Cannabinoid type-1 receptor gene polymorphisms are associated with central obesity in a Southern Brazilian population

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Abstract. The CB1 cannabinoid receptor and its endogenous ligands, the endocannabinoids, are involved in energy balance control, stimulating appetite and increasing body weight in wasting syndromes. Different studies have investigated the relationship between polymorphisms of the cannabinoid receptor 1 (\textit{CNR1}) gene and obesity with conflicting results. In the present study, we investigated the 1359G/A (rs1049353), 3813A/G (rs12720071) and 4895A/G (rs806368) polymorphisms in the \textit{CNR1} gene in a Brazilian population of European descent. To verify the association between these variants and obesity-related traits in this population, 756 individuals were genotyped by PCR-RFLP methods. The 4895G allele was associated with waist to hip ratio (WHR) ($P = 0.014$; $P = 0.042$ after Bonferroni correction). An additive effect with the GAA haplotype was associated with WHR ($P = 0.028$), although this statistical significance disappeared after Bonferroni correction ($P = 0.084$). No significant association was observed between the genotypes of the 1359G/A and 3813A/G polymorphisms and any of the quantitative variables investigated. Our findings suggest that \textit{CNR1} gene polymorphism is associated with central obesity in this Brazilian population of European ancestry.

Keywords: Obesity, \textit{CNR1}, polymorphisms, WHR, BMI

1. Introduction

The endocannabinoid (EC) system is an endogenous and physiological system that plays a key role in the regulation of food intake and fat accumulation. It can also modulate glucose and lipid metabolism through its effects on peripheral tissues. At least two 6-protein-coupled cannabinoid receptors have been identified [7, 26]. The first cannabinoid receptor, which is the most abundant in the brain, was named CB1 after the cloning of the second cannabinoid receptor subtype CB2, mostly present in immune cells [15]. CB1 is expressed abundantly in several areas of the central nervous system. These areas include the hippocampus, ganglia, cerebro cortex, cerebellum, limbic system and hypothalamus [16]. Early studies showed a scattered presence of CB1 receptors in peripheral organs, such as the adipose tissue and gastrointestinal system, which are key organs involved in the regulation of food intake [3, 25]. On the other hand, CB2 does not play a part in this regulation system.

Obesity is a major predisposing factor for several chronic diseases including coronary artery disease and diabetes. It arises from complex interactions between
genetics and environment that alter the delicate mechanism regulating energy expenditure and food intake. Feeding control determines the sensation of satiety and hunger through an interplay mechanism between internal signals (i.e. leptin) and environmental factors. The endocannabinoid system may influence food intake by regulating the expression and/or action of several hypothalamic anorectic and orexigenic mediators [6,14]. Signals of peripheral origins modulate the neurochemical activation of hypothalamic neurons. CB1 and endocannabinoids are able to cross-talk to those signals, such as leptin and adiponectin, secreted by adipocytes. In the central nervous system (CNS), endocannabinoids can act as neurotransmitters, representing an important modulatory mechanism of neuronal transmission. In the hypothalamus, the changes in endocannabinoid levels seem to be inversely correlated with some changes that are known to occur in leptin blood levels [21]. Indeed, leptin decreases endocannabinoid levels in the hypothalamus, as much as it does for other orexigenic mediators. On the other hand, animal models of obesity (ob/ob, db/db mice or fa/fa Zucker rats) have increased endocannabinoid levels [8]. Moreover, ventromedial hypothalamic administration of anandamide (one CB1 endogenous ligand) in pre-satiated rats resulted in CB1 dependent hyperphagia [17]. These findings are in agreement with the devastating impact that the disruption of CB1 signaling has on feeding and growth in young mice [9]. Recently, it has been demonstrated that CB1 mRNA co-expresses with corticotrophin releasing hormone (CRH), cocaine-amphetamine related transcript (CART), melanin concentrating hormone (MCH) and prepro-orexin in hypothalamic neurons [6].

