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Effects of Polymorphism rs3123554 in the Cannabinoid Receptor Gene Type 2 (*Cnr2*) on Body Weight and Insulin Resistance after Weight Loss with a Hypocaloric Mediterranean Diet

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Abstract

Background: There is few evidence of CNR2 SNPs and obesity. The role of CNR2 gene variants on weight loss after a dietary intervention remained uninvestigated.

Objective: Our aim was to analyze the effects of rs3123554) of *CNR2* receptor gene polymorphism on body weight, metabolic parameters and serum adipokine levels after a Mediterranean hypocaloric diet.

Design: A Caucasian population of 82 obese patients was analyzed before and after 3 months on a Mediterranean hypocaloric diet.

Results: In non A allele carriers, the decrease in weight -3.2 ± 1.9 kg (-2.2 ± 1.0 kg : p=0.02), BMI -1.0 ± 0.1 kg (-1.2 ± 0.5 kg : p=0.01), fat mass -2.5 ± 1.0 kg (-1.2 ± 0.8 kg : p=0.003), waist circumference -2.9 ± 1.1 cm (-2.1 ± 3.1 cm : p=0.004), systolic blood pressure were -5.9 ± 3.9 mmHg (-2.9 ± 2.2 mmHg), total cholesterol -25.1 ± 5.3 mg/dl (-6.4 ± 4.7 mg/dl : p=0.005), LDL- cholesterol -19.1 ± 9.5 mg/dl (-6.2 ± 8.5 mg/dl : p=0.003), glucose -5.2 ± 2.5 mg/dL (-0.1 ± 1.1 mg/dL : p=0.004), insulin -2.8 ± 1.3 mUI/L (-0.2 ± 1.0 mUI/L:p=0.01), HOMA-IR -0.9 ± 0.3 (± 0.1 ± 0.1 : p=0.01), IL-6 -0.7 ± 0.5 ng/dL (-0.1 ± 0.4 ng/dL:p=0.02) and CRP -2.8 ± 1.5 ng/dL (± 0.1 ± 0.2 ng/dL:p=0.02) were higher than A allele carriers.

Conclusion: Non A allele carriers has a better improvement after a Mediterranean hypocaloric diet in body weight, fat mass, waist circumference, level of insulin, HOMA-IR, total cholesterol, LDL cholesterol, IL-6 and CRP than A allele carriers.

Keywords: Adipokines; Cannabinoid receptor gene type 2; Obesity; Mediterranean diet; Rs3123554

Introduction

Obesity is a major health problem in industrialized countries, leading to increased morbidity from metabolic syndrome, diabetes mellitus type 2, stroke, coronary heart disease and cancer [1]. Genetic and environmental factors play important roles in the development of obesity. The increasing prevalence of obesity is caused by the excessive calorie intake and diminished physical activity in our modern environment. However, available evidence also suggests a significant genetic contribution to adiposity [2]. During the last decades, increasing numbers of genetic loci associated with obesity and/or body mass index (BMI) have been identified as a result of the genome-wide association study (GWAS). These loci include; fat related obese gene (FTO), melanocortin receptor subtype 4 (Mc4R), brain derived neutrophic factor (BDNF), cannabinoid receptors (CNRs) and so on [3].

The important role played by the endocannabinoid system is an interesting area of investigation. The cannabinoid receptor system consists of two receptors (CNR1 and CNR2). CNR1 is mainly located in the brain: its role in eating behavior is well-established [4]. In contrast, CNR2 has been referred to as the peripheral cannabinoid receptor isoform that is mainly expressed in cells of the immune system. This endogenous cannabinoid system consists of endogenous ligands 2-arachidonoylglycerol (2-AG) and anandamide (ADA), and the above-mentioned two types of G-protein-coupled cannabinoid receptor. A greater insight into the endocannabinoid system has been derived from studies in animals with a genetic deletion of the CNR1 receptor, which have a lean phenotype and are resistant to diet-induced obesity [5]. Otherwise, a single nucleotid polymorphism (SNP) rs1049353 of the *CNR1* gene resulting in the substitution of the

G to A at nucleotide position 1359 in codon 435 (Thr), was reported as a common SNP in Caucasian population [6], with metabolic implications. Few SNPs have been described in *CNR2* gene [7]. For example, Ketterer et al. [7] have found that carriers of minor allele (A) of this SNP showed an inverse relation with body weight. As far as we know the role of *CNR2* gene variants on weight loss after a dietary intervention remained uninvestigated.

