questionnaire adapted from Francis *et al.* (21). Although this questionnaire has not been presently validated, according to Bijkerk *et al.* (22), it shows good psychometric and methodologic qualities, and it seems particularly suitable to obtain adequate information on specific IBS symptoms. The questionnaire is based on score values ranging from zero to 96. In particular, the following symptoms were evaluated: abdominal pain, abdominal bloating, changes in bowel habits, change in symptoms with evacuation or flatus or both, urgency at defecation, and passing mucus during a bowel movement. The frequency was scored as 0 = never; 1 = rare (once/wk); 2 = occasional (two to three times/wk); 3 = recurrent (four to six times/wk); and 4 = extremely recurrent (seven times/wk). The severity was scored as: 0 = absent; 1 = light (not affecting usual activities); 2 = moderate (lightly affecting usual activities); 3 = severe (strongly affecting usual activities); and 4 = extremely severe (rest in bed).

Preparation of Platelet Membranes and [^{3}H]paroxetine Binding Assay

A quantity of 25 ml of blood was withdrawn from an antecubital vein into plastic tubes containing 5 ml of anticoagulant (sodium citrate, 2.2%, and citric acid, 1.2%). Platelet-rich plasma was obtained by low-speed centrifugation (1,500 *g* for 15 min at 23°C). Platelets were precipitated from platelet-rich plasma by centrifugation at 1500 *g* for 15 min at 23°C and then stored at −80°C until assay, which was performed within 1 wk. At the time of assay, platelets were washed in 10 ml of ice-cold 50 mmol/L Tris-HCl buffer (pH 7.4) containing 150 mmol/L of NaCl and 20 mmol/L of ethylenediaminetetraacetate and were centrifuged at 10,000 *g* for 10 min at 4°C. The resulting pellet was homogenized by an Ultraturrax homogenizer in 10 volumes of 5 mmol/L of Tris-HCl buffer (pH 7.4) containing 5 mmol/L ethylenediaminetetraacetate and were centrifuged at 30,000 *g* for 10 min at 4°C. The supernatant was discarded, and the final membrane pellet was resuspended in 10 ml of 50 mmol/L Tris-HCl buffer (pH 7.4), containing 120 mmol/L NaCl and 5 mmol/L KCl, and centrifuged as reported above.

The binding of [^{3}H]paroxetine to SERT on platelet membranes was determined according to the method of Marazziti *et al.* (23). The [^{3}H]paroxetine binding assays were performed in an incubation mixture consisting of 100 μl of platelet membranes (50–100 μg protein/tube), 50 μl of [^{3}H]paroxetine (Perkin-Elmer, Life Science, Milan, Italy; specific activity: 15.5 Ci/mmol) at concentrations ranging from 0.01 to 1 nmol/L, and 1850 μl of assay buffer. Specific binding was estimated as the binding remaining in the presence of 10 μmol/L fluoxetine (kindly provided by Eli-Lilly, Indianapolis, IN), used as the unlabeled competitor for SERT binding site. All samples were assayed in duplicate and incubated at 22°C for 1 h. The reaction was then halted by adding 5 ml of cold assay buffer, followed by immediate filtration under vacuum through glass fiber filters GF/C of 2.5 cm in diameter (Whatman International, Maidstone,

Table 1. Age, Binding Parameters of [^{3}H]paroxetine to Platelet SERT, and Symptom Score Values of D-IBS Patients at Baseline and at End of Treatment With Alosetron

		B_{max} (fmol/mg)		K_d (nmol/L)		Score	
Patient No.	Age (yr)	T0	T1	T0	T1	T0	T1
1	31	701.1	572.3	0.19	0.26	30	14
2	73	380.3	461.3	0.24	0.15	70	18
3	33	624.2	548.1	0.17	0.12	40	19
4	61	801.7	593.2	0.25	0.24	20	8
5	23	644.9	547.7	0.17	0.24	44	20
6	41	366.3	525.6	0.27	0.32	70	13
7	21	412.3	482.5	0.13	0.09	60	11
8	22	421.4	486.4	0.19	0.14	56	16
9	34	570.7	490.3	0.28	0.19	36	10
10	65	342.7	502.2	0.16	0.12	78	12
11	45	352.6	518.7	0.14	0.10	70	16
12	44	596.5	544.1	0.13	0.09	37	16

T0 = B_{max} and K_d values before treatment. T1 = B_{max} and K_d values after treatment.

