Research Article

Single Nucleotide Polymorphism of *ADIPOQ* Gene (Rs822393) Is Associated with Lipid Profile, Adiponectin Levels and Ratio of Adiponectin/Leptin ratio in Adult Obese Subjects

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Received: 26 December 2020; **Accepted:** 04 January 2020; **Published:** 11 January 2021

Citation: Daniel de Luis, Olatz Izaola, David Primo, Rocio Aller. Single Nucleotide Polymorphism of *ADIPOQ* Gene (Rs822393) Is Associated with Lipid Profile, Adiponectin Levels and Ratio of Adiponectin/Leptin ratio in Adult Obese Subjects. Cardiology and Cardiovascular Medicine 5 (2021): 61-72.

Summary

Objectives: Some studies in the literature indicate that *ADIPOQ* rs822393 polymorphism functionally regulates adiponectin levels. The aim of this study was to describe the association of rs822393 with adiponectin levels, cardiovascular risk factors, diabetes mellitus type 2 and metabolic syndrome.

Patients and Methods: The study involved a population of 1004 Caucasian adult obese subjects. Anthropometric parameters, blood pressure, fasting blood glucose, C-reactive protein (CRP), insulin concentration, insulin resistance (HOMA-IR), lipid profile, adipokines levels, ratio adiponectin/leptin ratio and prevalence of MS were recorded. Genotype of *ADIPOQ* gene polymorphism (rs822393) was evaluated.

Results: The distribution of the rs822393 polymorphism in this adult population was 62.1% (n=623) (CC), 32.1% (n=322) (CT) and 5.8% (n=59) (TT). We observed an association between the minor T allele and lower HDL cholesterol levels (CC vs. CT+TT: delta: 6.6+1.2 mg/ml;p=0.03) and lower adiponectin levels (CC vs. CT+TT: delta: 19.9+3.2 mg/ml;p=0.02). T allele carriers showed lower adiponectin/leptin ratio (CC vs. CT+TT: delta: 0.29+0.1 mg/ml;p=0.02) than non T allele carriers.

Logistic regression showed that the T allele increased the risk of low HDL cholesterol (OR=2.75, 95% CI=1.87-4.02, p=0.03), after adjusting by BMI and age.

Conclusions: In conclusion, we observed an association between rs822393 polymorphism with HDL-cholesterol, adiponectin and adiponectin/leptin ratio in Caucasian adult obese subjects.

Keywords: Adiponectin; Adiponectin/leptin ratio; HDL-cholesterol; Metabolic Syndrome; rs822393

1. Introduction

Obesity is a main factor in the development of diseases such as hypertension, diabetes mellitus type 2, dyslipidemia and cardiovascular diseases [1]. Adipose tissue is a relevant organ that secretes adipokines and many proteins produced by adipose tissue have been described, which may provide the link between obesity, insulin resistance and lipid profile [2]. Adiponectin is the most quantitatively abundant adipokine produced by adipocytes, and concentrations of adiponectin are reduced in obese subjects [3]. Adiponectin is encoded by the adiponectin C1Q and collagen domain containing (*ADIPOQ*) gene, which is located on chromosome 3 at q27. In this area, genome wide scans have detected a susceptibility locus for obesity and diabetes mellitus type 2 [4]. In humans, many polymorphisms in *ADIPOQ* gene have implied on adiponectin levels, insulin resistance and obesity [5], too.

One common single nucleotide polymorphism (SNP) of this gene is rs822393 (-4522C/T), it is located in the proximal promoter region of the *ADIPOQ* gene. Interesting SNPs candidates would be those that have a wide range of physiological effects like lipid and glucose homeostasis. Some studies in the literature indicate that *ADIPOQ* rs822393 polymorphism functionally regulates adiponectin promoter activity and is associated with hypoadiponectinemia [6]. This *ADIPOQ* variant has been identified to be associated with adiponectin levels and is related with lipid profile in an adolescent population [7]. In some ethnic groups (Indian), this SNP has been associated with the risk of diabetes mellitus [8]. Despite these previously mentioned relationships, there is no study evaluating the relationship of this SNP with the metabolic syndrome or any of its components in adult obese Caucasian populations. Metabolic syndrome (MS) is defined by the clustering of several factors; abdominal obesity, glucose intolerance and/or insulin resistance, dyslipidemia and increased blood pressure [9].

