0021-972X/07/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 92(6):2382–2386
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doi: 10.1210/jc.2006-2523

BRIEF REPORT

Genetic Variations at the Endocannabinoid Type 1 Receptor Gene (*CNR1*) Are Associated with Obesity Phenotypes in Men

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Context: The endocannabinoid system modulates food intake and body weight in animal models. Treatment with the cannabinoid type 1 receptor blocker, rimonabant, reduces body weight in obese individuals.

Objective: The aim of this study was to determine whether single nucleotide polymorphisms of the gene encoding cannabinoid type 1 receptor, *CNR1*, are associated with body fat mass and distribution in two independent samples of white European adult men.

Design, Setting, and Participants: The 3813A/G and 4895A/G single nucleotide polymorphisms at the exon 4 of *CNR1* were genotyped in 930 participants to the Olivetti Prospective Heart Study (OPHS) in Southern Italy and in 216 participants to the Wandsworth Heart and Stroke Study in the United Kingdom. Retrospective analysis was also performed on an OPHS subsample (n = 360) for which anthropometric data from 1987 and 1994–1995 examinations were available.

Main Outcome Measures: *CNR1* genotypes and anthropometric measures of body fat distribution were determined.

Results: In the OPHS study, the 3813G allele was associated with increased subscapular skinfold thickness (24.2 ± 9.1 *vs.* 22.8 ± 7.7 mm; $P = 0.031$) and waist circumference (WC) (99.1 ± 8.8 *vs.* 97.7 ± 8.8 cm; $P = 0.050$). No association was observed with 4895A/G variant. Haplotype analysis confirmed that the unique haplotype carrying the 3813G was associated with increased WC and subscapular skinfold thickness. Similar results were observed in the OPHS retrospective subsample and the Wandsworth Heart and Stroke Study sample. In the latter, the 3813G was associated with increased WC (96.8 ± 11.3 *vs.* 91.6 ± 10.4 cm; $P = 0.006$).

Conclusions: Genetic variants at *CNR1* are associated with obesity-related phenotypes in men. The detection of polymorphic variants in genes involved in the process of fat accumulation may help identify specific targets for pharmacological treatment of obesity and related metabolic abnormalities. **(*J Clin Endocrinol Metab* 92: 2382–2386, 2007)**

AN EMERGING BODY of evidence suggests that the cannabinoid type 1 receptor (CB1) and its endogenous ligands, the endocannabinoids, are involved in the regulation of feeding behavior and body weight, thus contributing to the regulation of energy balance (1–3). The endocannabinoid system modulates appetitive behavior and energy homeostasis through the activation of the G protein-coupled CB1 located in the central nervous system and in various peripheral tissues, including adipose tissue, muscle, the gastrointestinal tract, and liver (1–3). The orexigenic effect of endocannabinoids is antagonized by CB1 blockade. Recent data suggest that rimonabant, a selective CB1 blocker, prevents weight gain and associated metabolic disorders in overweight individuals (4–6), thus revealing a promising strategy for the treatment of obesity and related cardiometabolic disorders (7).

The endocannabinoid receptor type 1 gene, *CNR1*, located on 6q14-q15, might thus represent a strong candidate to harbor variants that may contribute to individual differences in susceptibility to obesity, body fat distribution, and related metabolic disorders. Several human *CNR1* polymorphisms have been recently described in European-American, African-American, and Japanese individuals with regard to human addiction vulnerability (8).

We made the hypothesis that genetic variations in *CNR1* would be associated with abnormal body mass index (BMI) and body fat distribution. To test this hypothesis we genotyped two exon 4 variants of the *CNR1*, 3813A/G(rs12720071), and 4895A/G (rs806368), on the 2002–2004 follow-up examination of the population of the Olivetti Prospective Heart Study (OPHS). These two polymor-

First Published Online April 3, 2007

Abbreviations: AA, $A_{3813}A_{4895}$; AG, $A_{3813}G_{4895}$; BMI, body mass index; CB1, cannabinoid type 1 receptor; GA, $G_{3813}A_{4895}$; GG, $G_{3813}G_{4895}$; LD, linkage disequilibrium; OPHS, Olivetti Prospective Heart Study; SNP, single nucleotide polymorphism; SS, subscapular skinfold thickness; WC, waist circumference; WHSS, Wandsworth Heart and Stroke Study.

