Effects of C358A polymorphism of the endocannabinoid degrading enzyme fatty acid amide hydrolase (FAAH) on weight loss, adipocytokines levels, and insulin resistance after a high polyunsaturated fat diet in obese patients

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ABSTRACT. ***Background and aims:*** **The C385A polymorphism of FAAH gene (rs324420C>A) has been associated with obesity. We investigate the role of this polymorphism on anthropometric and insulin resistance responses to a high polyunsaturated fat hypocaloric diet.** ***Methods:*** **Obese individuals (no.=99) were assessed at baseline and after 3 months of a high polyunsaturated fat hypocaloric diet.** ***Results:*** **Seventy-one patients (71.7%) had the genotype *C385C* and 28 (28.3%) patients had the *C385A* (26 patients, 26.3%) or *A358A* (2 patients, 2.0%) (A allele carriers group) genotype. In A allele carriers and after dietary intervention, total cholesterol (–16.3±37.4 mg/dl) and LDL-cholesterol (–12.9±6.5 mg/dl) levels decreased. In subjects with C385C genotype, the decreases were significant in total cholesterol (–12.3±27.4 mg/dl), LDL-cholesterol (–7.5±20.5 mg/dl), insulin (–2.2±6.2 mUI/l), and homeostasis model assessment of insulin resistance (HOMA-R) (–0.79±1.15 units) levels. The weight loss was similar in both genotype groups (–4.1±3.8 kg *vs* –4.2±3.2 kg). Only leptin levels had a significant similar decrease in both genotypes.** ***Conclusion:*** **Subjects with C385C genotype of the FAAH showed an improvement on insulin and HOMA-R levels with a high polyunsaturated fat hypocaloric diet after weight loss during 3 months.**

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INTRODUCTION

The endocannabinoid system is involved in the control of food intake and body weight. Obesity and overweight are major public health problems that are estimated to affect >50% of the population and have been linked as risk factors for many common diseases (1).

This endocannabinoid system comprises of a number of proteins involved in endocannabinoid synthesis, degradation, and signaling and has been demonstrated to play a role in appetite and body weight, as mentioned above (2). Fatty acid amide hydrolase (FAAH) inactives the orexigenic effect of the endocannabinoid N-arachidonoylethanolamine (andamide) by a rapid hydrolysis to ethanolamine and arachidonic acid (3, 4). Recently, a polymorphism (cDNA 385 C->A) (rs324420) has been described (5) in this enzyme.

A study in obese and dislypidemic subjects (6) showed that carriers of the A allele had a significantly greater improvement in lipid profile compared to wild type when following a low fat diet. A further study (7) has shown with a conventional hypocaloric diet that in carriers of the A allele this was associated with larger improvements in HDL cholesterol, body weight, and glucose levels. However, in another study (8), the allele A385 of FAAH was associated with a lack of improvement on metabolic parameters after two different hypocaloric diets (low carbohydrate *vs* low fat). Perhaps, the percentage of macronutrients in hypocaloric diets and the type of dietary fat, considering previous studies, may influence secondary metabolic responses to weight loss as a function of this polymorphism. The effect on chronic inflammation in obese patients of polyunsaturated fatty acid intake has been demonstrated (9), and the interaction with polymorphism of this gene has not been evaluated yet. Taking into account the effect of this polymorphism on the lipid pattern and cardiovascular risk of obese patients, it seems necessary to clarify intervention studies potential benefits.

The aim of our study was to investigate the role of this polymorphism on anthropometric and insulin resistance responses to a high polyunsaturated fat hypocaloric diet.

SUBJECTS AND METHODS

Subjects and procedures

A sample of 99 subjects (23 males/76 females) with obesity [body mass index (BMI) >30] was enrolled in a prospective way. The local Ethics Committee (CEIC-HURH) approved the protocol (2-2012 CEIC HURH) and patients approved the use of their genetic material for this study. All patients were recruited in a Nutrition Clinic Unit and signed an informed consent. Exclusion criteria included total cholesterol >300 mg/dl, triglycerides >300 mg/dl, blood pressure >140/90 mmHg, fasting plasma glucose >110 mg/dl, as well as the use of drugs with potential metabolic effects as metformin sulphonilurea, thiazolidinedions, insulin, glucocorticoids, antineoplastic agents, angiotensin receptor blockers, ACE inhibitors, and psychoactive medications.

