Are endocannabinoid type 1 receptor gene (*CNR1*) polymorphisms associated with obesity and metabolic syndrome in postmenopausal Polish women?

Short title: CNR1 polymorphism and postmenopausal obesity

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Abstract

Objective: The aim of this study was to determine whether genetic variation at the cannabinoid receptor-1 (CNR1) locus could have an effect on adiposity, fat distribution and obesity-related metabolic disorders in Polish postmenopausal women.

Design and Subjects: The A3813G, G1422A and A4895G single nucleotide polymorphisms of CNR1 were genotyped in 348 randomly selected postmenopausal women aged 50-60 years recruited from the Wroclaw city population.

Measurements: CNR1 genotypes, anthropometric measures (BMI, WC, body fat distribution by DEXA) and metabolic parameters (glucose, lipid profile, insulin FIRI) were determined.

Results: The 3813G allele was not significantly associated with higher body mass, BMI, WC, total fat, or fat percentage, but was associated with higher android fat deposit (2971.78 ± 1655.08 ± 2472.64 ± 1300.53, $p = 0.007$) and percentage of android fat (37.59 ± 8.45 *vs*. 35.66 ± 7.63, $p = 0.062$). The 1422A allele was associated with higher total fat (31587.72 ± 9161.28 g *vs*. 26078.26 ± 7552.14 g, $p = 0.019$), fat percentage (40.51 ± 5.66% *vs*. 37.51 ± 4.99%, $p = 0.052$), and percentage of android fat (40.86 ± 9.73% *vs*. 36.09 ± 7.70%, $p = 0.047$). No associations were observed for the A4895G variant.

Conclusions: There is an association of variants of CNR1 with obesity-related phenotypes in Polish postmenopausal women. As CB1 is a drug target for obesity, pharmacogenetic receptor gene analysis of obesity treatment by endocannabinoid blockade may be of interest to identify the best responders.

Key words: CNR1 polymorphism, obesity, metabolic syndrome, postmenopausal women

Introduction

Menopausal obesity is a multifactorial disease. The energy imbalance that results in menopausal obesity occurs as a result of genetic background, hormonal changes, and acquired changes in eating behavior and physical activity (1).

The endocannabinoid system comprises two cannabinoid receptor subtypes, CB1 and CB2, their endogenous ligands and the enzymes for ligand biosynthesis and degradation (2). The CB1 receptor, encoded by the *CNR1* gene, has the greatest expression in the central nervous system but also in several peripheral organs including liver, muscle, and adipose tissue (3-6). Evidence suggests that CB1 and its endogenous ligands, the endocannabinoids, are involved in the regulation of energy balance, influencing feeding behavior and body weight (4-6).

The endocannabinoid receptor type 1 gene, CNR1, is located on 6q14-q15. Several human *CNR1* polymorphisms have been recently described in European-American, African- American, and Japanese individuals in association with polysubstance abuse (7). Thus polymorphic variants of this gene may contribute to individual differences in susceptibility to obesity, changes in body fat distribution, and related metabolic disorders. Russo et al. showed that genetic variants of *CNR1* were associated with obesity related phenotypes in men (8), Peeters et al. demonstrated that G1422A polymorphism in the CNR1 gene was associated with increased abdominal adiposity in obese men (9) and Benzinou et al. showed that CNR1 variations increased the risk for obesity and modulated BMI in a European population (10). These observations led us to hypothesize that genetic variation at the cannabinoid receptor-1 (CNR1) locus could have an effect on adiposity, fat distribution and obesity-related metabolic disorders in postmenopausal women.

Materials and Methods

The study population consisted of 348 randomly selected postmenopausal women aged 50-60 years (mean age 55.42±2.73 years) recruited from the Wroclaw city population with BMIs ranging from 17.5 to 46.38 kg/m^2 (mean 27.48±4.78 kg/m^2). Only women with a history of natural menopause not taking hormonal replacement therapy were included. None of the women smoked, were on special diets, or used medications influencing lipid profile or glucose metabolism. Women with diabetes

mellitus, previously recognized dyslipidemia, or liver dysfunction were excluded from the study. The patients were informed of the aim and the methods of the study and gave their informed consent in writing. The study protocol was approved by the Ethics Committee of Wroclaw Medical University, Poland.

