

# Cannabinoid receptor 1 gene and irritable bowel syndrome: phenotype and quantitative traits

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<sup>1</sup>Clinical Enteric Neuroscience Translational and Epidemiological Research (CENTER), Mayo Clinic, Rochester, Minnesota; and <sup>2</sup>Division of Biomedical Statistics and Informatics, Department of Health Sciences Research, Mayo Clinic, Rochester, Minnesota; and <sup>3</sup>Division of Gastroenterology and Hepatology, Mayo Clinic, Jacksonville, Florida

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**Camilleri M, Kolar GJ, Vazquez-Roque MI, Carlson P, Burton DD, Zinsmeister AR.** Cannabinoid receptor 1 gene and irritable bowel syndrome: phenotype and quantitative traits. *Am J Physiol Gastrointest Liver Physiol* 304: G553–G560, 2013. First published January 10, 2013; doi:10.1152/ajpgi.00376.2012.—Genetic variations in metabolism of endocannabinoids and in *CNR1* (gene for cannabinoid 1 receptor) are associated with symptom phenotype, colonic transit, and left colon motility in irritable bowel syndrome (IBS). Our aim was to evaluate associations between two variations in *CNR1* genotype (rs806378 and [AAT]<sub>n</sub> triplets) with symptom phenotype, small bowel and colonic transit, and rectal sensations in 455 patients with IBS and 228 healthy controls. Small bowel and colonic transit were measured by scintigraphy, rectal sensation by isobaric distensions. Associations with genotype were assessed by  $\chi^2$  test (symptom phenotype) and ANCOVA (quantitative traits) based on a dominant genetic model. Significant association of *CNR1* rs806378 (but not *CNR1* [AAT]<sub>n</sub>) genotype and symptom phenotype was observed ( $\chi^2$   $P = 0.028$ ). There was significant association of *CNR1* rs806378 ( $P = 0.014$ ; CC vs. CT/TT) with colonic transit in IBS-diarrhea (IBS-D) group; the TT group had the fastest colonic transit at 24 and 48 h. There was significant overall association of *CNR1* rs806378 with sensation rating of gas ( $P = 0.025$ ), but not pain; the strongest associations for gas ratings were in IBS-D ( $P = 0.002$ ) and IBS-alternating ( $P = 0.025$ ) subgroups. For *CNR1* (AAT)<sub>n</sub>, gene-by-phenotype interactions were observed for colonic transit at 24 ( $P = 0.06$ ) and 48 h ( $P = 0.002$ ) and gas ( $P = 0.046$ , highest for IBS-D,  $P = 0.034$ ), but not pain sensation; the strongest association with transit was in controls, not in IBS. These data support the hypothesis that cannabinoid receptors may play a role in control of colonic transit and sensation in humans and deserve further study as potential mediators or therapeutic targets in lower functional gastrointestinal disorders.

colon; transit; sensation; genetics; variation

ENDOCANNABINOIDS ARE LIPIDS that can act on two different 7-transmembrane receptors that are members of the superfamily of G protein-coupled receptors, CB1 and CB2 (18). The ligands of these receptors are anandamide and 2-arachidonyl glycerol, and their respective ligand-inactivating enzymes are fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (10, 16, 23). CB1-receptor immunoreactivity is present in normal colonic epithelium, smooth muscle, and the submucosal myenteric plexus and in plasma cells that influence mucosal inflammation (19, 29). There is evidence of a direct role for endocannabinoids in the modulation of motor activity

in the human colon. Activation of CB1 receptors coupled to cholinergic motor neurons inhibits excitatory nerve transmission in human colonic circular muscle (13). CB2 receptors are predominantly located on immune cells and in brain areas implicated in emesis (15).

Endocannabinoid modulation with pharmacological agents can alter colonic motor function. Esfandyari et al. (11, 12) have shown that a nonselective CB receptor agonist, dronabinol, inhibits gastric emptying and colonic motility in humans.

The potential role of cannabinoid mechanisms in the pathophysiology of functional gastrointestinal disorders is supported by prior studies that explored associations of genetic variation in metabolism or receptors of endocannabinoids and gastrointestinal motor functions in humans.

Genetic variations in endocannabinoid metabolism are associated with alterations in colonic transit in diarrhea-predominant irritable bowel syndrome (IBS-D). We previously showed that C385A variation in the gene of the *FAAH* is significantly associated with IBS, particularly in patients with mixed or alternating bowel movements (IBS-A) or IBS-D (4). Moreover, there was a borderline association with chronic abdominal pain and a significant association with the quantitative trait colonic transit at 24 h in patients with IBS-D.

