5-HTTLPR and *STin2* polymorphisms in the serotonin transporter gene and irritable bowel syndrome: effect of bowel habit and sex

Beate Niesler[a], Johannes Kapeller[a], Catherine Fell[b], Wendy Atkinson[b], Dorothee Möller[a], Christine Fischer[c], Peter Whorwell[b] and Lesley A. Houghton[b]

Background **Conflicting data exist on the association between functional polymorphisms in the serotonin reuptake transporter (*SERT*) gene (*SLC6A4*) and irritable bowel syndrome (IBS). This may be partly because of small participant numbers and varying ethnic origin and sex within the cohorts studied.**

Aim **To reassess the potential association between the *SERT* polymorphisms *5-HTTLPR* and *STin2* in both male and female IBS patients with diarrhoea (IBS-D) and constipation (IBS-C) compared with healthy volunteers.**

Methods **In this case–control study, 196 Caucasian Rome II IBS patients [97 IBS-D (aged 18–66 years; 67 female) and 99 IBS-C (aged 18–65 years; 95 female)] and 92 Caucasian healthy volunteers (aged 18–63 years; 60 female) from the UK had genomic DNA extracted from peripheral blood and the *5-HTTLPR* and *STin2* polymorphisms genotyped.**

Results **The frequency of the *5-HTTLPR* (ss) genotype was slightly lower in both IBS-D (16.5%) and IBS-C (14.3%) patients compared with controls (23.9%), although not significantly ($P \leq 0.191$). This seemed to be related to a reduction in the frequency of the *5-HTTLPR* (ss) genotype in male patients, particularly those with IBS-D [IBS-D 10%, IBS-C 25%, controls 37.5%; $P = 0.01$ for IBS-D vs. controls; odds ratio (95% confidence interval) for *5-HTTLPR* (ss) vs. *5-HTTLPR* (non-ss) = 0.185 (0.046–0.744)] than in female patients (IBS-D 19.4%, IBS-C 13.8%, controls 16.7%). There were no differences in the frequencies of either the *5-HTTLPR* (ll) or (ls), or *STin2* genotypes between any of the three groups.**

Conclusion **Our finding that male IBS-D patients have a reduced frequency of the *5-HTTLPR* (ss) genotype contradicts three earlier studies of a similar size, which did not take sex into account. Therefore, replication studies in even larger cohorts, stratifying for sex and endophenotypes, after assessing physiological and psychological traits, are required to unravel the contribution of *SERT* polymorphisms to the IBS phenotype.** *Eur J Gastroenterol Hepatol* **22:856–861 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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[a]Department of Human Molecular Genetics, University of Heidelberg, Heidelberg, Germany, [b]Neurogastroenterology Unit, School of Translational Medicine – GI Sciences, University of Manchester, Wythenshawe Hospital, Manchester, UK and [c]Institute of Human Genetics, University of Heidelberg, Heidelberg, Germany

Correspondence to Dr Lesley A. Houghton, Neurogastroenterology Unit, School of Translational Medicine – GI Sciences, University of Manchester, Wythenshawe Hospital, Southmoor Road, Manchester M23 9LT, UK
Tel: +44 161 291 4186; fax: +44 161 291 4184;
e-mail: Lesley.Houghton@manchester.ac.uk

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Introduction

Irritable bowel syndrome (IBS) is a sensorimotor disorder, which affects twice as many women as men [1,2]. Aberrant serotoninergic signalling is thought to play a key role in this sensorimotor dysfunction [3–5]. Serotonin (5-hydroxytryptamine, 5-HT) is located in the enterochromaffin cells of the epithelium of the gut, and to a lesser extent, in enteric neurons of the submucosal and myenteric plexuses. Passage of food through the intestine leads to 5-HT release, and activation of the intrinsic primary afferent neurons and extrinsic sensory neurons modulating gastrointestinal function. 5-HT also enters the circulation from where it activates distant targets [3–5]. In patients with IBS, platelet depleted plasma 5-HT concentration has been shown to be abnormal, with IBS patients with diarrhoea (IBS-D) having elevated [6,7] and IBS patients with constipation (IBS-C) [6,8] reduced concentrations compared with healthy volunteers.