Our aim was to analyze the effects of rs3123554) of *CNR2* receptor gene polymorphism on body weight, metabolic parameters and serum adipokine levels after a Mediterranean hypocaloric diet in obese subjects.

Subjects and Methods

Subjects and procedure

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and our Hospital ethics committee approved all procedures involving patients. All participants provided

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informed consent. A population of 82 Caucasians subjects with obesity (body mass index >30) and without diabetes mellitus was analyzed in an interventional study. The recruitment of subjects was a non-probabilistic method of sampling among patients send from Primary Care Physicians with obesity. Inclusion criteria were body mass index > 30 and absence of a diet during the 6 months previously to the study. Exclusion criteria included history of cardiovascular disease or stroke during the previous 24 months, total cholesterol > 200 mg/dl, triglycerides > 250 mg/dl, blood pressure > 140/90 mmHg, as well as the use of metformin, sulfonylurea, dypeptidil type IV inhibitors drugs, thiazolidinedions, insulin, glucocorticoids, antineoplasic agents, angiotensin receptor blockers, angiotensin converting enzyme inhibitors, psychoactive medications, statins and other lipid drugs.

Procedure

Basal fasting glucose, c-reactive protein (CRP), insulin, insulin resistance (HOMA-IR), total cholesterol, LDL-cholesterol, HDL-cholesterol, plasma triglycerides concentration and adipokines (leptin, adiponectin, resistin, interleukin 6 and TNF-alpha) were measured within the start of the trial and repeated after 3 months of dietary intervention. Weight, height, and blood pressure measures were measured within the start of the trail and repeated 3 months of intervention. A tetrapolar bioimpedance was realized in order to measure fat mass. These measures were realized at same time of the day (morning). Genotype of CNR2 receptor gene polymorphism was studied.

Dietary intervention: The lifestyle modification program was a Mediterranean hypocaloric diet (1508 calories per day) during three months, the distribution of macronutrient was; 52% of carbohydrates, 25% of lipids and 23% of proteins. Distribution of fats was: 50.7% of monounsaturated fats, 38.5% of saturated fats and 11.8% of polyunsaturated fats. Diet was enriched with olive oil (30 ml per day), 3 servings of oily fish a week, 30-40 g 3 servings nuts days a week, vegetables 3-4 servings per day. The adherence of this diet was assessed each 14 days with a phone call by a dietitian in order to improve compliment of the calorie restriction, macronutrient distribution and Mediterranean diet recommendations. All enrolled subjects received instruction to record their daily dietary intake for three days including a weekend day. Records were reviewed by a dietitian and analysed with a computer-based data evaluation system. National composition food tables were used as reference [8].

Biochemical assays: Serum total cholesterol and triglyceride concentrations were determined by enzymatic colorimetric assay (Technicon Instruments, Ltd., New York, N.Y., USA), while HDL cholesterol was determined enzymatically in the supernatant after precipitation of other lipoproteins with dextran sulfate-magnesium. LDL cholesterol was calculated using Friedewald formula [9]. Plasma glucose levels were determined by using an automated glucose oxidase method (Glucose analyser 2, Beckman Instruments, Fullerton, California).

Insulin was measured by RIA (RIA Diagnostic Corporation, Los Angeles, CA) with a sensitivity of 0.5 mUI/L (normal range 0.5-30 mUI/L) [10] and the homeostasis model assessment for insulin resistance (HOMA-IR) was calculated using these values [11]. CRP was measured by immunoturbimetry (Roche Diagnostics GmbH, Mannheim, Germany), with a normal range of (0-7 mg/dl) and analytical sensivity 0.5 mg/dl.