Anthropometric measurements

Anthropometric parameters, such as body mass, body height, and waist circumference were measured in all subjects. Body mass and height were measured to the nearest 0.1 kg, and height was measured to the nearest centimeter crown to heel without shoes. Waist circumference was measured at half distance between the costal angle and the iliac crests to the nearest 0.1 cm with a flexible inextensible plastic tape, with the subject standing straight with the abdomen relaxed, arms at the sides, and feet together. The body mass index (BMI) was calculated from the equation: body mass [kg]/height-squared [m^2].

The percentage of body fat and visceral fat deposit were assessed using the dual-energy X-ray absorptiometry (DXA) method using a DPX (+) Lunar device (USA). The percentage of abdominal fat (android fat deposit) was calculated using a computerized method after measuring fat tissue volume in the area between the upper edge of the L2 disc and the lower edge of the L4 disc. This region is characterized by a high amount of visceral and subcutaneous fat, which play a crucial role in insulin resistance and the development of metabolic syndrome (11,12).

Biochemical measurements

Blood for laboratory tests was collected between 7:00 and 9:00 a.m. after overnight fasting. Centrifuged serum for biochemical analyses and whole blood for genomic studies were stored until examination at -20°C. Serum concentrations of glucose (G), total cholesterol, HDL cholesterol, and triglycerides were measured by routine enzymatic methods (Olympus Au 560; bioMerieux, France). LDL cholesterol was calculated according to the Friedewald formula: cholesterol LDL [mg/dl] = total cholesterol [mg/dl] – HDL cholesterol [mg/dl] – (triglycerides [mg/dl]/5). The serum insulin (I) concentration was measured by a radioimmunological method using commercial kits (Diagnostic

Products Corporation, USA). For the estimation of insulin resistance, Fasting Insulin Resistance Index (FIRI) was calculated as fasting glucose [mmol/l] x fasting insulin [(mU/l]/25.

Genetic studies

Whole genomic DNA was isolated from blood leukocytes using standards methods. *CNR1* genotyping (A3813G, G1422A, and A4895G) was performed by polymerase chain reaction (PCR) and minisequencing.

To amplify the three fragments of *CNR1* gene (347-bp, 346-bp, and 231-bp) was used multiplex PCR mix was employed containing: the specific three pairs of primers (see Table 1), 1x PCR buffer, 1.5mM $MgCl_2$, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 200 µM dTTP, 1x Q solution, 2 polymerase units (QIAGEN), 200 ng genomic DNA, and water for a total volume of 20 µl.

The DNA was denatured at 95^{o}C for 3 minutes followed by 35 cycles of denaturation at 95^{o}C for 30 seconds, annealing at 58^{o}C for 30 seconds, and extension at 72^{o}C for 45 seconds.

The amplified fragments were purified from oligonucleotides and free dNTPs by SAP and ExoI treatment (Fermentas).

The minisequencing method was based on the incorporation of single fluorescence-labeled dideoxynucleotides to the 3' end of the oligonuclotide that was correctly paired to the specific template DNA fragment using a SNaPshot kit (Applied Biosystems). The SNaPshot reaction was carried out using the oligonucleotides:

- A3813G: 5'-CTTGTTATGGTAGAAAAATTTCACG-3'
- G1422A: 5'-TGCAGCCAGTGTTCACAGGGCCGCAGAAAGCTGCATCAAGAGCAC-3'
- A4895G: 5'-TTAAGATGCCACGGCAATGTAAAGAAACTCTCCCA-3'

designed so that it ended immediately before the polymorphic side .The SNaPshot reaction consisted of 25 cycles: denaturation at 96^{o}C for 10 seconds, annealing at 50^{o}C for 5 seconds, and extension at 60^{o}C for 30 seconds. The product was analyzed by an ABI 310 sequencer (Applied Biosystems).