Weight, blood pressure, basal glucose, c-reactive protein (CRP), insulin, total cholesterol, LDL-cholesterol, HDL-cholesterol, blood

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triglycerides and adypokines [leptin, adiponectin, resistin, tumor necrosis factor α (TNF-α), and interleukin 6 (IL-6)] levels were measured in fasting condition at basal time and at three months, after dietary intervention. During 3 months, the enriched polyunsaturated (PUFA) fat hypocaloric intervention consisted in a diet of 1459 kcal (45.7% of carbohydrates, 34.4% of lipids, and 19.9% of proteins). The distribution of fats was: 21.8% of saturated fats, 55.5% of monounsaturated fats, and 22.7% of polyunsaturated fats (7 g per day of w-6 fatty acids, 2 g per day of w-3 fatty acids, and a ratio w6/w3 of 3.5). A tetrapolar bioimpedance (BIA) and a prospective serial assessment of nutritional intake with 3 days written food records were realized at both times (at basal time and after 3 months). Patients were motorized every 7 days with phone calls and visits every 14 days with attendance from diet adherence. Genotype of FAAH gene polymorphism was studied.

Assays

Plasma glucose levels were determined by using an automated glucose oxidase method (Glucose analyser 2, Beckman Instruments, Fullerton, California) [coefficients of variation of intra-assay (IACV) (1.5%) and inter-assay (IECV) (2.1%)]. Insulin was measured by radioimmunoassay (RIA Diagnostic Corporation, Los Angeles, CA) with a sensitivity of 0.5mUI/l (normal range 0.5-30 mUI/l) (IACV: 1.8% and IECV: 2.5%) (10), and the homeostasis model assessment for insulin resistance (HOMA-R) 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 sensitivity of 0.5 mg/dl with a IACV of 2.1% and a IECV of 2.3%. Leptin was measured by enzyme-linked immunosorbent assay (ELISA) (Diagnostic Systems Laboratories, Inc., Texas, USA) (IACV: 2.4% and IECV: 2.6%). Adiponectin was measured by ELISA (R&D systems, Inc., Minneapolis, USA) with a IACV of 2.3% and a IECV of 2.7%. IL-6 and TNF-α were measured by ELISA (R&D systems, Inc., Minneapolis, USA) (IACV: 2.7% and IECV: 2.1%; and IACV: 3.1% and IECV: 2.4%, respectively). Normal values of IL-6 were 1.12-12.5 pg/ml and TNF-α 0.5-15.6 pg/ml.

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 (12).

Genotyping of FAAH gene polymorphism

Oligonucleotide primers and probes were designed with the Beacon Designer 4.0 (Premier Biosoft International®, LA, CA). PCR was carried out with 50 ng of genomic DNA, 0.5 µl of each oligonucleotide primer (primer forward: 5'-ATG TTG CTG GTT ACC CCT CCT C -3'; primer reverse: 5'-CAG GGA CGC CAT AGA GCT G-3'), and 0.25 µl of each probes (wild probe: 5'-Fam-CTG TCT CAG GCC CCA AGG CAG G-BHQ-1-3') and (mutant probe: 5'-Hex-CTG TCT CAG GCC ACA AGG CAG G -BHQ-1-3') in a 25 uL final volume [Termociclador iCycler IQ (Bio-Rad®), Hercules, CA]. DNA was denaturized at 95 C for 3 min; this was followed by 50 cycles of denaturation at 95 C for 15 sec, and annealing at 59.3 C for 45 sec. The PCR were run in a 25 µl final volume containing 12.5 µl of IQTM Supermix (Bio-Rad®, Hercules, CA) with hot start Taq DNA polymerase. Hardy Weinberg equilibrium was assessed.

Anthropometric measurements and blood pressure

Tetrapolar body electrical bioimpedance (EFG, Akern, It) was used to determine body composition with an accuracy of 50 g (13). Resistance and reactance were used to calculate total body water, fat and fat-free mass. The same investigator measured patients. Precautions taken to insure valid BIA measurements were: no alcohol within 24 h of taking the test, no exercise or food for 4 h before taking the test.

Body weight was measured to an accuracy of 0.1 kg and BMI computed as body weight/(height^2). 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. Blood pressure was measured twice after a 10 min rest with a random zero mercury sphygmomanometer and a large cuff size was, and averaged.