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The additional observations that the postprandial relative to fasting 5-HT concentration (i.e. the increase with meal ingestion) is no different between IBS-D patients and healthy volunteers, and that the ratio of 5-hydroxyindoleacetic acid (5-HIAA), a metabolite of 5-HT, to 5-HT is significantly reduced compared with healthy controls, however, would suggest that these patients may have a disorder of metabolism and/or reuptake rather than synthesis and/or release of 5-HT [6]. Interestingly, plasma 5-HT concentration directly correlates with colonic motility [9], possibly explaining, at least in part, the increased and decreased motility reported in IBS-D and IBS-C patients, respectively, compared with healthy volunteers [2].

Inactivation of 5-HT and prevention of overstimulation and/or desensitization of the sensorimotor response is mediated by the serotonin reuptake transporter (SERT) protein, also known as the 5-HT transporter (5-HTT) or solute carrier family 6 member 4 (SLC6A4) [10]. The gene encoding SERT, *SLC6A4* (GenBank NM_001045), resides on chromosome 17q11 within the human genome, spanning more than 30 kb, consisting of 14 exons and encoding a 630-amino acid protein [11]. To date, two polymorphisms have mainly been investigated: (i) the serotonin transporter gene-linked polymorphic region *5-HTTLPR*, which is characterized by a 44 bp insertion within the *SERT* promoter, resulting in two different *5-HTTLPR* alleles – the long (l) and the short (s) variant; and (ii) a variable number of 17-bp tandem repeats located in intron 2 (*SERT STin2*), of which the four alleles, 9, 10, 11 and 12, have been described [11,12]. The presence of the short allele within the genotype (sl or ss) of *5-HTTLPR* has been shown to be associated with lower transcriptional activity, and thus lower levels of *SERT* mRNA and consequently 5-HT reuptake [11–14]. Moreover, the haplotype harbouring the short allele of *5-HTTLPR* plus the 12-repeat allele of *SERT STin2* (s12) has been shown to have stronger enhancer-like properties on transcription of the *SERT* gene than the haplotype with the 10-repeat allele (haplotype s10) [15,16].

To date, seven studies have investigated whether there is an association between IBS and *5-HTTLPR* polymorphisms [17–23], with three of these including the *STin2* polymorphism [19,21,23]. In case of the *STin2* polymorphism, no association has been found, whereas contradictory data exist for polymorphisms of *5-HTTLPR*. Four of the *5-HTTLPR* studies, however, have only used a small number of patients in each IBS bowel habit subgroup, significantly underpowering the studies and probably contributing to the variability of data found [18,19,21,22]. In the remaining studies [17,20,23], patient numbers were closer to or higher than 100 per subgroup, although numbers still tended to be low for the alternating group (less than 40 patients). The larger studies showed either an increased frequency of the *5-HTTLPR* (ss) genotype in IBS-D patients [20,23] or no difference [17] compared with healthy controls. A meta-analysis of all studies, however, has suggested no association between *5-HTTLPR* polymorphisms and IBS [24].

Only one earlier study has differentiated between males and females, and has shown that although the distribution of the *5-HTTLPR* genotypes in males did not differ between IBS and control groups, irrespective of bowel habit subgroup, females with IBS-D did tend to have slightly higher frequencies for the *5-HTTLPR* (ss) than *5-HTTLPR* (non-ss) genotype [20], supporting findings from one of the larger all female studies [23]. Hence, sex might have been a confounder in earlier studies, especially those with very small numbers. An additional confounder may be the ethnicity of the populations studied, as the *5-HTTLPR* (ss) genotype seems to be a lot less common and the *5-HTTLPR* (ll) genotype more common in Caucasians than Asians [24].

The aim of this study was, therefore, to reexamine the association between IBS phenotype and both the *5-HTTLPR* and *STin2* polymorphisms in a reasonably large group of Caucasian IBS and healthy individuals, taking into consideration the sex of the individuals.