Adipokine assays: Resistin was measured by ELISA (Biovendor Laboratory, Inc., Brno, Czech Republic) with a sensitivity of 0.2 ng/

ml with a normal range of 4-12 ng/ml [12]. Adiponectin was measured by ELISA (R&D systems, Inc., Mineapolis, USA) with a sensitivity of 0.246 ng/ml and a normal range of 8.65-21.43 ng/ml [13]. Leptin was measured by ELISA (Diagnostic Systems Laboratories, Inc., Texas, USA) with a sensitivity of 0.05 ng/ml and a normal range of 10-100 ng/ml [14]. Interleukin 6 and TNF alpha were measured by ELISA (R&D systems, Inc., Mineapolis, USA) with a sensitivity of 0.7 pg/ml and 0.5 pg/ml, respectively. Normal values of IL6 was (1.12-12.5 pg/ml) and TNF-alpha (0.5-15.6 pg/ml) [15].

Genotyping: Oligonucleotide primers and probes were designed with the Beacon Designer 5.0 (Premier Biosoft International ®, LA, CA). The polymerase chain reaction (PCR) was carried out with 50 ng of genomic DNA, 0.5 uL of each oligonucleotide primer (primer forward: 5'- ACGTTGGATGATTGTACCGAGGAGGGAACT-3' and reverse 5'- ACGTTGGATGGAGACACGTATTCTAGTCCC-3' in a 2 uL final volume (Termociclador Life Tecnologies, LA, CA). DNA was denaturated at 95ºC for 3 min; this was followed by 45 cycles of denaturation at 95ºC for 15 s, and annealing at 59.3º C for 45 s). The PCR were run in a 25 uL final volume containing 12.5 uL of IQTM Supermix (Bio-Rad®, Hercules, CA) with hot start Taq DNA polymerase Hardy Weimberg equilibrium was assessed with a statistical test (Chi-square) to compare our expected and observed counts. The two variants were in Hardy Weimberg equilibrium (p=0.03).

Blood pressure determination and anthropometric measurements: The same investigator evaluated all patients. Blood pressure was measured twice after a 10 minutes rest with a random zero mercury sphygmomanometer, and averaged (Omrom, LA, CA). Body weight was measured to an accuracy of 0.1 Kg and body mass index computed as body weight/(height²). Waist (narrowest diameter between xiphoid process and iliac crest) and hip (widest diameter over greater trochanters) circumferences to derive waist-to hip ratio (WHR) were measured, too. Tetrapolar body electrical bioimpedance was used to determine body composition with an accuracy of 50 g [16].

Statistical analysis

Sample size was calculated to detect differences over 3 kg in body weight loss with 90% power and 5% significance (n=80). The Kolmogorov–Smirnov test was used to determine variable distribution. The results were expressed as average ± standard deviation. Quantitative variables with normal distribution were analyzed with a two-tailed Student's-t test. Non-parametric variables were analyzed with the U-Mann-Whitney test. Qualitative variables were analyzed with the chi-square test, with Yates correction as necessary, and Fisher's test. A Chi square test was used to evaluate the Hardy–Weinberg equilibrium. The statistical analysis was performed for the combined *AA* and *AG* as a group (mutant) and GG genotype as second group (wild), with a dominant model. A p-value <0.05 was considered significant.

Results

Eighty-two obese subjects gave informed consent and were enrolled in the study. The mean age was 49.1 ± 7.3 years and the mean body mass index (BMI) 34.8 ± 4.1 kg/m². Thirty five patients (42.7%) had the genotype *GG* and 47 (57.3%) subjects had the next genotypes; *GA* (29 patients, 35.4%) or *GG* (18 study subjects, 22.0%) (second group). Age was similar in both groups (49.3 ± 8.2 years vs 48.9 ± 4.2 years: ns). Sex distribution was similar in both groups, males (20.0% vs 27.7%) and females (80.0% vs 72.3%). All patients completed the 3-month follow-up period without drop-outs.