Dietary intake and habits

All enrolled subjects received instruction to record their daily dietary intake for three days including a weekend day. Handling of the dietary data was by means of a personal computer equipped with personal software, incorporating use of food scales and models to enhance portion size accuracy. Records were reviewed by a nutritionist and analyzed with a computer-based data evaluation system. National composition food tables were used as reference (14). Regular aerobic physical activity (walking was allowed, no other exercises) was maintained during the period study for at least 4 times per week (60 min each).

Statistical analysis

Sample size was calculated to detect differences over 3 kg in body weight with 90% power and 5% significance (no.=99). The distribution of variables was analyzed with Kolmogorov-Smirnov test. Quantitative variables were analyzed with a 2-way analysis of variance model with genotype as the intergroup factor and intervention as the intragroup intervention. 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 C385A and A385A as a group and C385C as second group (dominant model). A *p*-value under 0.05 was considered statistically significant.

RESULTS

Ninety-nine patients gave informed consent and were enrolled in the study. The mean BMI was 37.9±5.3 kg/m^2 and mean age was 46.8±10.3 yr, with 23 males (23.2%) and 76 females (76.8%). All patients completed the 3-month follow-up period.

Seventy-one patients (71.7%) had the genotype *C385C* and 28 (28.3%) patients had the *C385A* (26 patients, 26.3%) or *A358A* (2 patients, 2.0%) (A allele carriers group) genotype. Sex distribution was similar in both groups, males (26.8% *vs* 15.4%) and females (73.2% *vs* 84.6%). Age was similar in both groups (C385C genotype: 47.0±17.7 yr *vs* A carriers group: 45.3±14.6 yr: ns). In subjects with C385C genotype, basal assessment of nutritional intake with 3 days written food records showed a caloric intake of 1787.4±497.7 kcal/day, a carbohydrate intake of 194.9±81.7 g/day (43.5% of calories), a fat in-

Table 1 - *Changes in anthropometric variables.*

Characteristics	*C385C*		*C385A* or *A385A*	
	Baseline	At 3 months	Baseline	At 3 months
BMI	37.4±5.3	35.4±5.1*	38.4±7.6	35.9±5.1*
Weight (kg)	95.4±16.3	91.4±16.7*	99.7±17.4	95.5±17.3*
Fat mass (kg)	39.3±11.3	35.4±11.5*	41.2±12.3	37.6±10.2*
Waist circumference	113.2±14	109.6±13*	112.8±11	109.3±11*
Waist to hip ratio	0.92±0.01	0.91±0.08	0.92±0.08	0.90±0.09
Systolic BP (mmHg)	127.7±14.8	122.7±12.4*	127.9±11	123.3±9.8*
Diastolic BP (mmHg)	82.4±9.2	79.1±7.4*	84.1±8.1	80.5±9.6*

BMI: Body mass index. BP: Blood pressure. Data presentation: mean±SD. 2-way analysis of variance model (*)significantly different compared to baseline $p<0.05$.

take of 72.3±29.5 g/day (36.4% of calories), and a protein intake of 85.3±22.1 g/day (21.1% of calories). During dietary intervention, these patients reached the recommendations of diet: 1481.2 calories (45.4% of carbohydrates, 33.8% of lipids and 20.8% of proteins). The distribution of dietary fats was: 20.8% of saturated fats, a 54.2% of monounsaturated fats and a 23.0% of polyunsaturated fats (6.9 g per day of w-6 fatty acids, 2.1 g per day of w-3 fatty acids and a ratio w6/w3 of 3.3).

In carriers of A allele, basal assessment of nutritional intake with 3 days written food records showed a caloric intake of 1833.2±522.7 kcal/day, a carbohydrate intake of 189.9±41.2 g/day (41.4% of calories), a fat intake of 79.4±34.1 g/day (38.9% of calories) and a protein intake of 85.7±21.3 g/day (19.7% of calories). These patients reached the recommendations of diet; 1316 kcal, 45.8% of carbohydrates, 34.2% of lipids and 20.0% of proteins). The distribution of fats was; 21.2% of saturated fats, 55.5% of monounsaturated fats and 23.3% of polyunsaturated fats (7.1 g. per day of w-6 fatty acids, 2.2 g per day of w-3 fatty acids and a ratio w6/w3 of 3.2). The final dietary intakes were similar in both genotypes, no statistical differences were observed in macronutrient distribution and percentage of dietary fat.