All these data taken together highlight the role of the endocannabinoid system in feeding and energy balance regulation. Indeed, it was reasonable to hypothesize that from a pharmacological point of view, inhibition of the endocannabinoid system through antagonism of the cannabinoïd CB1 receptor may be an effective therapeutic approach to treat human obesity.

The cannabinoid receptor 1 (CNR1) gene was mapped to chromosome 6q14-15 [13]. Several human CNR1 gene polymorphisms have been reported. Among them a silent mutation resulting in the substitution from G to A nucleotide position 1359 (rs1049353) in codon 453 (Thr) [11] and two exon 4 variants in the 3’UTR region, 3813A/G (rs12720071) and 4895A/G (rs806368) [33]. Although two previous studies have shown association of the 1359G/A variant with obesity-related traits in European populations [12,23], two other studies failed to confirm this association [1,22]. The two exon 4 polymorphisms were recently associated with abnormal body mass index (BMI) and body fat distribution in Italian middle-aged man [28]. Since the importance of the aforementioned gene variants to the development of obesity in human populations is still a matter of controversies, the aim of this study was to determine whether genetic polymorphisms at the CNR1 gene contribute to variation in obesity-related phenotypes in an European-derived Southern Brazilian population, and to examine these results in the context of current findings related to the proposed functions for these endocannabinoids.

2. Material and methods

2.1. Subjects

The study was performed in 756 individuals of European descent residing in Porto Alegre, the capital of Brazil’s southernmost state. This city was founded in 1752 by 60 Caucasian couples from the Azores Islands. Currently the population is still mainly of Portuguese descent, but Italians, Spaniards and Germans have also contributed to its gene pool [29]. The 447 women and 309 men most of them of low socio-economic status, were selected at random at the Clinical Analysis Laboratory of the Pharmacy School of the Federal University of Rio Grande do Sul among those who came from several city health centers for free routine blood determinations. The sample collection was performed between 2001–2002. The Ethics Committee of the Federal University of Rio Grande do Sul approved the study protocol and all individuals gave their written informed consent prior to the investigation. A questionnaire was completed, including smoking habits, alcohol consumption and regular physical activity in leisure time. Exclusion criteria were occasional smoking, secondary hyperlipidemia due to renal, liver or thyroid disease, and pregnant women. In addition, individuals with a previous history of diabetes mellitus or fasting glucose levels above 6.9 mmol/l [2] were excluded from the analyses to avoid the confounding effects of diabetes and its treatment on obesity-related traits.

2.2. Anthropometric measurements and biochemical analysis

The subjects attended the laboratory in the morning after a 12 h fast. Blood samples were collected and weight and height were measured. Height was
measured to the nearest centimeter using a rigid stadiometer. Weight was measured to the nearest 0.1 Kg using an electronic calibrated balance scale. Body mass index (BMI) was expressed as weight (Kg) divided by the square of height (m²). Waist circumference was measured at mid-level between the lower rib margin and the iliac crest [31], and hip circumference at the widest trochanters to the nearest mm; all measurements were performed by the same person. Overweight was defined for a BMI over 25 Kg/m² and obesity for a BMI over 30 Kg/m², according to the World Health Organization criteria [32]. Total cholesterol (TC), HDL cholesterol, triglycerides (TG) and glucose were determined by conventional enzymatic methods. LDL cholesterol was calculated according Friedewald et al. [10].

2.3. DNA analysis

Genomic DNA was extracted from 5ml anticoagulated venous blood samples using a salting out method [19]. The polymorphic sites 1359G/A, 3813A/G and 4895A/G at the CNR1 gene were genotyped by PCR-RFLP methods as previously described [11,28]. Oligonucleotides used to amplify these variants were: forward 5'-GAAAGCTGCAATCAAGA GCC-3' and reverse 5'-TTTTCTGTGCTGCAGG C-3' for the 1359G/A variant; forward 5'-GATGAAGG CTCAGGGTGCTAGAGG-3' and reverse 5'-TAGTGCT TGTCAGCCCCATGTCCC-3' for the 3813A/G variant; forward 5'-GAGACACCCCATATCATGCACAC A-3' and reverse 5'-AATCTGATCCCCAGTGGCC TAG-3' for the 4895A/G variant. The amplicons were digested with restriction endonucleases MspI (for 1359G/A), HinfI (for 3813A/G) and FokI (for 4895A/G).