After 3 months of intervention, all patients achieved dietary recommendations in both genotype groups without statistical differences

between calorie intake (wild genotype: 1503.1 ± 287.1 kcal/day vs mutant genotype: 1490.3 ± 212.8 kcal/day). Macronutrient intakes were similar; (wild genotype: 50.4% from carbohydrates vs 50.6% mutant genotype), (wild genotype: 25.2% from fats (50.1% of monounsaturated fats, 37.9% of saturated fats and 13.0% of polyunsaturated fats) vs 25.1% mutant genotype (50.2% of monounsaturated fats, 39.0% of saturated fats and 11.8% of polyunsaturated fats) and (wild genotype: 24.4% from proteins vs 24.3% mutant genotype).

Anthropometric characteristics of participants at baseline and at month 3 of intervention are shown in Table 1. In wild and mutant genotype groups, body weight, body mass index (BMI), fat mass, waist circumference and systolic blood pressure decreased. Before and after dietary intervention, body weight, BMI, fat mass and waist circumference were higher in A allele carriers than non A allele carriers. In non A allele carriers, the decrease in body weight was -3.2 ± 1.9 kg (A allele group -2.2 ± 1.0 kg:p=0.02), BMI -1.0 ± 0.1 kg (A allele group -1.2 ± 0.5 kg:p=0.01), fat mass -2.5 ± 1.0 kg (A allele group -1.2 ± 0.8 kg : p=0.003) and waist circumference -2.9 ± 1.1 cm (A allele group -2.1 ± 3.1 cm : p=0.004). These improvements were higher in non A allele carriers than A allele carriers. In non A allele carriers, the decrease in systolic blood pressure were -5.9 ± 3.9 mmHg (A allele carriers -2.9 ± 2.2 mmHg). These improvements were higher in non A allele carriers than A allele carriers, too. No differences were detected among other variables after weight loss.

Table 2 shows the classic cardiovascular risk factors. No differences were detected among baseline and post-treatment values of variables between both genotypes. In non A allele carriers and after dietary treatment, glucose, total cholesterol, LDL cholesterol, insulin and HOMA-IR decreased. Total Cholesterol and LDL cholesterol improved in A allele carriers, too. In non A allele carriers, the decrease in total cholesterol was -25.1 ± 5.3 mg/dl (A allele carriers -6.4 ± 4.7 mg/dl:p=0.005) and LDL- cholesterol decrease was -19.1 ± 9.5 mg/dl (A allele carriers -6.2 ± 8.5 mg/dl:p=0.003). Glucose -5.2 ± 2.5 mg/dL (A allele group -0.1 ± 1.1 mg/dL:p=0.004), insulin -2.8 ± 1.3 mUI/L (in A allele -0.2 ± 1.0 mUI/L:p=0.01) and HOMA-IR -0.9 ± 0.3 (A allele group ± 0.1 ± 0.1:p=0.01) improvement was higher in non A allele carriers than A allele carriers, too.

Table 3 shows levels of adipokines and inflammatory status. No differences were detected among baseline and post-treatment values of adipokines and inflammatory parameters between both genotypes. Leptin levels decrease in both genotypes after dietary treatment. IL-6 (-0.7 ± 0.5 ng/dL in non A allele carriers vs -0.1 ± 0.4 ng/dL in A allele carriers:p=0.02) and CRP (-2.8 ± 1.5 ng/dL in non A allele carriers vs ± 0.1 ± 0.2 ng/dL in A allele carriers:p=0.02) improvement were higher in non A allele carriers than A allele carriers. Other adipokines and inflammatory parameters remained unchanged in both groups.

Discussion

To the best of our knowledge, this is the first study that analyzes the effects of a Mediterranean hypocaloric diet and the *CNR2* gene variant rs3123554 on body weight loss and subsequent changes of metabolic parameters and adipokines. In addition, non A-allele carriers showed a better response of glucose, total cholesterol, LDL cholesterol, HOMA-IR, insulin, IL-6 and CRP levels than A-carriers. Secondly, A allele carriers had higher body weight, fat mass and waist circumference than non-carriers.