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phisms are tag single nucleotide polymorphisms (SNPs) of *CNR1* for Caucasian populations (HapMap-CEU) in the Seattle SNPs database (http://gvs.gs.washington.edu/GVS/) and were thus selected as maximally informative sites for *CNR1,* according to the tag SNPs approach in the evaluation of gene candidacy for common diseases (9).

In addition, we analyzed retrospective findings for a subset of OPHS participants who had undergone the 1987 and 1994–95 follow-up visits. Finally, the findings obtained from the OPHS database were verified in an independent sample of white British males participating in the Wandsworth Heart and Stroke Study (WHSS).

Subjects and Methods

Methods and populations of each survey have been described in detail elsewhere (10–12). The respective local Ethics Committee approved the study protocols, and all participants gave their informed consent.

Study populations

OPHS. The OPHS population was made of unselected white adult male individuals who were or had been part of the Olivetti factory workforce in Campania, a region of Southern Italy. The data used for the present analysis were collected between January 2002 and May 2004, and derived from the examination of 997 participants. For the purpose of the present work, 67 individuals were excluded because of an incomplete data set, thus resulting in a sample of 930 individuals. Of these, a subgroup of 360 men had also been examined in 1987 and 1994–1995.

WHSS. The WHSS was a population-based survey performed in South London from 1994–1996 to estimate the prevalence of the major cardiovascular risk factors in men and women of different ethnic background. The sample included 1578 men and women, aged 40–59 yr (524 white, 549 of African descent, and 505 of South Asian origin). Only white men (n = 216) were considered in the present analysis, as independent validation series.

Anthropometric measurements

Body weight and height were measured on a standard beam balance scale with an attached ruler. Body weight was measured at the nearest 0.1 kg, and height was measured at the nearest centimeter, with subjects wearing only light indoor clothing without shoes. The BMI was calculated as weight in kilograms divided by the square of the height in meters. The waist circumference (WC) was measured at the umbilicus level at the nearest 0.1 cm with a flexible inextensible plastic tape, with the subject standing erect with the abdomen relaxed, arms at the sides, and feet together. WC was taken as reference measure of abdominal obesity (13). In the OPHS, subscapular skinfold thickness (SS) was also measured with standard methods (10).

Genotyping

Genotyping of 3813A/G (rs12720071) and 4895A/G (rs806368) polymorphisms in the 3′UTR region of the exon 4 of *CNR1* was performed using PCR-restriction fragment length polymorphism methods and 2% agarose gels (8). Oligonucleotides used to amplify 3813A/G and 4895A/G variants were: 5′-GATGAAGGCTCAGGGTGCTAGAGG (forward) and 5′-TAGTGCTGTCAGCCCCATTGTCCC (reverse) for the 3813A/G variant; 5′-GAGACCACCCATATCATGCACACA (forward); and 5′- AACTCTGATCCCCAGTAGGCCTAG (reverse) for 4895A/G variant. PCRs were both performed using Taq DNA polymerase (Life Technologies, Inc.-Invitrogen, Carlsbad, CA) with supplied buffer and 58 C annealing temperatures. The amplicons were then digested with an appropriate restriction endonuclease that specifically cleaved one of the two alleles. The restriction endonucleases used were *Hin*fI (for 3813A/G) and *Fok*I (for 4895A/G) (Promega, San Luis Obispo, CA). The restriction fragment length polymorphism sizes were 233 and 114 for 3813A/G variant, and 271 and 75 for 4895A/G variant.

A 10% random sample of the study populations was double genotyped in a blinded fashion with concordant results.

Statistical analysis

Standard statistical genetic methods were used to verify the assumption of Hardy-Weinberg equilibrium. ANOVA was used to compare mean values of phenotypic variables across genotype combinations adjusting for covariates (age; alcohol intake: OPHS, categories of wine consumption; WHSS, grams/day; physical activity: OPHS, categories of time spent daily for walking or biking). The analyses were performed using the Statistical Package for Social Sciences (SPSS-PC version 11; SPSS Inc., Chicago, IL). Results are expressed as means ± SD or 95% confidence intervals, as specified.