Materials and methods

Irritable bowel syndrome and healthy individuals

The *SERT* genotype analysis was carried out on 97 IBS-D patients (aged 18–66 years; mean age 41.7 years; 67 female), 99 IBS-C patients (aged 18–65 years; mean age 40.6 years; 95 female) and 92 healthy volunteers (aged 18–63 years; mean age 36 years; 60 female). IBS patients with a mixed bowel habit were excluded from the study. IBS patients were recruited from the Outpatient Departments of the University Hospitals of South Manchester (tertiary patients excluded), local general practices, advertisement in regional newspapers and an existing departmental volunteer pool of patients. All satisfied the Rome II criteria for IBS and predominant bowel habit subtype [25], and were classified by either W.A. or C.F. after interviewing using a simple questionnaire detailing the Rome II IBS criteria and bowel habit requirements [25]. All patients underwent appropriate investigations to exclude organic disease, and did not show any functional disorder of the upper gastrointestinal tract that was more prominent than their IBS. In addition, no participant had a history of major psychiatric disorder, history of alcohol or substance abuse or was taking any drugs that might modify the 5-HT system, such as antidepressants. Healthy volunteers were recruited by advertisement and underwent similar investigations to exclude organic disease. No healthy

volunteer presented with or had a history of any functional gastrointestinal disorder, including IBS. All participants were Caucasian and drank below the recommended safe alcohol limit (< 21 U/week), smoked less than five cigarettes per day and had not participated in a clinical trial of any drug within the past 30 days. Written consent was obtained from all the participants, and the study was approved by the South Manchester Medical Research Ethics Committee.

Preparation of genomic DNA

Genomic DNA was isolated using 5 ml blood samples from all IBS and healthy participants according to standard protocols [26].

Polymerase chain reaction

Polymerase chain reactions (PCRs) were performed in 25 µl volumes containing 50 ng of genomic DNA as template, 10 pmol of each primer, 200 µmol/l dNTPs (MBI Fermentas, St. Leon-Rot, Germany), in 1x PCR buffer containing 1.5 mmol/l $MgSO_4$ and 1.25 U of HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany). Thermal cycling was performed in a PTC-200 (MJ Research, Waterdown, Massachusetts, USA) or Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany). PCR reactions were performed using the following primers: *5-HTTLPR* (forward: gtcagtatcacaggctgcgag, reverse: tgttcctagtcttacgccagtg) and *STin2* (forward: 6-Fam-gtcagtatcacaggctgcgag, reverse: tgttcctagtcttacgccagtg). Cycling conditions were: initial denaturation at 94°C for 15 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s. The final extension step was at 72°C for 5 min.

Genotyping

Genotyping of the *5-HTTLPR* polymorphism was performed by analysing the PCR products on a 3.5% NuSieve (3 : 1) agarose gel (Cambrex BioScience Rockland, Rockland, Maine, USA). The expected product sizes for the deletion (del or s) and insertion (ins or l) alleles are 484 and 528 bp. For *STin2* genotyping, a 5 µl aliquot of each PCR product was analysed on a 4% NuSieve test gel before automated genotype analysis. The product sizes for alleles 9, 10, 11 and 12 are 253, 270, 287 and 304 bp, respectively. The genotyping itself was carried out on a MEGABACE system (GE Healthcare, Freiburg, Germany) using the Genetic Profiler Software Version 2.2 as recommended by the manufacturer. Analysis was carried out by laboratory staff blinded to the case–control status.

Statistical analysis

Tests for deviation from the Hardy–Weinberg equilibrium (HWE) and tests for association in IBS patient groups and control samples were conducted using a tool provided from the Institute of Human Genetics, TU Munich, Germany (*http://ihg.gsf.de/cgi-bin/hw/hwa1.pl*) and Phase V2.0. Haplotype estimation and statistical analysis were carried out using Phase V2.0. Pointwise *P* values are reported; a *P* value of less than 0.05 was considered significant. Test for the HWE in case of more than two alleles and sparse cell counts were carried out as indicated by Guo and Thompson with tailored software [27].