The observed percentage of minor allele carriers over 50% was similar than other studies [7]. Actually, the scientific evidence supports that endocannabinoid system with both receptors (CNR1 and CNR2)

is been positioned for regulation of endocannabinoid levels that could influence craving and reward behaviors through the relevant neuronal circuitry and metabolic parameters [17]. CNR2 has long been referred to as the peripheral cannabinoid receptor isoform. Interestingly, there is now evidence of CNR2 expression in different areas of the brain [18]. In our interventional study, we found the significant association of the SNP with obesity and metabolic parameters in the cross sectional cohort. Recently, a potential role of cerebral CNR2 receptors has been hypothesized in the modulation of body weight. For example, central CNR2 over expression leads to a lean phenotype in mice [19] and secondly, CNR2 activation in humans seems to influence eating behavior [20].

Surprisingly, we found that the minor allele of rs31235554 – that was associated with higher body weight in the baseline- led to a significantly reduced loss of body weight during dietary intervention, and this effect was independent of the body weight at baseline. Perhaps, the effect of this SNP on central insulin action could explain our results

Parameters	GG		(GA or AA)	
	0 time	At 3 months	0 time	At 3 months
BMI(kg/m²)	34.3 ± 5.5	32.8 ± 5.1*	35.0 ± 4.9$	33.7 ± 4.2*$
Weight (kg)	87.6 ± 18.7	84.2 ± 17.3*	90.1 ± 16.1$	88.4 ± 16.8*$
Fat mass (kg)	37.2 ± 11.2	35.9 ± 10.8*	38.3 ± 16.1$	36.8 ± 11.8*$
WC (cm)	105.8 ± 15.4	102.1 ± 15.3*	106.4 ± 9.6$	104.3 ± 9.1*$
WHR	0.92 ± 0.08	0.91 ± 0.09	0.90 ± 0.1	0.89 ± 0.1
SBP (mmHg)	131.9 ± 9.9	128.6 ± 10.3*	133.5 ± 11.7	126.7 ± 12.5*
DBP (mmHg)	82.4 ± 7.5	80.4 ± 9.1	84.4 ± 7.0	84.2 ± 9.3

SBP: Systolic Blood pressure. DBP: Diastolic Blood Pressure. WC: Waist circumference. Waist to hip ratio ($) (p<0.05) Statistical differences between genotype groups. (*) (p<0.05) Statistical differences in the same genotype group after dietary intervention.

Table 1: Changes in anthropometric variables after a hypocaloric meditteranean diet.

Parameters	GG		(GA or AA)	
	0 time	At 3 months	0 time	At 3 months
Glucose (mg/dl)	100.6 ± 9.5	95.1 ± 8.8*	92.3 ± 8.4	92.1 ± 7.2
Total ch. (mg/dl)	212.5 ± 40.6	188.3 ± 21.3*	218.8 ± 31.7	212.4 ± 20.1*
LDL-ch. (mg/dl)	129.5 ± 24.7	103.1 ± 21.8*	146.0 ± 33.2	139.1 ± 21.3*
HDL-ch. (mg/dl)	53.5 ± 10.1	54.0 ± 9.8	53.9 ± 12.4	54.1 ± 11.5
TG (mg/dl)	130.5 ± 30.3	128.1 ± 23.4	127.5 ± 22.1	116.9 ± 31.3
Insulin (mUI/L)	13.6 ± 7.2	10.8 ± 7.1*	14.5 ± 6.2	14.3 ± 8.1
HOMA-IR	3.5 ± 2.0	2.6 ± 1.8*	3.8 ± 2.1	3.9 ± 2.0

Ch: Cholesterol. TG: Triglycerides CRP: C reactive protein. HOMA-IR: Homeostasis model assessment. (*) (p<0.05) Statistical differences in the same genotype group after dietary intervention. No statistical differences between genotype groups.