UK). The filters were washed three times with 5 ml of the assay buffer and dried, and the SERT-bound radioactivity trapped on the filters was counted in 4 ml of scintillation fluid by a scintillation spectrometry counter 1600 TR (Packard Bioscience, Groningen, The Netherlands). Protein concentration was determined according to the method of Lowry, as modified by Peterson (24).

Evaluation of Binding Parameters

Equilibrium-saturation binding data, the maximal binding capacity (B_{max}, fmol/mg protein), and the dissociation constant (K_d, nmol/L) were estimated by means of iterative curve-fitting computer programs EBDA and LIGAND (Biosoft, Cambridge, U.K.) (25). It may be noted here that radioligand binding experiments allow direct measurements of the interactions between drugs and their receptors. The technique is based on the assumptions that 1) the drug interacts reversibly with a single molecular site on its receptor and 2) the formation of the drug-receptor complex obeys the law of mass action. Under these circumstances, the relationship between drug concentration and receptor occupancy at the equilibrium is described by the Hill-Langmuir equation: $B = B_{max} \times F / (F + K_d)$, where B is the drug amount bound to receptors, B_{max} is the maximal binding capacity, F is the free drug concentration, and K_d is the dissociation constant. A plot of B/F (ordinate) against B (abscissa) generates a straight line, designated as a Scatchard plot, where B_{max} and K_d correspond to the abscissa intercept and the reciprocal of slope, respectively. B_{max} provides an estimate of the total number of binding sites expressed on the cell membrane preparation. K_d is numerically equal to the concentration of drug required to occupy 50% of receptors at equilibrium; it represents an estimate of drug affinity for its receptor inasmuch as, the higher the affinity, the lower the K_d value (26). Accordingly, in the present study, a reduction in B_{max} values was assumed as a lowered expression of the SERT protein on platelet membranes, whereas an increase in K_d values was taken as an index of decreased affinity of the SERT binding site for its ligand.

Statistical Analysis

Data are expressed as mean ± SD. Statistical analysis was carried out using the Spearman rank test and the Mann-Whitney test. Values of $p < 0.05$ were considered to be significant.

RESULTS

At baseline, mean B_{max} and K_d values of [^{3}H]paroxetine binding in D-IBS patients accounted for 518.7 ± 155.9 fmol/mg and 0.19 ± 0.05 nmol/L, respectively (Table 1). A comparison of these parameters with those in healthy volunteers (Table 2) indicated significant differences: the mean B_{max} value was significantly higher in healthy volunteers (1151.9 ± 187.4 fmol/mg; $p < 0.001$), whereas opposite results were obtained with regard to mean K_d (0.06 ± 0.02 nmol/L; $p < 0.001$). Figure 1 displays representative Scatchard plots obtained for patient 3 and healthy volunteer 2. Age did not seem to influence the binding parameters of platelet SERT in either D-IBS patients or healthy volunteers. Indeed, a lack of significant correlation was found between age and B_{max} or K_d values in D-IBS patients (B_{max}: $r = -0.167$, $p = 0.603$; K_d: $r = 0.270$, $p = 0.394$) as well

Table 2. Age and Binding Parameters of [^{3}H]paroxetine to Platelet SERT in Healthy Volunteers

Patient No.	Age (yr)	B_{max} (fmol/mg)	K_d (nmol/L)
1	26	1231.2	0.05
2	68	1215.3	0.07
3	36	1315.7	0.06
4	64	850.2	0.06
5	28	1201.6	0.04
6	38	1261.3	0.09
7	24	1185.3	0.08
8	27	1230.8	0.03
9	30	1330.2	0.03
10	60	1300.1	0.07
11	50	825.4	0.05
12	39	875.7	0.09