2.4. Statistical analysis

Allele frequencies were estimated by gene counting. The agreement of genotype frequencies to Hardy-Weinberg expectations was tested using the goodness of fit chi-square test. Allele and genotype frequencies between normal-weight (BMI<25 kg/m²) and overweight/obese (BMI≥25 kg/m²) groups were compared by chi-square tests.

Multiple linear regression analyses were used to model BMI and WHR on genotypes. These analyses were performed with BMI and WHR as dependent variables and age, physical activity, smoking, alcohol consumption, gender, HDL and LDL cholesterol, triglycerides and glycaemia as covariates. Age and serum lipids as well as glucose concentrations entered as continuous variables, and lifestyle factors were included as dichotomic variables. Interactions between SNPs and all covariates were tested. The statistical analysis was performed using the SPSS® 13.0.0 package.

Haplotype frequencies and linkage disequilibrium were estimated using the Multiple Locus Haplotype Analysis (version 2.0) [20]. Haplotype associations with BMI and WHR were performed by multiple linear regression analysis as described above. Two sided p-values <0.05 were considered for statistical significance. Bonferroni correction for multiparametric testing was applied when appropriate.
observed in normal weight individuals (as expected, a smaller waist to hip ratio (WHR) were observed between male and female subjects but, in genotype and allele frequencies, BMI and smoking

vestigated (Table 2). The same result was obtained

weight/obese groups for the three polymorphisms in-

cies were observed between normal-weight and over-

founders are shown in Tables 3, 4 and 5 for 1359 G

3813 A

polymorphisms and other potential con-

CNR1

G

A

A

G

G

A

A

A

G

Table 2

Genotype and allele frequencies of CNRI 1359G/A, CNRI 3813 A/G and CNRI 4895 A/G gene variants in normal-weight (BMI <25) and obese plus overweight (BMI ≥25) individuals

| Genotypes   | BMI <25 | BMI ≥25 | Alleles | BMI <25 | BMI ≥25 |
|-------------|---------|---------|---------|---------|---------|
| CNRI 1359 G/A |         |         |         |         |         |
| A/A         | 16 0.05 | 18 0.04 | A 0.22  | 0.22    |
| A/G         | 106 0.34| 152 0.36| G 0.78  | 0.78    |
| G/G         | 199 0.61| 255 0.60| P-value 1.0 |
| CNRI 3813 A/G |       |         |         |         |         |
| A/A         | 248 0.79| 335 0.78| A 0.89  | 0.89    |
| A/G         | 62 0.20 | 92 0.21 | G 0.11  | 0.11    |
| G/G         | 3 0.01  | 2 0.01  | P-value 1.0 |
| CNRI 4895 A/G |       |         |         |         |         |
| A/A         | 167 0.53| 217 0.50| A 0.73  | 0.71    |
| A/G         | 130 0.41| 179 0.42| G 0.27  | 0.23    |
| G/G         | 20 0.06 | 34 0.08 | P-value 0.45 |

n = number of individuals.

f = genotype frequencies.

a χ²-test between allele frequencies.

3. Results

Anthropometric, biological and lifestyle characteristics of the investigated subjects are summarized in Table 1. The individuals were between 15 and 85 years old (44.1 ± 15.1), being 40.9% males. Most individuals (42.6%) had a normal weight (BMI <25 kg/m²), while 36.9% could be classified as overweight and 20.5% were obese (BMI ≥30 kg/m²). No differences in genotype and allele frequencies, BMI and smoking were observed between male and female subjects but, as expected, a smaller waist to hip ratio (WHR) was observed in normal weight individuals (P < 0.0001).