Genetic variation of *CNR1* (gene for cannabinoid 1 receptor) has shown that *CNR1* rs806378 (CC vs. CT/TT) has a potential effect on fasting proximal left colonic motility in IBS-D and IBS-A patients (27). In addition, the nonselective cannabinoid agonist dronabinol preferentially delayed colonic transit in IBS patients with the *CNR1* rs806378 CT/TT genotypes (28). Alternative splicing is known to occur at *CNR1*, and five transcripts have been identified (31). The two most common variants are present in either exon 3 (5' untranslated region) of the alternate transcript (CB1-E) or in intron 1 of the transcript (CB1-A). The functional significance of the T allele of *CNR1* polymorphism rs806378 was demonstrated through alteration of nuclear protein binding in an electrophoretic mobility shift assay (24). The potential associations with gastrointestinal motor functions were also demonstrated by Vazquez-Roque et al. (25) who reported that rs806378 CC genotype was associated with reduced fasting gastric volume, and a nonsignificant association with gastric emptying of solids, compared with the CT/TT group.

The gene that codes for the *CNR1* contains a polymorphic (AAT)<sub>n</sub> triplet (14); a higher number of AAT triplets may induce a Z-shape conformation in the DNA (21), thereby altering gene transcription. The expression of this gene can be inversely proportional to the number of repeats (6). A Korean group found an association between genetic variants of AAT

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repeats located in the 3' flanking region of the *CNR1* gene and IBS (17). The >10/>10 AAT triplet repeats occurred with greater frequency in IBS patients than in healthy controls, although the allele frequencies and the *CNR1* genotypes did not differ between the IBS subtypes based on bowel function; there was significant association with higher abdominal discomfort or pain in patients with the *CNR1* >10/>10 genotype.

Our hypothesis is that genetic variation in *CNR1* at the rs806378 locus and higher numbers of AAT triplet repeats in *CNR1* are associated with IBS, compared with healthy controls from the Midwest USA, and that these genetic variants are associated with small bowel and colonic transit or sensation ratings of gas and pain following isobaric pressure-based rectal distensions.

Our overall aim was to evaluate associations between variations in the *CNR1* genotype, that is rs806378 and (AAT)*n* triplets, with symptom phenotype, small bowel and colonic transit, and rectal sensations in previously identified patients with functional gastrointestinal disorders who reside in Midwest USA. The significance of such associations with phenotype and quantitative traits is that they support a potential role of cannabinoid mechanisms in the pathophysiology of functional gastrointestinal disorders.

## METHODS

**Study design.** We had a total of 683 subjects (455 patients and 228 healthy controls) with prior research authorization to use their genetic material for analysis. The analysis of the genetic material was investigated with prior collected information regarding IBS phenotype, small bowel and colonic transit, and sensation ratings in response to isobaric rectal distensions in patients with IBS and lower functional gastrointestinal disorders.

**Participants.** Participants who were eligible for evaluation of the association between symptom phenotypes and genotypes have been extensively described in previous publications (5). All participants were recruited in studies of symptom phenotype and genotype from 2000 to 2012. All participants resided within 150 miles of Rochester, Minnesota. Participants had been recruited for the original studies by means of letters or public advertisements and had signed informed consent for the respective studies; and all patients fulfilled Rome II criteria for IBS phenotypes. The study was approved by the Mayo Clinic Institutional Review Board, and all participants had provided written permission for research studies based on their medical records and DNA samples. Experienced coordinators used the validated Bowel Disease Symptom Questionnaire, electronic medical record, and information from direct physician interview and examination (M. Camilleri) to characterize the subtype of lower functional gastrointestinal disorders.

**Small bowel and colonic transit by scintigraphy.** We used an adaptation of our established scintigraphic method to measure small bowel and colonic transit in our subjects, which has been extensively validated (8, 9). Transit measurements were performed after an overnight fast. In summary, indium-111 (<sup>111</sup>In) adsorbed on activated charcoal particles was delivered to the colon via a methacrylate-coated, delayed-release capsule that was administered by the oral route. Following emptying of the capsule from the stomach, its position was documented relative to radioisotopic markers placed on the anterior iliac crests. After this, a breakfast meal of two scrambled eggs radiolabeled with technetium-99m (<sup>99m</sup>Tc) along with one slice of whole wheat bread and one glass of whole milk (300 kcal total) was ingested. This meal enabled the measurement of the small bowel transit. Standardized meals for lunch and dinner at 4 and 8 h, respectively, were ingested by the subjects after the radiolabeled breakfast meal. Abdominal scans were obtained every hour for the

first 6 h for colonic filling at the 6-h time point (CF6%) and at 8, 24, and 48 h after ingestion of the capsule containing the <sup>111</sup>In-charcoal. The performance characteristics of this test have been summarized elsewhere (8, 9).