Results

Genotype analysis of *5-HTTLPR*

Genotype analysis showed that the frequency of the *5-HTTLPR* (ss) genotype was slightly lower in both IBS-D (16.5%) and IBS-C (14.3%) patients compared with controls (23.9%), but did not reach statistical significance (IBS-D vs. controls $P = 0.191$; IBS-C vs. controls $P = 0.09$; Table 1). This seemed to be related to a reduction of the *5-HTTLPR* (ss) genotype in male patients, particularly those with IBS-D [IBS-D 10%, IBS-C 25%, controls 37.5%; $P = 0.01$ for IBS-D vs. controls; odds ratio (95% confidence interval) for *5-HTTLPR* (ss) vs. *5-HTTLPR* (non-ss) = 0.185 (0.046–0.744)] than in female patients (IBS-D 19.4%, IBS-C 13.8%, controls 16.7%). There were no differences in the frequencies of either the *5-HTTLPR* (ll) (IBS-D 36.1%, IBS-C 33.7%, controls 29.3%) or *5-HTTLPR* (ls) (IBS-D 47.4%, IBS-C 52.0%, controls 46.8%) genotypes between any of the three groups (Table 1). For the *5-HTTLPR* genotypes, no deviation from the HWE was detected in the IBS patients or the healthy controls (data not shown).

Table 1 ***5-HTTLPR*** **genotype frequencies in IBS patients and healthy controls**

Genotype	IBS-D	IBS-C	IBS	Controls
Total no. of participants, *N* (%)	97 (100.0)	98 (100.0)	195 (100.0)	92 (100.0)
ll	35 (36.1)	33 (33.7)	68 (34.9)	27 (29.3)
ls	46 (47.4)	51 (52)	97 (49.7)	43 (46.8)
ss	16 (16.5)	14 (14.3)	30 (15.4)	22 (23.9)
P	NS	NS	NS	
Female, *N* (%)	67 (100.0)	94 (100.0)	161 (100.0)	60 (100.0)
ll	27 (40.3)	33 (35.1)	60 (37.3)	22 (36.7)
ls	27 (40.3)	48 (51.1)	75 (46.6)	28 (46.7)
ss	13 (19.4)	13 (13.8)	26 (16.1)	10 (16.7)
P	NS	NS	NS	
Male, *N* (%)	30 (100.0)	4 (100.0)	34 (100.0)	32 (100.0)
ll	8 (26.7)	0 (0)	8 (23.5)	5 (15.6)
ls	19 (63.3)	3 (75.0)	22 (64.7)	15 (46.9)
ss	3 (10.0)	1 (25.0)	4 (11.8)	12 (37.5)
P	IBS-D versus controls *P*=0.0115 OR=0.185, 95% CI: 0.046–0.744	NS	IBS versus controls *P*=0.0147 OR=0.222, 95% CI: 0.063–0.787	

Values indicate number of patients and healthy controls with the respective genotype (relative frequencies in % are listed in brackets).
95% CI, 95% confidence interval; IBS, irritable bowel syndrome; IBS-C, IBS patients with constipation; IBS-D, IBS patients with diarrhoea; l, long; NS, not significant; OR, odds ratio; s, short.
P values calculated using χ^2 test (ss vs. non-ss *5-HTTLPR* genotypes).

Table 2 ***STin2*** **genotype frequencies in IBS patients and healthy controls**

Genotype	IBS-D	IBS-C	IBS	Controls
Total no. of participants, *N* (%)	97 (100.0)	99 (100.0)	196 (100.0)	92 (100.0)
9/10	0 (0)	2 (2.0)	2 (1.0)	0 (0)
9/11	1 (1.0)	0 (0)	1 (0.5)	0 (0)
9/12	5 (5.2)	3 (3.0)	8 (4.1)	3 (3.3)
10/10	18 (18.6)	13 (13.1)	31 (15.8)	13 (14.1)
10/12	36 (37.1)	48 (48.5)	84 (42.9)	44 (47.8)
12/12	37 (38.1)	33 (33.3)	70 (35.7)	32 (34.8)
P	NS	NS	NS	
Female, *N* (%)	67 (100.0)	95 (100.0)	162 (100.0)	60 (100.0)
9/10	0 (0)	2 (2.1)	2 (1.2)	0 (0)
9/11	1 (1.5)	0 (0)	1 (0.6)	0 (0)
9/12	4 (6.0)	3 (3.2)	7 (4.3)	1 (1.7)
10/10	11 (16.4)	13 (13.7)	24 (14.9)	7 (11.7)
10/12	23 (34.3)	47 (49.5)	70 (43.2)	32(53.3)
12/12	28 (41.8)	30 (31.6)	58 (35.8)	20 (33.3)
P	NS	NS	NS	
Male, *N* (%)	30 (100.0)	4 (100.0)	34 (100.0)	32 (100.0)
9/10	0 (0)	0 (0)	0 (0)	0 (0)
9/11	0 (0)	0 (0)	0 (0)	0 (0)
9/12	1 (3.3)	0 (0)	1 (2.9)	2 (6.3)
10/10	7 (23.3)	0 (0)	7 (20.6)	6 (18.8)
10/12	13 (43.3)	1 (25.0)	14 (41.2)	12 (37.5)
12/12	9 (30.0)	3 (75.0)	12 (35.3)	12 (37.5)
P	NS	NS	NS	