The aim of the present investigation was to describe the association of rs822393 with adiponectin levels, cardiovascular risk factors, diabetes mellitus type 2 and metabolic syndrome in an adult obese Caucasian population.

2. Materials and Methods

2.1 Subjects and clinical investigation

A total of 1004 unrelated study subjects were recruited from obese patients sent by the primary care physicians of our Health Area, obesity is defined by a body mass index (BMI) $\geq$30 kg/m^2. These obese Caucasian subjects were enrolled in a non-probabilistic consecutive method of sampling. The enrolled subjects fulfilled the following inclusion criteria; body mass index $\geq$ 30 kg/m^2, had no a history of cardiovascular disease, lack of severe renal or hepatic disorders, had no history of alcoholism, active malignant tumor, previous bariatric surgery,

and within the 6 months before the study were not receiving medications known to influence lipid profile (statins, hormonal therapy, glucocorticoids and anti-inflammatory drugs). The exclusion criteria were ages under 20 years or older than 70 years, BMI over 40 kg/m^2 years old, and a hypocaloric dietary intervention 3 months prior to the current study.

Informed consent was obtained from all study participants. Our Ethics Committee (HCUVA Committee) in accordance with the guidelines laid down in the Declaration of Helsinki approved the study. After signed consent was obtained, all participants underwent a medical evaluation including physical examination and complete medical history.

Classical anthropometric parameters (weight, height, body mass index (BMI) and waist circumference) were collected. Body fat mass was assessed by impedanciometry. We collected blood samples after a 10-hour overnight fasting state with the consequent biochemistry assays and genetic analysis. The following parameters were measured; lipid profile (total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides), plasma C reactive protein (CRP), adipokine levels (leptin, total adiponectin and resistin) and insulin.

To evaluate the prevalence of diabetes mellitus type 2, we used American Diabetes Association criteria (fasting glucose above 126 mg/dl or HBA1c >6.5% or glucose oral overload with a glucose levels at 120 minutes above 200 mg/dl) [10]. In order to calculate the prevalence of Metabolic Syndrome (MS), the definitions of the ATPIII was used [1]. Subjects need to fulfill at least 3 of the following 5 criteria in order to be diagnosed of MS; elevated systolic or diastolic blood pressure (>130/85 mmHg or antihypertensive treatment), hyperglycaemia elevated fasting glucose or treatment for diabetes as hyperglycemia, elevated triglycerides (>150 mg/dl) or treatment for dyslipidemia, low HDL cholesterol < 40 mg/dl (males) or <50 mg/dl (females), and increased waist circumference (>94 cm (males) or >80 cm (females)).

2.2 Phenotype measurements

Weight and height were measured on the subjects barefooted and lightly clothed. Weight was measured using scales (Omrom, LA, CA, USA) and recorded to the nearest 50 g. Height was measured with a tape measure (Omrom, LA, CA, USA) while patients were standing with shoulders in normal alignment and no wearing shoes. Body mass index (BMI) was calculated as body weight (in kg) divided by height (in m^2), and obesity was defined as BMI >30 kg/m^2. Waist circumferences (WC) were measured at the umbilical level with the use of an upstretched tape measure while the subjects were standing after normal expiration. Impedanciometry was used to determine body composition with an accuracy of 5 g [11] (EFG, Akern, Pisa, It). Blood pressure was measured three times from the left arm of seated subjects with a blood pressure monitor (Omrom, LA, CA, USA) after 30 minutes of rest. The average of the two last measurements were recorded for each subject.