**Transit data analysis.** A variable region of interest program was used to quantitate the counts in the stomach and each of four colonic regions: ascending, transverse, descending, and combined sigmoid and rectum. These counts were corrected for isotope decay, tissue attenuation, and downscatter of <sup>111</sup>In counts in the <sup>99m</sup>Tc window. The overall colonic transit was summarized as the colonic geometric center (GC) at specified times. The GC is the weighted average of counts in the different colonic regions [ascending (AC), transverse (TC), descending (DC), rectosigmoid (RS), and stool, respectively 1 to 5]. Thus, at any time, the proportion of counts in each colonic region is multiplied by its weighting factor as follows:

$$(\%AC \times 1 + \%TC \times 2 + \%DC \times 3 + \%RS \times 4 + \%stool \times 5) / 100 = \text{genometric center}$$

Hence, higher GC represents a faster colonic transit. The primary transit end points were the colonic filling at 6 h and colonic geometric center at 24 h (GC24). A secondary end point was colonic transit at 48 h (GC48). A total of 190 of the 683 participants had transit data.

**Rectal sensation by isobaric barostat-based balloon distensions.** For this analysis, we incorporated information recorded in prior studies of rectal sensation performed at baseline (pretreatment) in 102 participants (of the 683) who subsequently received clonidine treatment to investigate the associations of adrenergic and serotonergic mechanisms and effects of  $\alpha$ -2 adrenergic modulation of gastrointestinal and rectal functions, as published elsewhere (2, 3).

The method and performance characteristics have been extensively described elsewhere (1, 7). The focus of the present study was on sensation ratings. Subjects self-administered the bowel preparation (Fleet phosphate enema) at least 1 h before reporting to the Clinical Research Center after an overnight fast. Random-order phasic distensions above baseline operating pressure were used to assess sensory ratings, as in prior studies (1, 26). Phasic distensions of 12, 24, 30, and 36 mmHg above baseline operating pressure were each applied once in random order; each distension was maintained for 60 s with an interstimulus interval of 2 min, during which the balloon was deflated to the baseline operating pressure. Thirty seconds after onset of the distension, subjects marked four separate 100-mm visual analog scales (VAS, anchored at each end by the descriptions "unnoticeable" and "unbearable") for the sensations of gas, urgency, discomfort, and pain. For the purpose of the analysis of the sensations of gas and pain and to avoid multiple comparisons and multiple end points, the primary end points for analysis were the sensation ratings of gas and pain at the 36 mmHg (above baseline operating pressure) isobaric distensions.

**Genotyping.** Genomic DNA was isolated from blood using standard methods. Genotyping of *CNR1* rs806378 was performed with TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions using 10–20 ng of genomic DNA for each sample. Primers and probes were from Assay-by-Design (Applied Biosystems). Following polymerase chain reaction (PCR) amplification, end reactions were read on the 7900 Real-Time PCR System by use of Sequence Detection Software (Applied Biosystems).

Although there are variations in the number of AAT triplet repeats in different races, we chose the 10 repeats as the cutoff to attempt to replicate the study of Park et al. (17); in addition, (AAT)10 and (AAT)14 are the most common allele types in European Americans (22), who constituted the vast majority of participants in our study. To determine the number of (AAT)*n* triplet repeats, DNA samples were amplified with LA Taq with GC buffers (TaKaRa Shuzo) using the following primers: 5'-VIC-CATGCAGCACACCAACATGGCA-3' and 5'-TGTGTACAAAATGCTGCTTCTGTA-3' (17). VIC is a

Table 1. *CNR1* rs806378: frequency distribution of symptom phenotypes (%) among different genotypes

	IBS-C	IBS-D	IBS-A	CAP	Dyspepsia	Healthy
Total number	154	173	80	18	30	228
CC ( <i>n</i> = 358)	21.2	29.0	11.7	2.2	4.5	31.3
CT ( <i>n</i> = 264)	25.8	21.2	13.3	1.9	4.2	33.7
TT ( <i>n</i> = 61)	16.4	21.3	4.9	8.2	4.9	44.3

There is a significant association of this *CNR1* rs806378 genotype and symptom phenotype. ( $\chi^2 P = 0.027$ ). IBS-C, constipation-predominant irritable bowel; IBS-D, diarrhea-predominant irritable bowel syndrome (IBS); IBS-A, mixed or alternating irritable bowel syndrome; CAP, chronic abdominal pain.

fluorescent dye labeled to one of the primers (17) and it was also used in the design of the primers for this assay (Applied Biosystems). An aliquot of PCR product was mixed with Hi-Di formamide and GeneScan-500ROX size standard (Applied Biosystems) and run on an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems) at the Mayo Clinic Sequencing Core. Allele sizes were determined by using GENESCAN software version 2.1 (Applied Biosystems) and typed by comparing them with the allelic ladder using GENOTYPER software version 2.1. Hardy-Weinberg equilibrium calculations were performed to verify that each marker was within allelic equilibrium in controls.