The THESIAS program was used to calculate allele frequency, to test for pairwise linkage disequilibrium (LD) and perform haplotype-phenotype association analysis. This program is based on the maximum likelihood model described by Tregouet *et al.* (14). Pairwise LD coefficients between polymorphisms were expressed in term of D′, the ratio of the unstandardized coefficients to their maximal value. Haplotype associations were performed adjusting for age. In all haplotype analyses, the haplotype combining the most frequent alleles at each locus was used as the reference. Because the two SNPs generated three main haplotypes, for the haplotype analysis, P values < 0.025 were considered statistically significant.

Results

OPHS

Table 1 shows the allele and genotype distribution of *CNR1* in the 2002–2004 follow-up examination of the OPHS. For both polymorphisms, genotype distribution was in Hardy-Weinberg equilibrium. The examination of pair-wise LD indicated that the two polymorphisms were in strong LD ($D' = 0.87$; $P < 0.001$). Demographic and anthropometric characteristics of the 360 individuals for whom retrospective data were available did not differ from the whole population, as well as the allele, genotype, and haplotype distribution of *CNR1*.

The 3813A/G polymorphism. Because the GG genotype was present only in 10 individuals, the analysis was performed according to the G dominant model (AG + GG *vs.* AA). Univariate analysis, adjusted for covariates, showed that the 3813G allele was associated with increased WC (99.1 ± 8.8 *vs.* 97.7 ± 8.8 cm; $P = 0.050$), increased SS (24.2 ± 9.1 *vs.* 22.8 ± 7.7 mm; $P = 0.031$), and increased BMI ($P = 0.095$), although not significantly (Table 2). A significant association between the G allele and WC was observed at both 1987 ($P = 0.007$) and 1994–1995 ($P = 0.044$) examinations. When BMI and WC changes over time were evaluated on the subsample previously examined in 1987 and 1994–1995, no significant association with genotype was observed.

TABLE 1. Genotype and allele frequencies of *CNR1* polymorphisms

Localization	Polymorphism	Genotypes			Allele frequency	
		AA	AG	GG	A	G
OPHS						
Exon 4	3813A>G	761	159	10	0.90	0.10
Exon 4	4895A>G	480	347	81	0.72	0.28
WHSS						
Exon 4	3813A>G	175	36	5	0.89	0.11
Exon 4	4895A>G	141	62	13	0.80	0.20

TABLE 2. Anthropometric variables according to the *CNR1* alleles in the OPHS population

	3813A>G			4895A>G		
	AA	AG+GG	*P* value	AA	AG+GG	*P* value
2002–04 follow-up						
No.	759	169		478	428	
Age (yr)	59.5 ± 6.6	60.3 ± 6.7	0.145	59.7 ± 6.6	59.6 ± 6.8	0.762
BMI (kg/m^2)	27.3 ± 3.4	27.8 ± 3.4	0.095	27.3 ± 3.3	27.6 ± 3.4	0.322
WC (cm)	97.7 ± 8.8	99.1 ± 8.8	0.050	97.7 ± 8.7	98.6 ± 8.7	0.123
SS (mm)	22.8 ± 7.7	24.2 ± 9.1	0.031	23.0 ± 7.9	23.3 ± 8.2	0.644
Retrospective analysis						
1987 follow-up						
No.	276	67		195	148	
Age (yr)	46.7 ± 5.6	46.5 ± 5.7	0.734	46.5 ± 5.6	46.9 ± 5.9	0.437
BMI (kg/m^2)	26.3 ± 3.0	27.3 ± 4.8	0.034	26.5 ± 3.9	26.5 ± 2.9	0.992
WC (cm)	92.7 ± 7.3	95.2 ± 6.6	0.007	92.7 ± 7.3	93.9 ± 7.2	0.131
1994–95 follow-up						
No.	286	71		198	159	
Age (yr)	51.3 ± 6.9	52.2 ± 7.1	0.136	51.4 ± 6.8	51.5 ± 7.3	0.939
BMI (kg/m^2)	27.0 ± 3.1	27.9 ± 2.9	0.215	27.1 ± 3.2	27.0 ± 2.9	0.846
WC (cm)	95.1 ± 8.5	97.3 ± 7.6	0.044	95.4 ± 8.5	95.8 ± 8.1	0.716

Except for number, values are mean ± SD. *P* value for ANOVA (adjusted for age, physical activity, and alcohol intake).

The 4895A/G polymorphism. No association was observed between the genotypes of the 4895A/G polymorphism and any of the quantitative variables investigated (whatever the genetic model, dominant, recessive, or additive) (Table 2).