Statistical analysis

Means and standard deviations of some physical and biochemical variables were calculated for genotypes of analysed polymorphisms. To compare means between genotypes the ANOVA and post-hoc test (LSD) were used. Frequencies of risk factors in genotypes were compared by the chi-square test.

Results

We investigated, whether specific CNR1 polymorphisms are associated with anthropometric markers of obesity and fat distribution as well as parameters describing metabolic syndrome in Polish postmenopausal women.

The A3813G polymorphism. Genetic analysis for this polymorphism was performed in 346 women. The AA genotype was found in 70%, AG in 22% and GG in only 8% of the women. The frequency of the A allele was p= 0.81 and of the G q= 0.19 (p+q=1).

Because the GG genotype was present in only 8% of the individuals, the analysis was performed according to the G dominant model (AG + GG *vs.* AA). The whole group was divided into two subgroups according to genotype (AG + GG *vs.* AA). The anthropometric and metabolic characteristics of these subgroups are presented in Tables 2 and 3.

Although ANOVA analysis, adjusted for covariates, indicated that the G allele was associated with higher body mass, BMI, WC, total fat, and fat percentage, statistical significance was not observed. A significant positive association between the G allele and fat distribution was observed, with the G allele carriers having significantly higher android fat deposit (2971.78 ± 1655.08 ± 2472.64 ± 1300.53, $p = 0.007$) and percentage of android fat (37.59 ± 8.45 *vs*. 35.66 ± 7.63, $p = 0.062$) (Table 2). The latter difference did not achieve statistical significance.

No significant association between genotype and metabolic parameters was observed (Table 3).

The G1422A polymorphism. Genetic analysis for this polymorphism was performed in 333 women. The GG genotype was found in 62%, GA in 33% and AA in only 5% of the women. The frequency of the G allele was p= 0.79 and of the A q= 0.21 (p+q=1).

Because the AA genotype was present in only 5% of the individuals, the analysis was performed according to the G dominant model (AG + AA *vs.* GG), and indicated no association between the genotypes and any of the quantitative variables investigated (data not shown).

Analysis was also performed according to the A recessive model (AG + GG *vs.* AA). The whole group was divided into two subgroups according to genotype (AG + GG *vs.* AA). The anthropometric and metabolic characteristics of these subgroups are presented in Tables 4 and 5.

ANOVA analysis, adjusted for covariates, indicated that the G allele was associated with lower body mass, although not statistically significantly. There were no differences in BMI between the subgroups, although it appeared that AA carriers were significantly taller, which could have affected differences in BMI.

A significant positive association between the AA genotype and fat distribution was observed: the AA carriers had higher total fat (31587.72 ± 9161.28 *vs*. 26078.26 ± 7552.14 g, $p = 0.019$), fat percentage (40.51 ± 5.66% *vs*. 37.51 ± 4.99%, $p = 0.052$, on the border of statistical significance), and percentage of android fat (40.86 ± 9.73% *vs*. 36.09 ± 7.70%, $p = 0.047$).

The A4895G polymorphism . Genetic analysis for this polymorphism was performed in all women studied. The AA genotype was found in 57%, AG in 33% and GG in 10% of the women. The frequency of the A allele was p= 0.74 and of the G q= 0.26 (p+q=1).

No association was observed between the genotypes of the A4895G polymorphism and any of the quantitative variables investigated, whatever the genetic model, i.e. dominant or recessive (data not shown).