2.3 Biochemical measurements

Total cholesterol and triglyceride levels were determined by enzymatic colorimetric assay (Technicon Instruments, Ltd., New York, N.Y., USA), while HDL cholesterol was measured in the supernatant after precipitation of other lipoproteins by enzymatic methods. LDL cholesterol was calculated using Friedewald equation (LDL cholesterol= total

cholesterol-HDL cholesterol-triglycerides/5) [12]. Glucose levels were measured by an automated glucose oxidase method (Glucose analyser 2, Beckman Instruments, Fullerton, CA, USA). Insulin was determined by radioimmunoassay (RIA) (RIA Diagnostic Corporation, Los Angeles, CA, USA) with a sensitivity of 0.5mUI/L (normal range 0.5-30 mUI/L) [13] and the homeostasis model assessment for insulin resistance (HOMA-IR) was calculated using these parameters [14]. C-reactive protein (CRP) was measured by immunoturbimetry (Roche Diagnostics GmbH, Mannheim, Baden-Wurtemberg Germany), with a normal range of (0-7 mg/dl) and analytical sensivity 0.5 mg/dl. Adipokines were measured by enzyme linked immunosorbent assay (ELISA). Resistin was measured with the commercial kit assay (Biovendor Laboratory, Inc., Brno,Moravia, Czech Republic) (sensitivity of 0.2 µg/ml and a normal range of 4-12 µg/ml) [15]. Adiponectin was measured with (R&D systems, Inc., Mineapolis, Minnesota, USA) (sensitivity of 0.24 µg/ml and normal range of 8.63-21.42 µg/ml [16]. Finally, leptin was measured with (Diagnostic Systems Laboratories, Inc., Houston, Texas, USA) (sensitivity of 0.05 µg/ml and a normal range of 11-100 µg/ml) [17].

2.4 Genotyping *ADIPOQ* gene

Genomic DNA was isolated from peripheral blood leucocytes (300 uL of blood.) by QIAamp ® DNA blood kit following the manufacturer's instructions. Oligonucleotide primers and probes were designed with the Beacon Designer 5.0 (Premier Biosoft International ®, LA, CA, USA). The polymerase chain reaction (PCR) was realized with 50 ng of genomic DNA, 0.5 uL of each oligonucleotide primer (primer forward: 5'-ACGTTGGATGAAAGCATGACACGGAGCTTC -3' and reverse 5'-ACGTTGGATGAACCCTCACCCATGTCAGC -3' in a 2 uL final volume (Termociclador Life Tecnologies, LA, CA, USA). DNA was denatured at 90ºC for 2 min; this was followed by 50 cycles of denaturation at 90ºC for 30 s, and annealing at 56.1º C for 60 s). The PCR were run in a 30 uL final volume containing 15 uL of IQTM Supermix (Bio-Rad®, Hercules, CA, USA) with hot start Taq DNA polymerase. We used as internal standard for RT-PCR (GAPDH) with a forward sequence: GTCTCCTCTGACTTCAA and reverse sequence: ACCACCCTGTTGCTGTA. Hardy Weinberg equilibrium was assessed with a statistical test (Chi-square) to compare our expected and observed counts. The variant was in Hardy Weinberg equilibrium (p=0.28).

2.5 Statistical analysis

Statistical analyses were performed by SPSS for Windows, version 23.0 software package (SPSS Inc. Chicago, IL, USA). Sample size was calculated to detect differences over 3 mg/dl of HDL-cholesterol between genotype groups with 90% power and 5% significance. The biochemical parameter values were reported as mean±standard deviation and were compared by Student´s t-test if they were in Gaussian distribution and compared by non-parametric Mann-Whitney U-test. Logistic regression analyses adjusted by age, gender and BMI were used to calculated odds ratio (OR) and 95% confidence interval (CI) to estimate the association of the rs822393 SNP with the risk of Metabolic syndrome, components of MS and diabetes mellitus type 2. A *p*-value under 0.05 was considered statistically significant. All analysis were performed under a dominant genetic model with rs822393 T- allele as the risk allele (CC+CT vs. TT).

3. Results

A total of 1004 Caucasian obese subjects completed the study. The mean age was 46.9±6.2 years (range: 26-64) and a sex ratio of 733 females (73.0%) and 271 males (27.0%). The mean body mass index (BMI) 36.8±2.9 kg/m^2 (range:31.5-39.7). The distribution of the rs822393 polymorphism in this adult population was 62.1% (n=623) (CC), 32.1% (n=322) (CT) and 5.8% (n=59) (TT). The allele frequency was C (0.63) and T (0.37).