Values indicate number of patients and healthy controls with the respective genotype (relative frequencies in % are listed in brackets).
IBS, irritable bowel syndrome; IBS-C, IBS patients with constipation; IBS-D, IBS patients with diarrhoea; NS, not significant; OR, odds ratio.

Genotype analysis of *STin2*

There were no significant differences between any of the patient and healthy volunteer subgroups, or any effect of sex (Table 2). No deviation from the HWE was detected in the IBS patients or the healthy controls (data not shown).

SERT haplotype analysis

There were no differences in expected haplotypes between any of the patient and healthy volunteer subgroups (data not shown).

Discussion

This is only the second study to examine whether sex has any effect on the relationship between IBS phenotype and the serotonin transporter gene polymorphism *5-HTTLPR*, and the first one to determine any effect on the *STin2* polymorphism. Unexpectedly, the study showed that the *5-HTTLPR* (ss) genotype occurred at a significantly lower frequency in male patients with IBS-D than healthy males. No differences in the frequencies of *5-HTTLPR* genotypes were observed between any of the female IBS and healthy volunteer subgroups. Moreover, no significant differences in the frequencies of the 5-HTTLPR (ss, ls or ll) genotypes were observed between the sex combined IBS-D patients, IBS-C patients and healthy volunteer subgroups, probably because the majority of patients studied were female (IBS-D, 69%; IBS-C, 95%; healthy volunteers, 65%). These observations are in accordance with one of the earlier larger studies that also showed no association between IBS phenotype and *5-HTTLPR* polymorphisms in similar female/male ratio groups to ours (IBS-D, 77%; IBS-C, 92%; healthy volunteers, 79%), but in which the effect of sex was not assessed [17]. Our data are also consistent with that from one of the smaller studies, which suggested that the frequency of the *5-HTTLPR* (ss) genotype was lower in a group of 18 IBS-D patients compared with healthy controls (11 vs. 39.5%; $P = 0.01$) [21]. Interestingly, they reported very similar frequencies to ours, although the small number of IBS-D patients studied meant that the effects of sex could not be evaluated. The present data, however, are inconsistent with the other two larger studies [20,23] both of which showed that the *5-HTTLPR* (ss) genotype occurred at a higher frequency in IBS-D patients compared with healthy volunteers; with one study carried out in all female participants [23], and another in an undisclosed female/male ratio group [20]. Despite omitting information on the sex mix of participants, the latter study, however, did report a tendency for female but not male IBS-D patients to exhibit a higher frequency of the *5-HTTLPR* (ss) genotype compared with their corresponding healthy control group [20]. No differences were reported between the IBS-C and healthy volunteer subgroups, or with respect to sex [20], as was the case in our study. The effect of sex in the case of IBS-C could not be properly addressed in this study, as only four of 98 patients were male. Of the smaller studies, either no differences [18,22], or increased ss [21] or ll [19] 5-H*TTLPR* genotypes were reported in IBS-C patients compared with controls. Similar to earlier studies [19,21,23], we found no association between the *STin2* polymorphism and the IBS-D or IBS-C subgroups, or any effect of sex.