Haplotype analysis. Due to the strong LD between polymorphisms, three frequent haplotypes were inferred, $A_{3813}A_{4895}$ (AA), $A_{3813}G_{4895}$ (AG), and $G_{3813}G_{4895}$ (GG), with frequency 0.712, 0.193, and 0.086, respectively. The estimated frequency of the $G_{3813}A_{4895}$ (GA) haplotype was less than 1%. Therefore, the 3813G allele was mainly carried by the GG haplotype. In agreement with the univariate analysis, the GG haplotype was associated with increased WC (+1.78 cm, 95% confidence interval 0.29–3.28; $P = 0.019$) by comparison to the AA haplotype and with increased SS ($P = 0.027$).

When the haplotype analysis was repeated in the retrospective subgroup, the GG haplotype was significantly associated with higher WC at the 1987 ($P = 0.004$) and 1994–1995 ($P = 0.021$) follow-up.

WHSS

The genotype distribution did not deviate from Hardy-Weinberg equilibrium. Allele frequencies were similar to those observed in the OPHS sample (Table 1). The examination of pairwise LD indicated that the two polymorphisms were in LD ($D' = 0.67$; $P < 0.001$). No age differences were observed between genotypes (3813: AA, 50.4 ± 5.6 yr; AG+GG, 50.8 ± 5.5 yr; 4895: AA, 50.4 ± 5.8 yr; AG+GG, 50.6 ± 5.3 yr).

The 3813A/G polymorphism. The 3813G allele was found to be associated with WC and BMI. Carriers of the G allele had higher values of WC and BMI than noncarriers (96.8 ± 11.3 *vs.* 91.6 ± 10.4 cm, $P = 0.006$; and 27.4 ± 4.2 *vs.* 25.6 ± 4.0 kg/m^2, $P = 0.012$, respectively).

The 4895A/G polymorphism. Significant association was observed between the 4895G allele and WC (AG+GG: 94.6 ± 11.7 cm; AA: 91.5 ± 10.1 cm; $P = 0.047$), while the association with BMI did not reach statistical significance (AG+GG: 26.7 ± 4.6 kg/m^2; AA: 25.5 ± 3.8 kg/m^2; $P = 0.06$).

Haplotype analysis. The haplotype structure deriving from these two SNPs was very similar to that observed in the OPHS sample, with estimated haplotype frequencies of 0.769, 0.123, 0.028, and 0.078 for AA, AG, GA, and GG haplotypes, respectively. In agreement with the univariate analysis, the GG haplotype was associated with higher WC ($P = 0.045$), BMI ($P = 0.033$), although these *P* values are above the threshold of statistical significance for haplotype analysis ($P < 0.025$).

Discussion

This study provides novel demonstration of statistically significant associations between the 3813A/G polymorphism, that lies in sequences encoding 3′ untranslated regions of the exon 4 of *CNR1*, or another ungenotyped functional SNP in strong LD with it, and BMI and body fat distribution in man. These associations were observed in a relatively large sample of an adult white male population from Southern Italy and were confirmed in an independent, albeit smaller, adult white population sample in the United Kingdom. Carriers of the 3813G allele had a higher level of total body fat and central fat deposition. Even though the association of the 3813G allele was observed under the assumption of dominance, haplotype analysis, considering the LD between the two SNPs being studied, and assuming additive haplotype effects, only confirmed the results of the univariate analysis by showing that the unique haplotype carrying the 3813G allele was consistently associated with the same phenotypes. Although in this case haplotype analysis did not add any significant information compared with the univariate analysis, it was nevertheless useful to exclude the existence of interactions between genotypes.

These results suggest a contribution of genetic variations at the *CNR1* locus to the interindividual variability of body fat accumulation and body fat distribution, a process resulting from the interaction of multiple genetic and lifestyle factors (15).

The interest in the endogenous cannabinoid system his-

torically derives from its neuromodulatory properties, the molecular bases of which have been elucidated by the discovery of the first cannabinoid receptor, CB1, in the early 1990s (16). Several lines of evidence suggested that this receptor might be implicated in the vulnerability to drug addictions (8). Indeed, variants of the gene encoding for the human CB1 have been described to be associated with psychiatric conditions and substance abuse patterns in diverse ethnic groups. In particular, the exon 4 haplotype of *CNR1*, 1359A/3813A/4895G displayed a significant allele frequency difference between African-American abusers and controls (8).