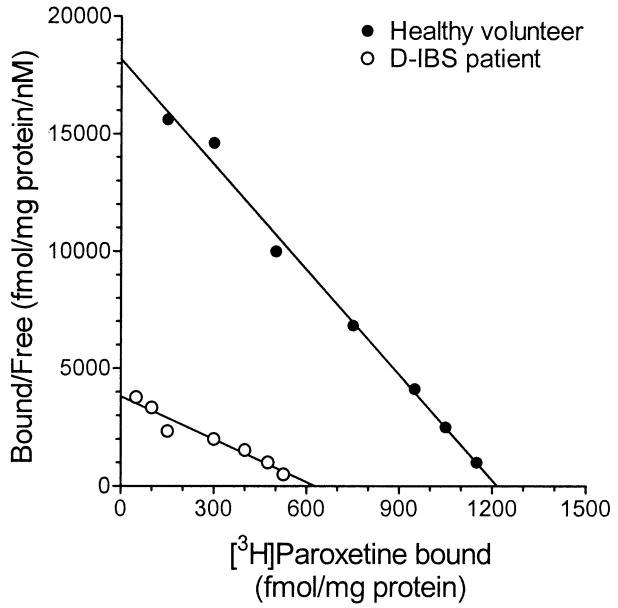

Figure 1. Representative Scatchard plots obtained for binding assay of [^{3}H]paroxetine to platelet SERT of patient 3 and healthy volunteer 2.

as in healthy volunteers (B_{max}: $r = -0.340$, $p = 0.263$; K_d: $r = 0.286$, $p = 0.365$).

The evaluation of symptom severity at baseline in D-IBS patients yielded score values ranging from 30 to 78, with a mean value of 50.9 ± 18.8. At this time, the severity of symptoms was shown to be strictly related to the maximal binding capacity of platelet SERT, inasmuch as the statistical analysis showed a marked negative correlation between symptom scores and the respective B_{max} values ($r = -0.964$, $p < 0.001$) (Fig. 2A). By contrast, no significant correlation was found when comparing symptom severity with K_d values ($r = -0.164$, $p = 0.609$) (Fig. 2B).

At the end of the 8-wk treatment with alosetron, all patients reported consistent relief of their symptoms (Table 1). Accordingly, the mean score value for symptom severity at the end of therapy was significantly lower than that recorded at baseline (14.4 ± 3.7 *vs* 50.9 ± 18.8, $p < 0.0001$). Mean B_{max} and K_d values after alosetron treatment accounted for 522.7 ± 39.7 fmol/mg and 0.17 ± 0.07 nmol/L, respectively; these values were not significantly different from baseline (Table 1). Furthermore, at the end of therapy no significant correlation was found between the binding parameters of [^{3}H]paroxetine and the score values of symptom severity (B_{max}: $r = -0.086$, $p = 0.789$; K_d: $r = -0.187$, $p = 0.560$).