The conflicting results obtained in this and the other larger studies [17,20,23] makes interpretation of any possible association between the *5-HTTLPR* polymorphism and IBS difficult. This is even more challenging, given the fact that in the larger studies different criteria were used to define IBS, and the effect of sex was not always explored, but taking the data as a whole and in line with a recent meta-analysis [24], it seems unlikely that there is a direct association between IBS and the *5-HTTLPR* or the *STin2* serotonin polymorphism. However, particular genotypes may increase the risk of causing or exacerbating particular symptoms associated with this disorder, such as depression and anxiety, or the severity of bowel habit disturbance. Indeed, it is well established that the *5-HTTLPR* polymorphism plays a key role in the aetiology of a variety of psychiatric and anxiety-related personality traits [28,29]. Thus, *5-HTTLPR* genotypes may influence the risk of psychopathology in

patients with IBS. This is supported by the observations that IBS patients carrying the *5-HTTLPR* (ss) genotype or the *STin2* allele 9 seem to be significantly more likely to have a history of depression [30], whereas those with *5-HTTLPR* (ll) and *5-HTTLPR* (ss) genotypes in combination with the variant allele of α_{2c}-adrenoceptor polymorphism α_{2c}-Del(322–325) have been shown to be more likely to report high somatic symptom scores (difficulty sleeping, fatigue, depression, headache, etc.) [17]. This rationale would be in line with the current thinking that IBS is best described by a biopsychosocial model, in which *SERT* genotypes seem to modify the risk for depressive episodes [30]. Unfortunately, however, we did not record either psychopathology or extraintestinal symptomatology in this study, and thus the influence of these factors cannot be determined.

As mentioned earlier, we have previously shown that both male and female IBS-D patients have elevated concentrations of platelet depleted plasma 5-HT compared with healthy controls, and speculated that this might be related to disordered metabolism/reuptake rather than synthesis/release of 5-HT [6]. These findings at first sight might seem to contradict our present observations on the *5-HTTLPR* polymorphism, as it might have been expected that we should have detected an increased frequency of the *5-HTTLPR* (ss) genotype, as reported by others [20,23]. However, recent studies have reported that SERT protein expression does not necessarily relate to the *5-HTTLPR* genotype [31]. Indeed, Coates and colleagues [32] have described reduced SERT transporter protein expression in both IBS-D and IBS-C patients. Moreover, *SERT* knockout mice show not only diarrhoea and enhanced colonic motility, but also sometimes constipation and alternating bowel habit over time [33]. The effect of a reduction in SERT transporter protein expression on bowel habit is consistent with the observations of Drossman and colleagues [34], who showed that bowel habit in many IBS patients changes over time. Thus, the abnormal plasma 5-HT concentration observed in IBS, particularly in IBS-D, may be related to decreased SERT transporter protein expression in the gastrointestinal tract caused by a *5-HTTLPR* genotype-independent mechanism, such as epigenetic factors. One possibility is gastrointestinal inflammation, which is often associated with IBS-D [2] and known to be linked with reduced transporter protein expression [35,36]. Nevertheless, a gastrointestinal-specific isoform of the serotonin transporter or usage of alternative promotors cannot be excluded to be influencing serotonin reuptake in the gut, and thereby contributing to the development of gastrointestinal-specific symptoms in IBS. In contrast, our observation that there were no differences in the *5-HTTLPR* (ss, ls or ll) genotypes between IBS-C patients and controls or with respect to sex is consistent with our earlier findings that these patients have reduced plasma 5-HT and 5-HIAA concentrations and a normal 5-HIAA/5-HT ratio suggesting a problem with synthesis/release rather than metabolism/reuptake of 5-HT in these patients. A reduced expression of the transporter protein in this subgroup might therefore not be expected to have much of an effect on plasma concentrations of 5-HT.

In conclusion, the investigation of the role of genetic factors in the aetiology of IBS is still in its infancy, and as this is a complex and heterogeneous disorder, different polymorphisms and additional factors may contribute to the manifestation of a particular phenotype. Thus, to determine which alleles contribute to specific phenotypes, even larger studies differentiating for sex in which endophenotypes are built from additional physiological and psychological traits are required. This should help to resolve the conflicting observations seen in this and other studies.

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