In the last years, a wealth of data indicated that the endogenous cannabinoid system is also an important homeostatic modulator of energy balance and weight control through appetite-stimulating mechanisms as well as other physiological pathways, such as peripheral lipogenesis (17, 18). More recently, growing evidence has accumulated in favor of the presence of the CB1 receptor at multiple peripheral sites, including adipose tissue, skeletal muscle, liver, pancreas, and gastrointestinal tract (1–3, 18). Recent reviews have discussed the evidence for endocannabinoids as potent determinants of feeding behavior and, ultimately, body weight regulation (1–3). The pharmacological blockade of the CB1 with rimonabant (SR141716) reduces food intake and body weight in animal models of obesity (19, 20). More recently, results of phase III clinical trials have shown that prolonged treatment with rimonabant was an effective additional measure to reduce body weight and abdominal fat, and to improve the cardiometabolic risk profile in obese patients (4–6).

Against this background, we have hypothesized that genetic variation at *CNR1* might play a role in interindividual differences in body fat mass and distribution, and focused our attention on two polymorphisms of the exon 4 haplotype previously described in association with substance abuse phenotypes (8).

The present study has some strengths and limitations. It was conducted on a large and well-characterized sample of Italian middle-aged men, the OPHS, and the findings were consistently replicated in a subsequent analysis of a sample of white United Kingdom men of the WHSS. In both the populations examined, the 3813A/G polymorphism, and the GG haplotype as well, were consistently associated with diverse measures of obesity. Although these measures are highly interrelated, they cover different aspects of body fatness. In particular, WC provides a reasonably accurate estimate of visceral fat and is helpful for the identification of individuals at higher risk for the metabolic syndrome (21). Of importance, the findings obtained in the OPHS cohort at 2002–2004 follow-up examination were corroborated by the data of retrospective analyses on a representative subsample examined on two previous occasions. The retrospective data suggest that the influence of genetic variations at *CNR1* occurs earlier in life, and support the evidence implicating this locus with fat deposition and distribution in men.

The first limitation of our study is inherent to the use of genetic association for the study of common multifactorial conditions, such as obesity (22). However, in the present study, the SNPs genotyped [two of three previously identified by Zhang *et al.* (8) as risk haplotype for polysubstance abuse in African-Americans] may be considered representative for variations at the *CNR1* locus because they are tag SNPs of this gene for Caucasian populations (http://gvs.gs.washington.edu/GVS/). Although we recognize that also genotyping 1359A/G would have possibly increased the power to identify a risk haplotype in our population samples, we selected 3813A/G and 4895A/G because these two tag SNPs are maximally informative sites at the *CNR1* locus, according to the tag SNPs approach (9), and show the higher degree of correlation (r^2 value) in Caucasian populations (*i.e.* the higher probability to belong to the same cluster).

Second, we studied only white men, so caution is needed in generalizing our conclusions to other populations.

In summary, this study has shown for the first time the association of variants of the CB1 gene (*CNR1*) with obesity related phenotypes in men. Although statistical associations do not prove cause-effect relationships, these data support the hypothesis that genetic variations at the *CNR1* locus might involve an alteration of endocannabinoid signaling that in turn may end in quantitative and qualitative differences in body fat accumulation ultimately accounting for the phenotypic expression of several metabolic risk factors.

Acknowledgments

We thank the research teams of the Olivetti Prospective Heart Study and the Wandsworth Heart and Stroke Study.

Received November 16, 2006. Accepted March 27, 2007.

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The OPHS was supported in part by MIUR (Italian Ministry of University and Research, COFIN 2004 and FIRB 2001). The WHSS has received support from the Wandsworth Health Authority, the South West Thames Regional Health Authority, the NHS R&D Directorate, the British Heart Foundation, the British Diabetic Association, and The Stroke Association. The funding agencies had no role in the design and conduct of the study; in the collection, analysis, and interpretation of the data; or in the preparation, review, or approval of the manuscript.

Disclosure Statement: P.R., D.A.T., F.L., M.L., G.B., M.V, and A.S. have nothing to declare. P.S. received lecture fees from Sanofi-Aventis. F.P.C. was on the advisory board of Sanofi, Sankyo, Pfizer, consults for Brita, and received lecture fees from Pfizer, Schering-Plough.

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