No differences in genotype and/or allele frequencies were observed between normal-weight and overweight/obese groups for the three polymorphisms investigated (Table 2). The same result was obtained when comparing normal and obese individuals (BMI ≥30 kg/m²; data not shown). All genotypes were in Hardy-Weinberg equilibrium.

Multiple linear regression analysis of BMI and WHR on the CNRI polymorphisms and other potential founders are shown in Tables 3, 4 and 5 for 1359 G>A, 3813 A>G 4895 A>G polymorphisms respectively. These analyses were performed with BMI and WHR as continuous traits using the whole sample. Since the less frequent alleles for the three CNRI polymorphisms were present only in few individuals, the analyses were performed according to a dominant model pooling heterozygous and less frequent allele homozygous individuals. When controlled by founders, the 4895G allele was associated with WHR (P = 0.014; P = 0.042 after Bonferroni correction; statistical pow-
er = 0.71), but not with BMI (P = 0.958) (Table 5). The multiple R² of the regression model with the CNRI 4895 polymorphism and covariates was 0.368; the partial R² for CNRI 4895 was 0.008. The effect of the gene can also be evaluated through the difference between the adjusted means of the genotypes. The WHR mean ± SE were: 0.877 ± 0.003 for homozygous AA individuals, and 0.889 ± 0.003 for AG + GG individuals. The difference between means was 0.012 (CI 95%: 0.0024–0.0211). No significant association was observed between the genotypes of the 1359G/A and 3813A/G polymorphisms and any of the quantitative variables investigated (Tables 3 and 4). No second order interactions were observed between each SNP and covariates (data not shown).

The examination of pair-wise linkage disequilibrium (LD) indicated that the three polymorphisms were in strong LD (1359G/A and 3813A/G – D’ = 0.87 and P < 0.0001; 1359G/A and 4895A/G – D’ = 0.88 and P < 0.0001; 3813A/G and 4895A/G – D’ = 0.76 and P < 0.0001). Due to this strong LD between polymorphisms, three frequent haplotypes accounted for 88% of the chromosomes investigated, G1359A3813A4895 (GAA), G1359A3813G4895 (GAG) and A1359A3813A4895 (AAA), frequencies were 0.489, 0.182 and 0.212, respectively. The other five haplotypes accounted together for less than 12% of the observed chromosomes. Because the haplotype derived from the three wild alleles corresponds to almost 50% of the haplotype frequencies, we tested the association assuming an additive effect of this haplotype GAA (absent, one copy or two copies) with BMI and WHR using a multiple linear regression analysis. An association
Table 3
Multiple Linear Regression of BMI and WHR with CNR1 1359G/A genotypes (GG versus AG plus AA) and potential confounders

| Variables          | BMI         |          | WHR         |          |
|--------------------|-------------|----------|-------------|----------|
| CNR1 1359A        | 0.234       | 0.336    | 0.487       | 0.003    | 0.005    | 0.519 |
| Age (years)       | 0.021       | 0.012    | 0.092       | 0.001    | 0.0001   | 0.0001 |
| Smoking           | −0.405      | 0.347    | 0.243       | 0.008    | 0.005    | 0.123 |
| Alcohol           | −0.024      | 0.352    | 0.946       | −0.011   | 0.005    | 0.029 |
| Physical inactivity| −0.186    | 0.344    | 0.589       | 0.015    | 0.005    | 0.004 |
| Gender            | −0.661      | 0.365    | 0.07        | 0.065    | 0.005    | 0.0001|
| HDL-Chol (mg/dL)  | −0.055      | 0.015    | 0.0001      | −0.0007  | 0.0001   | 0.002 |
| LDL-Chol (mg/dL)  | 0.008       | 0.004    | 0.054       | 0.00005  | 0.0001   | 0.415 |
| Triglycerides (mg/dL) | 0.011    | 0.002    | 0.0001      | 0.0001   | 0.0001   | 0.001 |
| Glycaemia (mg/dL) | 0.078       | 0.014    | 0.0001      | 0.0007   | 0.0001   | 0.001 |

with WHR was observed ($P = 0.028$) (Table 6), although this P-value is above the threshold of statistical significance after Bonferroni correction ($P = 0.084$). These results suggest that the presence of the block carrying the wild 4895A allele was associated with a smaller WHR, as the association at the single SNP level pointed to a higher WHR for 4895G allele carriers. Therefore a contribution of a genetic variant at CNR1 gene in central obesity is suggested although it does not exclude the effect of other polymorphisms not investigated in the present study.