Discussion

Recent data suggest that the endocannabinoid system controls food intake via central, and lipogenesis via peripheral CB1 receptors (4-6). Pharmacological blockade of endocannabinoid receptor 1 leads to weight loss and an improved metabolic risk profile in overweight and obese individuals (13-15). Thus it was hypothesized that genetic variation at the cannabinoid receptor-1 (CNR1) locus could have an effect on adiposity. Many investigators have suggested than an association exists between various CNR1 gene polymorphisms and parameters of obesity in various

populations (8,9,10,16,17). On the other hand, Lieb et al. who studied 18 single-nucleotide polymorphisms in the CNR1 gene in 2415 Framingham Offspring Study participants, found no evidence for an association of CNR1 polymorphisms with BMI, waist circumference, or visceral or subcutaneous adipose tissue (18).

In our study, we examined three CNR1 polymorphisms: A3813G, G1422A and A4895G. We observed that the G allele of the A3813G polymorphism was associated with higher body mass, BMI, WC, total fat, and fat percentage, although the differences were not statistically significant. Furthermore, G allele carriers had significantly higher android fat deposit. Similar observations were made in Italian and English men by Russo et al. (8), and in a French population by Benzinou et al. (10), while Jaeger et al. observed no significant association between the 3813A/G polymorphism and any of the obesity-related traits in a Brazilian population (16).

In our study carriers of the AA genotype of the G1422A polymorphism were characterized by higher total fat, fat percentage, and percentage of android fat. Peeters et al. found that the CNR1 A1422A genotype was significantly associated with higher WHR, WC, and fat mass percentage, but only in obese men. In obese women, no meaningful associations between CNR1 genotype and anthropometric parameters were found (9). Associations between the genotypes of the A4895G polymorphism and the investigated anthropometric variables were observedneither in our study nor in the studies of other authors (8). One explanation for the differences in results observed in the previously mentioned studies may relate to population differences. For example, in our study only white postmenopausal women were considered. Therefore generalization of these conclusions to other populations should be done with caution.

What is more, body fat accumulation and body fat distribution result from the interaction of multiple genetic and lifestyle factors (1,19,20).

Clinical trials have shown that prolonged treatment with rimonabant, a pharmacological blocker of the CB1, not only effectively reduced body weight and abdominal fat, but also improved the metabolic profile in obese patients (13-16). Thus we hypothesized that genetic variations at the

CNR1 locus might lead to an alteration of endocannabinoid signaling that in turn may lead to differences in body fat accumulation finally resulting in phenotypic expression of a metabolic disorder. However, no association between any of the polymorphic variants of CRN1 and metabolic parameters was observed in our study in contrast to the study of Aberle et al. (21). These workers observed that carriers of at least one A allele of the G1359A polymorphism in the CB1 gene following 3 months of a low-fat caloric restricted diet lost more weight and reduced LDL cholesterol more than wild-type patients (21). They concluded that genetic polymorphisms associated with obesity may become relevant only under the condition of a low-calorie diet and that the presence of certain genotypes may be associated with a more beneficial response to obesity treatment.

In summary, our study showed an association of variants of CNR1 with obesity-related phenotypes in Polish postmenopausal women. As CB1 is a drug target for obesity, pharmacogenetic receptor gene analysis may be of interest to identify the best responders to endocannabinoid blockade therapy for obesity.

Conflict of interest

Authors declare there are not any competing financial interests in relation to the work described.

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TABLE 1.

The specific pairs of primers used for the amplification of *CNR1* gene (347-bp and 346-bp).

	SNP	**Forward primer**	**Revers primer**
1	A3813G	5'-GATGAAGGCTCAGGGTGCTAGAGG-3'	5'-TAGTGCTGTCAGCCCCATTGTCCC-3'
2	G1422A	5'-CCTGCGACACGCTTTCCGGA-3'	5'-CTGCCAGGGAGGCATCAGGC-3'
3	A4895G	5'-GAGACCACCCATATCATGCACACA-3'	5'-AACTCTGATCCCCAGTAGGCCTAG-3'

TABLE 2. Anthropometric variables according to the *CNR1* A3813G alleles in Polish postmenopausal women.