**Statistical analysis.** Previously collected data in subjects meeting criteria for IBS and healthy non-IBS subjects were compiled for all identified subjects with general research authorization for use of their medical records. The associations of (AAT)n triplet repeats and rs806378 of *CNR1* with symptom phenotype were assessed by the  $\chi^2$  test (general genetic coding). The associations of (AAT)n triplet repeats of *CNR1* and rs806378 with small bowel and colonic transit were assessed by analysis of covariance (ANCOVA) models adjusted for symptom phenotype. A dominant genetic model coding was used in the ANCOVA models, i.e., both alleles with >10 repeats vs. 1 or 2 with  $\leq 10$  repeats, and for rs806378, CC vs. CT/TT. These models also included a symptom phenotype by genotype "interaction" term (i.e., cross-product term) to examine whether the associations with genotype differed by symptom phenotype.

The reported *P* values are unadjusted in deference to the hypothesis-generating aim of the analyses. An adjusted  $\alpha$  level can be specified by considering that the associations of two genes were tested for each of four physiological types of response [i.e., symptom phenotype, and 3 quantitative traits (small bowel transit, CF6%; colonic transit, GC at 24 h and 48 h; and VAS sensation ratings, gas and pain at 36 mmHg)]. Thus, for symptom phenotype and small bowel transit,  $\alpha = 0.025$  would be an adjusted  $\alpha$  level; for colonic transit and sensation ratings,  $\alpha = 0.0125$  would be an adjusted  $\alpha$  level. All analyses employed SAS software (version 9.3). Although the distension studies were conducted at four levels of distension, the analysis conducted here prespecified that the primary end points of interest (presumably reflecting the worst sensations) were the ratings observed with 36 mmHg distension. This strategy was also chosen to

Table 2. *CNR1* AAT triplet repeats: frequency distribution of symptom phenotypes (%) among different genotypes

	IBS-C	IBS-D	IBS-A	CAP	Dyspepsia	Healthy
Total number	154	173	80	18	30	228
<10/<10 ( <i>n</i> = 84)	19.1	28.6	15.5	3.6	8.3	25.0
<10/>10 ( <i>n</i> = 295)	23.4	29.2	11.5	2.4	3.0	30.6
>10/>10 ( <i>n</i> = 304)	22.7	20.7	10.9	2.6	4.6	38.5

There is a nonsignificant association of the *CNR1* AAT repeats and symptom phenotype ( $\chi^2 P = 0.105$ ); 10/10 refers to the number of AAT repeats in each allele.

Table 3. *CNR1* rs806378: distribution of demographics and transit data in the participants with transit measurements

	<i>n</i>	CC	CT	TT
Genotype distribution, <i>n</i>		100	76	14
Age, yr	190	37.5 $\pm$ 11.7	37.9 $\pm$ 12.7	40.9 $\pm$ 13.0
Female/Male (%Female)	190	88/12 (88)	69/7 (91)	10/4 (71)
BMI, kg/m <sup>2</sup>	186	27.5 $\pm$ 5.8	26.8 $\pm$ 4.8	26.3 $\pm$ 3.4
CF6, %	187	48.8 $\pm$ 31.4	50.1 $\pm$ 29.4	56.0 $\pm$ 30.0
GC24	190	2.6 $\pm$ 1.1	2.5 $\pm$ 1.2	3.0 $\pm$ 1.1
GC48	188	3.7 $\pm$ 1.2	3.6 $\pm$ 1.1	4.0 $\pm$ 1.1

Values are means  $\pm$  SD, unless otherwise indicated. *n*, Number of subjects; CF6, colonic filling at 6 h; GC24, geometric center (GC) at 24 h; GC48, GC at 48 h.

avoid multiple comparisons in the sensation domain, which would require further corrections for multiple comparisons.

## RESULTS

**Genetic analysis.** The genetic variations studied were in Hardy-Weinberg equilibrium and did not differ significantly from the frequency in Caucasian controls' minor allele frequency reported in the literature: rs806378, *P* = 0.188; (AAT)n, *P* = 0.661. The two genetic variants studied are not in linkage disequilibrium (*D'* score 0.447). The races of the participants were 1 American Indian, 4 Asian, 612 white, and 66 unreported.