4. Discussion

We have genotyped three SNPs in the CNR1 gene in a sample of individuals from Southern Brazil of European ancestry. Evidence of association of 4895A/G variant and WHR was detected which was consistent in single SNP and haplotype analyses. Although it is not expected for any of these three SNPs to be functional, it is possible that we have detected an association through linkage disequilibrium of nearby polymorphisms.

Obesity was measured using body mass index (BMI) and waist to hip ratio (WHR). The measurement of WHR was chosen instead of waist circumference (WC) because it has been suggested to be a superior predictor of cardiovascular disease associated with abdominal obesity, as long as it includes hip circumference, which is inversely associated to metabolic dysfunction, hypertension, and death [18].

Five previous publications [1,12,22,23,28] have examined the possible association of CNR1 polymorphisms with obesity. The association between the 1359A allele with lower BMI found by Gazzerro et al. [12] has not been replicated in two other investigations from different populations [1,22]. Russo et al. [28] reported an association of the 3813G allele with several obesity related traits including waist circumference but not with the 4895 A>G polymorphism. In the present study, we observed an association of the 4895 A>G polymorphism with waist to hip ratio (WHR). Peeters et al. [23] recently reported an association of the CNR1 1359G/A variant with abdominal adiposity. In obese men, CNR1 1359G/A genotype was significantly associated with higher WHR and waist circumference. In the current study, we demonstrated that genetic vari-
Table 5
Multiple Linear Regression of BMI and WHR with CNR1 4895A/G genotypes (AA versus AG plus GG) and potential confounders

| Variables          | BMI |                      |                      | WHR |                      |                      |
|--------------------|-----|-----------------------|----------------------|-----|-----------------------|----------------------|
|                    | B   | Std. Error            | P-value              | B   | Std. Error            | P-value              |
| CNR1 4895G         | 0.018 | 0.332               | 0.958               | 0.012 | 0.005               | 0.014               |
| Age (years)        | 0.024 | 0.012               | 0.052               | 0.001 | 0.0001               | 0.0001              |
| Smoke              | −0.367 | 0.349               | 0.294               | 0.009 | 0.005               | 0.076               |
| Alcohol            | 0.07 | 0.355               | 0.644               | −0.012 | 0.005               | 0.022               |
| Physical inactivity | −0.119 | 0.346               | 0.731               | 0.015 | 0.005               | 0.003               |
| Gender             | −0.579 | 0.368               | 0.116               | 0.067 | 0.005               | 0.0001              |
| HDL-Chol (mg/dL)   | −0.049 | 0.015               | 0.001               | −0.0006 | 0.0001               | 0.007               |
| LDL-Chol (mg/dL)   | 0.009 | 0.004               | 0.043               | 0.00006 | 0.0001               | 0.331               |
| Triglycerides (mg/dL) | 0.012 | 0.002               | 0.0001              | 0.0001 | 0.0001               | 0.0001              |
| Glycaemia (mg/dL)  | 0.04 | 0.011               | 0.0001              | 0.0004 | 0.0001               | 0.015               |