Table 2: Clasical cardiovascular risk factors after a hypocaloric meditteranean diet.

Parameters	GG		GA or AA	
	0 time	At 3 months	0 time	At 3 months
IL 6 (pg/ml)	1.50 ± 1.2	0.68 ± 0.9 *	1.31 ± 1.1	1.33 ± 1.2
TNF-œ (pg/ml)	7.01 ± 3.0	7.11 ± 2.1	7.12 ± 3.1	7.34 ± 2.2
CRP (mg/dl)	8.9 ± 1.1	6.1 ± 1.0*	5.3 ± 2.0	5.5 ± 1.1
Adiponectin (ng/ml)	26.7 ± 10.4	22.4 ± 9.8	26.1 ± 11.3	25.9 ± 11.3
Resistin (ng/ml)	4.1 ± 1.2	3.8 ± 1.2	4.20 ± 1.2	4.42 ± 1.3
Leptin (ng/ml)	81.9 ± 32.4	62.1 ± 23.1*	80.7 ± 19.8	69.9 ± 30.6

IL6: Interleukin 6, CRP: C reactive protein. TNF-œ: Tumor necrosis factor alpha (*) (p<0.05) Statistical differences in the same genotype group after dietary intervention. No statistical differences between genotype groups.

Table 3: Circulating adypocitokines and inflammatory status after a hypocaloric meditteranean diet.

[7], because insulin action is an important regulator of body weight [21]. Ketterer et al. [7] have found that carriers of minor allele of this SNP showed lower cerebral insulin sensitivity. In contrast of the insulin effects on peripheral tissues, cerebral insulin sensitivity rather facilities body weight loss during dietary interventions [22]. One could speculate that altered cerebral insulin sensitivity in carriers of the minor allele of rs3123554 may be responsible for lower body weight loss than non-carriers.

The different metabolic response in our study after body weight could be explained by a double mechanism. First of them, it is the better body weight loss in subjects without minor allele after a hypocaloric Mediterranean diet. Interactions between weight loss secondary to a hypocaloric Mediterranean diet and other polymorphism has been yet described [23]. A second hypothesis could be due to peripheral metabolic mechanisms, because CNR2 has been isolated in organs important for the control of metabolism like adipose tissue, liver and skeletal muscle [24]. Only one previous study has explored relation of rs3123554 with body weight or metabolic parameters [7]. Ketterer et al. [7] showed an inverse relation of minor allele of this SNP with body weight. However, this relationship has been described only in females and in a population of obese subjects for high risk for diabetes mellitus type 2 or diagnosis of impaired fasting glycaemia). Our sample has male, females, and diabetes/impaired glycaemia was an exclusion criterion.

In order to show the complex relationships of this receptor with metabolism and the inconsistent results in the literature in this topic area. Recently, the CNR2 rs35761398 polymorphism revealed a significantly earlier age of menarche in subjects carrying the Q63 allele, which was also found after adjusting for BMI. This study reported that patients homozygous for the Q allele had a 2.2-fold higher risk of presenting with an early menarche (age at menarche <12 years) [25]. Finally, rs3003336, rs2501431, rs2502992, rs2501432 SNPs of *CNR2* genes are related in the etiology of osteoporosis and suggest that it may be a genetic risk factor for bone density and osteoporosis in postmenopausal women [26].

Limitations of our study include the lack of a control group without intervention, however strengths are many others as the design of intervention and evaluate a healthy diet as it is a low calorie Mediterranean diet pattern.

In conclusion, our data showed an association between the rs3123554 polymorphism of (*CNR2*) gene and metabolic response after weight loss. Non A allele carriers has a better improvement after a Mediterranean hypocaloric diet in body weight, fat mass, waist circumference, level of insulin, HOMA-IR, total cholesterol, LDL cholesterol, IL-6 and CRP than A allele carriers. Further studies will be needed to explore the relationship of this SNP with the response to other interventions in obese subject such as drugs or bariatric surgery.

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