**Association of *CNR1* genetic variation and IBS symptom phenotype.** This analysis was based on the study of 683 participants, of whom 228 were healthy participants and the others had different functional gastrointestinal disorders. A significant association of *CNR1* rs806378 genotype and symptom phenotype was observed (Table 1;  $\chi^2 P = 0.027$ ). On the other hand, there was a nonsignificant association of the *CNR1* (AAT)n repeats and symptom phenotype (Table 2;  $\chi^2 P = 0.105$ ).

**Association of *CNR1* genetic variation and small bowel and colonic transit.** This analysis was based on data from 190 individuals: 187 had measurements of colonic filling at 6 h (a valid surrogate of small bowel transit time), and 190 participants underwent colonic transit measurement at 24 h. There were no associations of any genotype with small bowel transit in the entire cohort or subgroups of the cohort (Tables 3 and 4).

On average, the TT genotype of *CNR1* rs806378 was associated with a numerically faster colonic transit in the entire cohort with transit measurements (Table 3). There was a borderline *CNR1* rs806378 gene-by-subgroup interaction (*P* = 0.10) for colonic transit at 24 h, with significant (*P* = 0.024)

Table 4. *CNR1* AAT triplet repeats: distribution of data and demographics in the entire cohort of participants with transit measurements

	<i>n</i>	<10/<10	<10/>10	>10/>10
Genotype distribution, <i>n</i>		15	94	81
Age, yr	190	35.1 $\pm$ 11.5	39.9 $\pm$ 12.9	36.1 $\pm$ 11.1
Female/Male (%Female)	190	13/2 (87)	83/11 (88)	71/10 (88)
BMI, kg/m <sup>2</sup>	186	27.2 $\pm$ 5.1	26.5 $\pm$ 4.3	27.8 $\pm$ 6.1
CF6, %	187	46.2 $\pm$ 30.0	49.8 $\pm$ 32.4	50.6 $\pm$ 28.3
GC24	190	2.3 $\pm$ 1.0	2.7 $\pm$ 1.1	2.5 $\pm$ 1.1
GC48	188	3.2 $\pm$ 1.3	3.8 $\pm$ 1.1	3.6 $\pm$ 1.1

Values are means  $\pm$  SD, unless otherwise indicated. BMI, body mass index.

Table 5. *CNR1* rs806378: distribution of small bowel and colonic transit data according to genotype in different IBS symptom phenotype subgroups among 190 patients with colonic transit measured at 24 h

	IBS-C			IBS-D			IBS-A			Healthy		
	CC	CT	TT									
No.	28	25	5	43	23	4	12	10	2	17	18	3
CF6, %	49.2 ± 29.0	45.0 ± 27.8	60.0 ± 32.0	49.0 ± 35.7	64.3 ± 29.2	59.8 ± 25.5	50.1 ± 26.8	29.5 ± 15.0	31.0 ± 43.8	47.1 ± 29.2	50.4 ± 30.8	61.3 ± 33.5
GC24	2.2 ± 1.0	1.8 ± 0.6	2.3 ± 1.0	3.0 ± 1.2	3.4 ± 1.3	4.3 ± 0.4*	2.4 ± 0.6	2.4 ± 0.5	1.7 ± 0.6	2.3 ± 1.0	2.5 ± 1.1	3.0 ± 0.8
GC48	3.0 ± 1.1	3.0 ± 1.0	3.6 ± 1.2	4.2 ± 1.0	4.4 ± 1.0	4.8 ± 0.4	4.1 ± 0.8	4.0 ± 0.7	3.0 ± 1.4	3.2 ± 1.2	3.4 ± 1.2	4.4 ± 1.0

There was borderline gene-by-subgroup interaction ( $P = 0.10$ ) for colonic transit at 24 h, with a significant ( $P = 0.014$ ) association of rs806378 with colonic transit (GC at 24 h) in IBS-D group.

Table 6. *CNR1* AAT triplet repeats: distribution of small bowel and colonic transit data in different IBS symptom phenotype groups

	IBS-C			IBS-D			IBS-A			Healthy		
	<10/<10	<10/>10	>10/>10	<10/<10	<10/>10	>10/>10	<10/<10	<10/>10	>10/>10	<10/<10	<10/>10	>10/>10
AAT repeats	3	27	28	6	40	24	3	12	9	3	15	20
No.	20.7 ± 11.7	47.6 ± 32.2	52.2 ± 24.3	60.3 ± 28.0	54.4 ± 36.0	53.7 ± 32.0	33.7 ± 25.7	44.8 ± 26.6	35.4 ± 24.2	56.0 ± 40.3	45.6 ± 28.5	51.8 ± 30.2
CF6, %	1.6 ± 0.3	2.2 ± 1.0	2.0 ± 0.6	2.9 ± 1.4	3.3 ± 1.2	3.2 ± 1.3	2.3 ± 0.4	2.5 ± 0.5	2.0 ± 0.4	1.7 ± 0.2	2.0 ± 0.6	3.0 ± 1.1
GC24	2.0 ± 0.3	3.1 ± 1.1	3.0 ± 1.0	3.8 ± 1.4	4.5 ± 0.8	4.1 ± 1.2	4.2 ± 0.3	4.1 ± 0.7	3.6 ± 1.1	2.0 ± 0.4	3.0 ± 1.0	4.0 ± 1.0