Table 1 shows the differences in anthropometric variables. In both groups, weight, BMI, fat mass, waist circumference, systolic blood pressure, and diastolic blood pressure decreased. In A allele carriers, the decrease in these parameters were: weight –4.2±3.2 kg (decrease in C385C genotype group –4.1±3.8 kg), fat mass –3.6±3.8 kg (decrease in C385C genotype group –3.8±3.8 kg), waist circumference –3.5±3.8 cm (decrease in C385 genotype group –3.6±3.3 cm), diastolic blood pressure –2.8±7.4 mmHg (decrease in C385 genotype group –3.3±6.6 mmHg) and systolic blood pressure –4.7±9.4 mmHg (decrease in C385 genotype group –5.0±10.6 mmHg). No statistical differences were detected in the improvement of these parameters between both genotype groups.

Table 2 shows cardiovascular risk factors. In C385A genotype group, total cholesterol, LDL-cholesterol, insulin, and HOMA-R levels decreased significantly. In A allele carriers and after dietary intervention, total cholesterol (–16.3±37.4 mg/dl) and LDL-cholesterol (–12.9±6.5 mg/dl) levels decreased. In subjects with C385C genotype, the decreases were significant; total cholesterol (–12.3+27.4 mg/dl), LDL-cholesterol (–7.5+20.5 mg/dl), insulin (–2.2+6.2 mUI/l) and HOMA-R (–0.79+1.15 units), too. The amount of total cholesterol and LDL cholesterol decreases were similar in both genotype groups.

Table 3 shows differences between basal and after treatment levels of adipokines. Only leptin levels had a significant decrease in C383C genotype group (–6.8±11.7 ng/ml) and A allele carriers reached statistical differences (–11.46±19.9 ng/ml), too. IL-6, TNF-α, resistin, and adiponectin remained unchanged after weight loss in both groups.

No differences in basal parameters (biochemical, anthropometric and dietary) were detected between heterozygous and homozygous patients.

Table 2 - *Classical cardiovascular risk factors.*

Characteristics	*C385C*		*C385A* or *A385A*	
	Baseline	At 3 months	Baseline	At 3 months
Glucose (mg/dl)	100.6±10.1	99.8±12.6	99.9±11.2	102.1±11.5
Total ch. (mg/dl)	202.7±36.5	190.3±36.7*	217.3±64.7	200.9±32.6*
LDL-ch. (mg/dl)	129.3±31.9	121.8±28.8*	141.3±53.9	128.4±55.2*
HDL-ch. (mg/dl)	50.3±11.3	49.1±9.9	50.1±11.6	51.7±11.2
TG (mg/dl)	129.2±63.3	120.1±52.4	136.3±67.4	118.6±62.4
Insulin (mUI/l)	13.1±7.1	10.8±5.1*	11.8±7.2	11.9±11.2
HOMA	3.49±2.2	2.60±1.3*	3.22±1.8	3.12±3.2
CRP (mg/dl)	5.2±7.1	5.5±6.2	6.0±4.4	6.9±3.9

Data presentation: mean±SD. Ch: cholesterol; TG: triglycerides; HOMA: homeostasis model assessment; CRP: C reactive protein. 2-way analysis of variance model (*)significantly different compared to baseline $p<0.05$.

Table 3 - *Circulating adypocitokines.*

Characteristics	*C385C*		*C385A* or *A385A*	
	Baseline	At 3 months	Baseline	At 3 months
IL 6 (pg/ml)	2.45±4.8	1.45±1.0	2.35±1.1	2.23±0.6
TNF-α (pg/ml)	1.2±0.8	1.5±0.9	1.6±1.2	1.5±1.6
Adiponectin (ng/ml)	12.5±6.5	11.3±5.4	9.4±5.2	9.1±5.2
Resistin (ng/ml)	6.7±3.1	6.6±3.9	6.2±2.2	6.7±1.4
Leptin (ng/ml)	39.7±26.8	32.9±26.5*	50.8±40.1	39.2±34.1*

IL-6: interleukin 6; TNF-α: tumor necrosis factor α. 2-way analysis of variance model (*)significantly different compared to baseline $p<0.05$.

DISCUSSION

The main finding of our study is the association of the C385C genotype with an additional improvement on insulin and HOMA-R levels after a high polyunsaturated fat hypocaloric diet. A significant decrease of weight, fat mass, waist circumference, total cholesterol, and LDL-cholesterol were observed in subjects with both genotypes.