Table 6
Multiple Linear Regression of BMI and WHR with GAA haplotype (additive effect) and potential confounders

| Variables          | BMI |                      |                      | WHR |                      |                      |
|--------------------|-----|-----------------------|----------------------|-----|-----------------------|----------------------|
|                    | B   | Std. Error            | P-value              | B   | Std. Error            | P-value              |
| Haplotype GAA      | 0.021 | 0.234               | 0.929               | −0.008 | 0.003               | 0.028               |
| Age (years)        | 0.023 | 0.012               | 0.061               | 0.001 | 0.0001               | 0.0001              |
| Smoke              | −0.39 | 0.349               | 0.265               | 0.009 | 0.005               | 0.081               |
| Alcohol            | 0.042 | 0.355               | 0.905               | −0.012 | 0.005               | 0.025               |
| Physical inactivity | −0.132 | 0.346               | 0.703               | 0.015 | 0.005               | 0.003               |
| Gender             | −0.549 | 0.367               | 0.135               | 0.067 | 0.005               | 0.0001              |
| HDL-Chol (mg/dL)   | −0.048 | 0.015               | 0.001               | −0.0006 | 0.0001               | 0.007               |
| LDL-Chol (mg/dL)   | 0.009 | 0.004               | 0.037               | 0.00005 | 0.0001               | 0.375               |
| Triglycerides (mg/dL) | 0.012 | 0.002               | 0.0001              | 0.0002 | 0.0001               | 0.0001              |
| Glycaemia (mg/dL)  | 0.04 | 0.011               | 0.0001              | 0.0004 | 0.0001               | 0.016               

ation at CNR1 gene might play a role in abdominal obesity. Although we did not find an association with this polymorphism, these data reinforce the relevant effect of the CNR1 gene in abdominal fat accumulation and distribution. These inconsistencies among studies could be explained by different genetic backgrounds or environmental factors, as for other gene variants, the impact of environmental factors such as diet, exercise, and climatic temperatures may obscure the expression of detectable and consistent phenotypes across human populations. Nevertheless these results taken together suggest a role for these CNR1 polymorphisms on appetite control, although caution is needed in reporting which polymorphism might be involved.

A recent investigation showed that CNR1 mRNA expression was negatively correlated with visceral fat mass. They also identified endocannabinoid plasma concentrations, in addition to body fat mass, as significant predictors of CB1 receptor gene expression. Once CB1 gene expression in visceral and subcutaneous adipose tissues were closely correlated, it suggest that CB1 gene expression in subcutaneous adipose tissue may be an acceptable surrogate for CB1 gene expression in visceral adipose tissue [4]. Although it is more likely to expect that genetic variation contributes to decrease rather than increase the receptor activity, leading to underweight and anorexia instead of overweight and obesity, this finding suggests that the association of the 4895G allele with WHR observed in the present investigation could be due to a higher expression of the CNR1 gene. Meanwhile, no functional studies were carried out to test if this gene is differentially expressed according to 4895A/G or other gene variants. Because many of the regulatory regions of the gene are largely unknown, and the inconsistencies of which polymorphism is associated with obesity related traits, it is likely that the functional polymorphisms have yet to be identified.

The present study has some limitations. When studying polygenic traits, it is important to keep in mind that, generally, each gene contributes with a small to moderate percentage of the trait variance, which implies that the same disorder in different groups may be caused by different combinations of polygenes [5]. This fact could explain the small effect of the CNR1 4895 polymorphism in the regression model, in spite of the statistical significance. Although these polymorphisms may be considered representative for variations...
at this locus [11,33], the observed associations could be spurious (type I error) due to multiple statistical comparisons. We performed the Bonferroni procedure for multiple testing corrections. However, when the probability of the type I error (false positive) decreases, the probability of the type II error (false negative) increases. The appropriate application of multiple testing corrections is not always clear [24] and therefore the two P-values (before and after corrections) are shown. Indeed, independent replication of significant associations in different populations is an important aspect of a credible genetic association result.

In summary, the results presented here showed evidence for the association of CNR1 gene variation with central obesity phenotype. As only few recent studies showed association of the CNR1 polymorphisms and obesity-related traits, in the current study we preferred to consider our findings as exploratory. Despite of our results being consistent with the hypothesis that the CNR1 gene is a genetic contributor to abdominal obesity, replications in other populations are obviously warranted.

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