Values are means ± SD. There was a borderline gene-by-subgroup interaction ( $P = 0.06$ ) for GC at 24 h and significant ( $P = 0.002$ ) interaction for GC at 48 h, with significant associations of *CNR1* (AAT)n with colonic transit in the healthy participant subgroup, GC at 24 h ( $P = 0.027$ ) and at 48 h ( $P = 0.001$ ).

Table 7. *CNR1 rs806378: distribution of sensation ratings (on 100 mm visual analog scale) of gas and pain during rectal isobaric distensions at 36 mmHg above baseline operating pressure according to genotype in different IBS symptom phenotype subgroups*

rs806378	IBS-C			IBS-D			IBS-A			Healthy		
	CC	CT	TT	CC	CT	TT	CC	CT	TT	CC	CT	TT
<i>n</i>	17	13	2	17	8	1	8	10	2	11	12	1
Gas	67.4 ± 29.4	60.5 ± 25.0	43.0 ± 19.8	45.5 ± 32.8	79.3 ± 15.5	99	44.9 ± 28.7	74.1 ± 17.3	66.5 ± 20.5	72.6 ± 21.1	69.5 ± 21.0	0
Pain	58.2 ± 29.7	49.2 ± 30.0	43.0 ± 9.9	53.2 ± 25.4	83.0 ± 14.4	23	52.9 ± 30.8	62.5 ± 30.2	75.0 ± 2.8	66.3 ± 26.0	62.3 ± 16.5	0

Values are means ± SD. There was a significant overall association of *CNR1 rs806378* with gas sensation rating ( $P = 0.037$ ) and gene-by-phenotype interaction ( $P = 0.002$ ) with the strongest associations in the IBS-D ( $P = 0.001$ ) and IBS-A ( $P = 0.021$ ) subgroups. There were no significant overall associations of *CNR1 rs806378* with pain sensation ratings, although a modest association within IBS-D with genotype was found ( $P = 0.040$ ).

Table 8. *CNR1 AAT repeat: distribution of sensation ratings of gas and pain during rectal isobaric distensions at 36 mmHg above baseline operating pressure according to genotype in different IBS symptom phenotype subgroups*

AAT repeats	IBS-C			IBS-D			IBS-A			Healthy		
	<10/<10	<10/>10	>10/>10	<10/<10	<10/>10	>10/>10	<10/<10	<10/>10	>10/>10	<10/<10	<10/>10	>10/>10
No.	1	18	13	3	13	10	3	8	9	2	10	12
Gas	1.0	66.0 ± 27.3	58.7 ± 28.3	51.0 ± 48.9	48.3 ± 34.3	72.6 ± 22.6	75.0 ± 25.5	69.3 ± 25.4	50.4 ± 24.4	57.5 ± 47.4	74.3 ± 18.5	64.6 ± 27.1
Pain	2.0	57.0 ± 30.0	46.5 ± 27.3	62.3 ± 41.1	57.0 ± 27.4	66.4 ± 24.0	80.7 ± 18.2	53.8 ± 36.2	58.4 ± 23.3	54.0 ± 60.8	61.9 ± 16.9	62.5 ± 25.9

Values are means ± SD. There was a significant ( $P = 0.046$ ) gene-by-phenotype interaction for the sensation rating of gas at 36 mm with the strongest association observed in the IBS-D ( $P = 0.034$ ) subgroup, and no significant association with pain sensation ratings.

Table 9. Post hoc statistical power to detect 20–30% effect sizes for colonic transit

Response (overall mean ± SD)	Effect Size % (units)*	Power†	
		CC (n = 100) vs. CT/TT (n = 90)	>10 > 10 (n = 109) vs. ≤10/>10 (n = 81)
GC 24 h (2.6 ± 1.1)	20% (0.52)	88%	87%
	25% (0.65)	97%	97%
	30% (0.78)	>99%	>99%

Note that the actual numbers of participants with quantitative trait data and the observed allele frequencies were used to generate these power calculations. Therefore, our study had sufficient power to detect clinically meaningful differences according to genotype. \*As a percentage of listed overall mean; units are GC units for colonic transit. †Based on 2-sample comparison using a 2-sided alpha level of 0.05.

association of rs806378 with colonic transit in the IBS-D group for the comparison of CC and CT/TT groups. Table 5 shows that, in particular, the TT genotype group had faster colonic transit at 24 h compared with the CC and CT genotype groups. In the IBS-D group, the magnitude of the difference in mean colonic transit GC at 24 h was about 0.6 GC unit (least square means of 2.94 for the CC genotype and 3.52 for the CT/TT combined group); similarly, there was numerically faster colonic transit at 48 h.