	AA	AG + GG	*p* value
No. (%)	241 (70%)	105 (30%)	
Age (yr.)	55.59 ± 2.68	55.15 ± 2.84	0.167
Body mass (kg)	70.12 ± 11.31	71.74 ± 12.87	0.242
Height (m)	160.33 ± 5.28	160.50 ± 5.63	0.779
BMI (kg/m^2)	27.33 ± 4.57	27.92 ± 5.24	0.296
WC (cm)	87.13 ± 11.37	88.67 ± 12.21	0.281
Total fat (g)	25948.29 ± 7479.67	27323.31 ± 8394.61	0.174
Fat (%)	37.38 ± 4.87	38.20 ± 5.67	0.220
Android deposit (g)	2472.64 ± 1300.53	2971.78 ± 1655.08	**0.007**
Android deposit (%)	35.66 ± 7.63	37.59 ± 8.45	0.062
Gynoid deposit (g)	6639.84 ± 1759.95	6543.93 ± 2507.68	0.716

Except for number, values are mean ± SD. *p* value for ANOVA

TABLE 3. Metabolic variables according to the *CNR1* A3813G alleles in Polish postmenopausal women

	AA	AG + GG	*p* value
No. (%)	241 (70%)	105 (30%)	
Glucose [mg/dl]	87.68 ± 9.57	89.28 ± 11.97	0.193
Total cholesterol [mg/dl]	244.71 ± 40.85	246.42 ± 48.02	0.735
HDL cholesterol [mg/dl]	70.80 ± 17.57	69.74 ± 16.33	0.604
LDL cholesterol [mg/dl]	153.42 ± 39.27	153.73 ± 40.91	0.947
Triglycerides [mg/dl]	106.02 ± 47.54	114.86 ± 52.86	0.129
Insulin [µIU/ml]	6.38 ± 4.04	6.93 ± 4.69	0.279
FIRI	1.23 ± 0.88	1.35 ± 1.07	0.266

Except for number, values are mean ± SD. *p* value for ANOVA

TABLE 4. Anthropometric variables according to the *CNR1* G1422A alleles in Polish postmenopausal women.

	AA	AG + GG	*p* value
No. (%)	244 (70%)	104 (30%)	
Age (yr.)	54.93 ± 2.71	55.44 ± 2.73	0.465
Body mass (kg)	75.18 ± 15.29	70.19 ± 11.52	0.097
Height (m)	162.93 ± 6.19	160.12 ± 5.13	**0.035**
BMI (kg/m^2)	28.25 ± 5.08	27.44 ± 4.77	0.511
WC (cm)	91.12 ± 13.51	87.32 ± 11.48	0.201
Total fat (g)	31587.72 ± 9161.28	26078.26 ± 7552.14	**0.019**
Fat (%)	40.51 ± 5.66	37.51 ± 4.99	0.052
Android deposit (g)	3185.51 ± 1454.89	2604.58 ± 1433.83	0.189
Android deposit (%)	40.86 ± 9.73	36.09 ± 7.70	**0.047**
Gynoid deposit (g)	7893.93 ± 2422.82	6522.90 ± 1963.45	**0.025**

Except for number, values are mean ± SD. *p* value for ANOVA

TABLE 5. Metabolic variables according to the *CNR1* G1422A alleles in Polish postmenopausal women

	AA	AG + GG	*p* value
No. (%)	244 (70%)	104 (30%)	
Glucose [mg/dl]	85.73 ± 6.83	88.36 ± 10.61	0.342
Total cholesterol [mg/dl]	244.80 ± 47.84	244.93 ± 43.46	0.990
HDL cholesterol [mg/dl]	71.26 ± 15.80	70.25 ± 17.24	0.824
LDL cholesterol [mg/dl]	151.46 ± 46.28	153.34 ± 39.65	0.858
Triglycerides [mg/dl]	110.40 ± 45.09	109.14 ± 49.47	0.923
Insulin [µIU/ml]	7.19 ± 3.78	6.65 ± 4.32	0.625
FIRI	1.26 ± 0.83	1.30 ± 0.96	0.883

Except for number, values are mean ± SD. *p* value for ANOVA