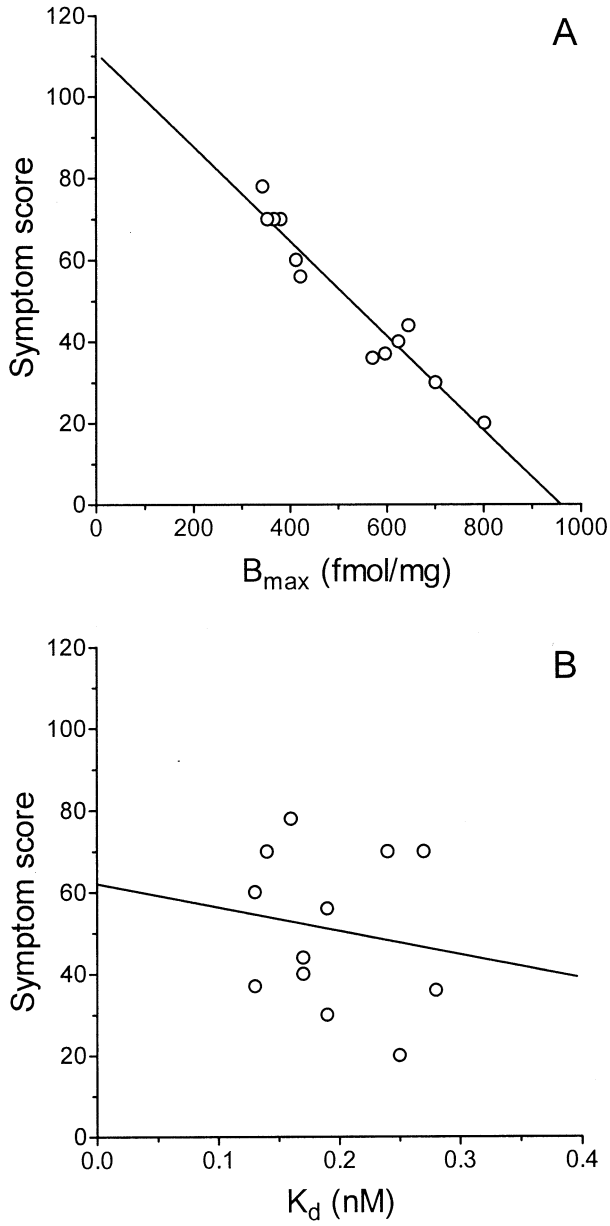

Figure 2. Linear correlation analysis between B_{max} (*A*) or K_d (*B*) values of [^{3}H]paroxetine binding to platelet SERT and symptom score values in D-IBS patients at baseline.

DISCUSSION

Receptor actions of 5-HT are influenced by its reuptake into serotonergic neurons or enterocytes, a process that is regulated by a specific SERT protein (12). Previous studies have demonstrated that the same SERT is expressed in the central nervous system, platelets, and digestive tract (12, 13). Possible implications of SERT in central nervous system disorders have been previously investigated (14, 15); more recently it has been proposed that altered expression of SERT may influence the symptom pattern or therapeutic response to alosetron in IBS patients (18). These findings prompted us to investigate the pharmacologic characteristics of SERT in platelets of D-IBS patients. B_{max} and K_d values of [^{3}H]paroxetine binding were assumed as estimates of SERT density and ligand binding affinity, respectively, and the most interesting observation was that both parameters differed significantly from those evaluated in healthy vol-

unteers. Although this observation was made in a small number of D-IBS patients and the study was performed exclusively in women, it is likely that the homogeneity of this group enabled us to detect significant changes in the kinetic properties of platelet SERT.

In the present investigation, SERT was found to be expressed on platelet membranes of D-IBS patients at a low density (decreased B_{max}) as well as to display a low degree of affinity (increased K_d) at its ligand binding site. In accordance with the general receptor theory (26), such combined changes in B_{max} and K_d values allow the inference that the efficiency of 5-HT uptake by platelet SERT is globally reduced. Because the SERT protein displays the same molecular properties at all known cellular locations (12, 13), it is conceivable that similar alterations in the 5-HT uptake efficiency may also occur at the intestinal level. According to this hypothesis, more 5-HT would be left in the synaptic space, not being subjected to reuptake into the presynaptic compartment and therefore remaining available for binding to postsynaptic receptors at overphysiologic concentrations for extended periods of time. Under these circumstances, it is expectable that changes in bowel motility, secretion, and sensitivity, which are responsible for the characteristic clinical pattern of D-IBS, reflect an increased intestinal availability of 5-HT and that the severity of symptoms might be related to the degree of altered 5-HT uptake. Consistent with this suggestion, in the present study the correlation analysis showed that the lower the density of SERT on platelet membranes, the higher the severity of D-IBS symptoms. Clearly, more extensive clinical studies, based on appropriate disease controls and including subjects with constipation-predominant IBS and other lower functional GI diseases, are needed to substantiate the hypotheses generated in the present investigation. On the other hand, although reliable preclinical models of IBS are currently lacking, it is noteworthy that in guinea pigs with experimental colitis a concomitant increment of 5-HT availability and decrease in mRNA SERT expression was detected in the inflamed colonic mucosa (27). Moreover, preliminary clinical evidence has been recently reported to suggest that similar alterations might also occur in patients with either IBS or inflammatory bowel disease (28).

A reduced uptake capacity is consistent with previous observations indicating that abnormal increments of postprandial 5-HT plasma levels occur in D-IBS patients (16). In addition, an excessive stimulation of peripheral 5-HT receptors would account for the onset of abdominal discomfort, pain, and diarrhea and might explain why the clinical use of 5-HT_3 receptor antagonists such as alosetron can promote the relief of disturbances associated with D-IBS (1, 7). The present results are also in keeping with data obtained from preclinical models showing that the inhibition of mucosal 5-HT uptake by pharmacologic blockade of SERT enhances the recruitment of submucosal primary afferent neurons, which initiate peristaltic and secretory reflexes in response to the mechanic stimulation of intestinal mucosa (5, 12). Moreover, targeted deletion of the SERT gene in transgenic mice resulted in an initial increment of intestinal motility with diarrhea, followed by a later onset of constipation (29).