For *CNR1* (AAT)n there was a significant gene-by-phenotype interaction for colonic transit GC at 24 h ( $P = 0.021$ ) and a significant interaction at 48 h ( $P = 0.002$ ). Particularly, in the healthy participant group, the association of *CNR1* (AAT)n was significant for GC at 24 h ( $P = 0.005$ ) and at 48 h ( $P < 0.001$ ), but there were no significant associations within the IBS subgroups (Table 6).

*Association of CNR1 genetic variation and rectal sensation ratings.* This analysis was based on 102 participants. As shown in Table 7, there were a significant overall association of *CNR1* rs806378 with sensation rating of gas at 36 mmHg distension ( $P = 0.037$ ) and a significant gene-by-phenotype interaction ( $P = 0.002$ ), with the strongest associations in the IBS-D ( $P = 0.001$ ) and IBS-A ( $P = 0.021$ ) subtypes. There were no significant overall associations of *CNR1* rs806378 with pain sensation ratings, although a modest association within IBS-D with genotype was found ( $P = 0.040$ ). For *CNR1* (AAT)n, there was a significant ( $P = 0.046$ ) gene-by-phenotype interaction for the sensation rating of gas at 36 mm, with the strongest association observed in the IBS-D ( $P = 0.034$ ) subgroup and no significant association with pain sensation (Table 8).

## DISCUSSION

The endocannabinoids are synthesized on demand in postsynaptic neurons and released in postsynaptic clefts where they act as retrograde messengers to act through many mechanisms (e.g., activation of CB1 receptors on cholinergic neurons, inhibition of cAMP production, and inhibition of acetylcholine release in the enteric nervous system). Thus CB1 receptors also modulate intestinal propulsion by an attenuation of the peristaltic reflex (30).

In humans, we recently showed that a nonselective cannabinoid agonist reduced fasting colonic motility in nonconstipated IBS patients (27). CB1 and CB2 receptor activation inhibits abdominal sensitivity to colorectal distension (a sur-

rogate of visceral pain) in rats under basal conditions (20); conversely, relatively low doses of the nonselective CB agonist, dronabinol, increased colonic sensation to distension in humans (11). In the present study, we explored the associations of genetic variations in *CNR1* and small bowel and colonic transit and rectal sensation in humans. The study demonstrates the association between variations in the *CNR1* gene, that is rs806378 and (AAT)n triplets, with symptom phenotype, small bowel and colonic transit, and rectal sensation in humans. The study was based on previously identified patients with functional gastrointestinal disorders ( $n = 455$ ) and healthy controls ( $n = 228$ ), all of whom reside in the Midwest USA. Cannabinoid receptors, especially *CNR1*, are reported to affect numerous gastrointestinal functions, including pain modulation, inflammation, and gastric and colonic motility (11, 12, 15).

Our study focused on *CNR1* gene variations at rs806378 locus and (AAT)n triplet repeats in the 3' flanking region, since these have been proposed as candidate genes based on their functional effects. We demonstrated a significant association of *CNR1* rs806378 genotype, but not of *CNR1* (AAT)n repeat with symptom phenotype. In addition, our studies support the hypothetical mechanism of the association of *CNR1* rs806378 with IBS because of the demonstrated associations with colonic transit and rectal sensation of gas, but not pain. Thus we found significant ( $P = 0.024$ ) associations of rs806378 with colonic transit in the IBS-D group for the comparison of CC and CT/TT groups and with sensation rating of gas at 36 mmHg distension ( $P = 0.037$ ), the strongest associations being observed in the IBS-D ( $P = 0.001$ ) and IBS-A ( $P = 0.021$ ) subtypes. The functional significance of the T allele at rs806378 is supported by the observation that, among IBS-D patients receiving the CB1 and CB2 receptor agonist, dronabinol, the CT/TT genotype subgroup, but not the CC genotype subgroup, showed slowing of colonic transit posttreatment with both 2.5- and 5-mg doses of dronabinol, compared with placebo treatment (28).

The T allele of *CNR1* rs806378 was shown to increase nuclear protein binding in an electrophoretic mobility shift assay (23), creating a binding site for arylhydrocarbon receptor translocator (ARNT), a member of the basic helix-loop-helix/Per-Arnt-Sim protein family. The ancestral C-allele does not bind to either ARNT or any other nuclear protein. It is unclear how this allelic change and the associated alteration in nuclear protein binding would ultimately result in the changes in the quantitative traits measured (transit and sensation).