The important data of our study is the association of the C385C genotype of FAAH with a better decrease in insulin and HOMA-R levels after a similar weight loss than A allele carriers. There are few intervention studies in this topic area. Three studies reported that obese adult carriers of the A allele of rs324420 had different improvements in several biochemical and anthropometric parameters after a dietary intervention. Aberle et al. (6) have shown that carriers of the A allele had a significantly greater improvement in cholesterol profile compared to non carriers of A allele when following a low fat diet during 6 weeks of intervention. De Luis et al. (7) have shown with a conventional hypocaloric diet during 3 months (52% of carbohydrates, 25% of lipids and 23% of proteins) that carriers of the A allele was associated with larger improvements in HDL cholesterol, body weight, and glucose levels. Finally, in other study (8), the allele A385 of FAAH was associated with a lack of improvement on metabolic parameters after two different hypocaloric diets during 3 months (low fat *vs* low carbohydrate). In the branch of low fat diet (8), a significant decrease of glucose, HOMA, and insulin levels were reported.

Recently, a new study in children has been published (15). Knoll et al. (15) did not detect evidence for an association of FAAH genotypes with weight reduction in overweight and obese children and adolescents.

Some reasons could explain these contradictories results. Firstly, age and initial average weight of populations are two factors that influence in the interaction of FAAH genotype and weight response. In the last study (15), a young overweight population (average 10.8 yr) was evaluated. In the other studies (6-8), a middle-age obese population (average 45-50 yr) was included. Secondly, the distribution of macronutrients in the prescribed diets and the type of dietary fat may influence on secondary metabolic responses to weight loss as a function of this polymorphism. For example, distribution of macronutrients and percentage of dietary fat were not reported in some studies (6, 15). In another study (7), percentage of monounsaturated fat acids was 30% of all dietary fat with 1520 kcal, 52% of carbohydrates, 25% of lipids, and 23% of proteins. The percentage of polyunsaturated fatty acids was around 10%, this data was lower than 23% of our present study. In the last study (8), low fat diet had the next distribution of macronutrients; 53% carbohydrates, 27% fats, and 20% proteins) and the percentage of polyunsaturated fatty acids was around 18%, a percentage close to that used in our study and this fact could explain the similarity in metabolic results on insulin and HOMA-R levels. Finally, duration of dietary intervention may influence secondary metabolic responses to weight loss as a function of this polymorphism. The duration of interventions has been around 1 yr (15) till only 6 weeks (6). Perhaps the interaction FAAH polymorphism and weight loss secondary to diet is modulated during the time. A limitation of our study may be the short duration of the intervention and the small number of patients.

The lack of association between this polymorphism and anthropometric parameters has been described by previous studies, too. The results of our study agree with those of Papazoglou et al. (16) or Jensen et al. (17) ,and contrast with those of Sipe et al. (18).

In order to understand all these previous data, we must consider that the endocannabioid system is a complex redundant system, in this way the mesolimbic addition and reward/craving circuit including the medial forebrain bundle projections to the nucleus accumbens shows a high correlation of FAAH enzyme expression and CB1 receptor density (19).

In our study, the improvement on levels of insulin and HOMA-R levels in C385C genotype group with a high polyunsaturated fat hypocaloric diet could be related with other unmeasured factors because adiponectin levels remained unchanged. Perhaps, the type of dietary fat is important in these metabolic effects as indicated with a favorable role of polyunsaturated fatty acids intakes on insulin resistance (20). Finally, the existence of complex unmeasured gene-gene or gene-environment interactions could explain this interesting interaction on insulin resistance and weight loss with rs324420, without effects on adipokines and lipid profile.

The limitations of our study are important. Short-term of observation and the limited number of patients included are two important potential biases. Indeed, a longer observation time may have given different results.

In conclusion, non-carriers of the allele A385 of FAAH showed an improvement on insulin and HOMA-R levels with a high polyunsaturated fat hypocaloric diet after weight loss during 3 months. Further studies are needed to explore the right macronutrient distribution and type of dietary fats to evaluate this gene-environment interaction.

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D.A. de Luis designed the study and wrote the article. R. Aller recruited patients and made dietary evaluation. M Gonzalez Sagrado realized laboratory test. R Conde realized laboratory test. O. Izaola recruited patients and made dietary evaluation.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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