Abnormal patterns of density or affinity for platelet SERT have been previously detected in patients with neurologic or psychiatric disorders. For instance, studies based on binding or immunoblotting assays showed a decreased expression of platelet SERT in patients with major depression and somatoform disorders (30, 31). On the other hand, other pathologic conditions, including schizophrenia and migraine, seem to be associated with increments of platelet SERT density (32, 33). In these cases, changes in the number of platelet SERT are unlikely to reflect the primary pathogenetic mechanisms accounting for the underlying disease, and they are instead regarded as nonspecific indexes of presynaptic serotonergic dysfunctions (34). In line with this view, the present results, indicating a decrease in both density and binding affinity of platelet SERT in D-IBS patients, do not allow us to hypothesize a specific role for intestinal SERT in the pathogenesis of D-IBS. However, we could observe a significant correlation between the altered binding profile of platelet SERT and the symptom severity, and therefore it is conceivable that a concomitant dysfunction of intestinal SERT might contribute to the severity of clinical picture in patients with D-IBS.

Beside its functions at the digestive level, SERT is regarded as a fine modulator of serotonergic neurotransmission in the central nervous system, and it is abundantly expressed in the cortical and limbic areas of the brain, which are involved in the emotional aspects of behavior (35). Studies performed by positron imaging tomography have suggested that these brain regions are malfunctioning in IBS patients and represent important target sites for the therapeutic action of alosetron in D-IBS (36, 37). Based on these findings, it should be considered that the altered binding profile of platelet SERT in D-IBS patients might also reflect a dysfunction of neuronal SERT in brain areas implicated in the pathophysiology of D-IBS symptoms.

Previous investigations have described a polymorphism of SERT gene, which gives rise to a short (S) and a long (L) allele, with S/S and L/S genotypes resulting in a reduced SERT protein expression and a less efficient 5-HT reuptake. Recently, Pata *et al.* (19), who examined a possible association between SERT polymorphisms and the different clinical patterns of IBS, observed that the L/S genotype was present in 18 D-IBS patients with a frequency of 88%. However, Camilleri *et al.* (18), in a study aiming to assess the influence of SERT polymorphisms on colonic transit in response to alosetron, found a different frequency (48%) for the L/S genotype. Analogously, Kim *et al.* (38) reported a prevalence of 50% for the L/S genotype in 100 D-IBS patients. Overall, the distribution of the L/S genotype seems to be largely heterogeneous in different populations, and on this basis, the potential role of this SERT polymorphism in the pathophysiology of IBS symptoms remains unclear.

In the present study, it was also observed that after 8 wk of treatment with alosetron, all patients reported a global improvement of their symptoms. Although this is an open label, nonrandomized study, our results are in accordance with data obtained in larger randomized, double-blind trials (11). After alosetron treatment, the binding parameters of platelet SERT remained unchanged with respect to baseline values. This observation was not unexpected, inasmuch as alosetron does not act on SERT and platelets do not express 5-HT_3 receptors, and it does not exclude the possibility that individual differences in the function of intestinal SERT may determine the therapeutic response to alosetron. However, it should be noted that clinical data relating the binding capacity of intestinal SERT to the outcome of alosetron treatment are currently lacking. Furthermore, in a previous study, Camilleri *et al.* (18) made the matter more intriguing by reporting that alosetron reduced the colonic transit with the lowest efficacy in D-IBS patients with L/S genotype. This finding would allow us to hypothesize that an overstimulation of 5-HT postsynaptic receptors, caused by an excess of 5-HT left in the synaptic space, might lead to their desensitisation. Clearly, additional studies specifically designed to correlate SERT gene polymorphisms, the binding profile of intestinal SERT, clinical manifestations of IBS, and the therapeutic response to pharmacological treatments in extended numbers of patients are needed to clarify this complex matter.

In conclusion, the results obtained in the present study indicate that SERT expressed on platelet membranes of D-IBS patients is characterized by low density and binding affinity and suggest a possible correlation between the reduced capacity of 5-HT reuptake and the severity of D-IBS symptoms.

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