Table 10. Post hoc statistical power to detect 20–30% effect sizes for gas sensation ratings

Gas Rating @ 36 mm (62 ± 28)	Effect Size % (units)*	Power†	
		CC(n = 53) vs. CT/TT (n = 49)	>10 > 10 (n = 58) vs. ≤10/>10 (n = 44)
	20% (12)	56%	57%
	25% (16)	82%	81%
	30% (19)	92%	92%

Note that the actual numbers of participants with quantitative trait data and the observed allele frequencies were used to generate these power calculations. Therefore, our study had sufficient power to detect clinically meaningful differences according to genotype. \*As a percentage of listed overall mean; units are mm on 100-mm visual analog scale for sensation ratings. †Based on 2-sample comparison using a 2-sided alpha level of 0.05.

Although a significant association of *CNR1* (AAT)n was identified in a Korean cohort of IBS patients, Park et al. (17) recommended that the identified association required replication, given the multiple comparisons, univariate associations, and relatively small sample size of the study. Our study demonstrated *CNR1* (AAT)n gene-by-phenotype interactions were observed for colonic transit at 24 h ( $P = 0.021$ ) and 48 h ( $P = 0.001$ ), and gas ( $P = 0.046$ ); the strongest association for IBS-D,  $P = 0.034$ ), but not pain sensation; however, the strongest association with transit was in controls, not in IBS patients.

The association of *CNR1* (AAT)n genotype with colonic transit at 24 and 48 h was mainly demonstrable in the healthy controls, not in IBS patients. Moreover, we did not identify a significant association with the symptom phenotype of predominantly Caucasian IBS patients, in contrast to the association reported in Koreans (17).

The strengths of our study include the associations with quantitative traits in addition to symptom phenotype and the consistency of the information regarding associations with symptoms and quantitative traits (positive with *CNR1* rs806378 and negative with *CNR1* [AAT]n). Tables 9 and 10 detail a post hoc analysis showing the power (based on 2-sample comparison using a 2-sided  $\alpha$  level of 0.05) to detect effect sizes of 20 to 30% in the quantitative traits, colonic transit at 24 h (Table 9) and sensation ratings of gas in response to 36 mmHg distension (Table 10). In each analysis, which assumed the data would be subjected to the dominant genetic model, the sample sizes are based on the observed major and minor allele frequencies for rs806378 and AAT(n) repeats, and the actual number of participants in whom the quantitative trait was available. Note from this analysis that there was >80% power to detect a clinically meaningful effect on transit, that is 0.5 GC units or above, and there was >80% power to detect at least a 25% difference in sensation rating of gas in response to the distension. Therefore, the absence of association of (AAT)n genetic variation with these quantitative traits in IBS patients is a true negative and is not due to a type 2 statistical error.

A similar analysis was conducted to appraise the power to detect association of the genetic variation with symptom phenotype. There were 359 subjects in the CC subgroup and 327 subjects in the CT/TT subgroup for the rs806378 variation, and 304 >10/>10 AAT repeat subjects and a total of 379 in the  $\leq 10/>10$  AAT repeats subgroup for the (AAT)n repeat variation. The study was significantly underpowered to detect differences by genotype in any symptom subgroup, except for the IBS-D subgroup (rs806378,  $P = 0.018$ ; >10/>10 AAT repeats,  $P = 0.013$ ).

The limitations of our study are the predominantly Caucasian cohort with insufficient numbers of other races or ethnic groups to make the findings generalizable. As in any such genetic association study, the associations with rs806378 genotype do not necessarily result from the effects of this genotype, since it may be in linkage disequilibrium with the variation that actually causes the alteration in the functions of the *CNR1* gene or the resulting proteins.

In conclusion, our study suggests that, in addition to the effects of genetic variation in *FAAH* metabolism of endocannabinoids, CB1 receptor-related mechanisms modify colonic transit and sensation and may influence the development of symptoms in Caucasian patients with IBS, particularly IBS-D.

These data support the hypothesis that CB1 receptors may play a role in the control of colonic transit and sensation in humans and deserve further study as potential direct therapeutic targets in lower functional gastrointestinal disorders.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

#### AUTHOR CONTRIBUTIONS

M.C. and M.I.V.-R. conception and design of research; M.C., D.D.B., and A.R.Z. analyzed data; M.C. and G.J.K. drafted manuscript; M.C., G.J.K., M.I.V.-R., P.C., and A.R.Z. edited and revised manuscript; M.C., G.J.K., M.I.V.-R., P.C., and A.R.Z. approved final version of manuscript; P.C. and D.D.B. performed experiments.

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