For the statistical analysis, the patients were grouped into two genotype groups (CC *vs.* CT+TT). Age was similar in both genotype groups (CC; 47.0±3.1 years *vs.* CT+TT; 46.8±5.2 years: ns). Sex ratio was equal in both genotype groups (CC 26.2% males *vs.* 73.8% females *vs.* CT+TT; 28.3% males *vs.* 71.7% females).

Applying a dominant genetic model, we did not observe a significant association between rs822393 T-allele and anthropometric parameters such as fat mass, weight, waist circumference BMI and blood pressure. As expected, males had higher weight, fat mass and waist circumference than females in both genotype groups. In the remaining analysis by sex groups, we did not detect statistically significant differences between both genotypes (Table 1).

	Total group (n=1004)		**Male (n=271)**		**Female (n=733)**	
Parameters	CC n=623	CT+TT n=381	CC n=163	CT+TT n=108	CC n=460	CT+TT n=273
BMI	36.9±3.0	36.7±4.0	36.1±3.1	36.2±3.2	36.9±3.1	36.8±3.0
Weight (kg)	94.9±7.0	94.0±8.7	106.1±9.1*	107.3±9.0*	90.9±8.2	89.3±5.7
Fat mass (kg)	39.6±6.1	38.8±7.0	33.4±3.1*	34.0±3.2*	41.9±4.1	40.7±4.3
WC (cm)	112.0±7.0	110.1±7	118.9±6.1*	118.7±5.9*	109.7±4.2	107.8±5.3
SBP (mmHg)	127.0±9.0	127.2±8.1	128.2±7.8	129.3±6.0	126.3±5.8	126.9±5.2
DBP (mmHg)	81.8±5.2	81.9±3.8	82.3±5.1	82.5±4.4	80.9±5.0	81.4±4.2

BMI: body mass index DBP, diastolic blood pressure; SBP, systolic blood pressure; WC, waist circumference; *p<0.05, between genders. No statistical differences between genotype groups.

Table 1: Anthropometric variables and blood pressure.

Biochemical characteristics according to genotype are shown in table 2. In the total group, there were no differences in the parameters related to glucidic metabolism (glucose, insulin, HOMA-IR) or CRP levels. We observed an association between the minor T allele of polymorphism and lower HDL cholesterol levels under the dominant model (CC *vs.* CT+TT: delta: 6.6±1.2 mg/ml;p=0.03). This significant difference in HDL cholesterol levels was maintained in male patients (CC *vs.* CT+TT: delta: 6.3±1.1 mg/ml;p=0.02) and also in females (CC *vs.* CT+TT: delta: 6.7±0.9 mg/ml;p=0.03). No significant differences were detected in the rest of the lipid profile parameters (total cholesterol, LDL-cholesterol and triglycerides).

	Total group (n=1004)		**Male (n=271)**		**Female (n=733)**	
Parameters	CC n=623	CT+TT n=381	CC n=163	CT+TT n=108	CC n=460	CT+TT n=273
Fasting Glucose (mg/dl)	102.0±7.9	101.6±7.1	105.4±7.1	104.5±6.2	100.8±5.1	99.8±4.1
Total cholesterol (mg/dl)	209.3±20.8	201.1±18.2	204.5±12.6	199.7±20.1	210.5±19.6	204.1±20.1
LDL-cholesterol (mg/dl)	128.1±10.2	127.6±9.1	127.2±12.3	125.3±12.1	128.5±12.1	128.9±10.1
HDL-cholesterol (mg/dl)	53.3±3.0	45.7±2.9*	50.1±2.4	43.8±7.2*	56.7±3.0	50.1±2.1 *
Triglycerides (mg/dl)	128.2±38.1	125.6±21.7	142.1±31.9	144.9±31.9	118.1±17.1	118.4±13.1
Insulin (mUI/l)	13.7±5.9	14.4±5.9	16.3±4.0	17.0±5.2	12.8±4.5	12.4±5.1
HOMA-IR	3.5±1.8	3.7±1.9	4.1±1.1$	5.0±1.3	3.2±1.0	3.1±1.1

HOMA-IR (homeostasis model assessment of insulin resistance).; *p<0.05, in CC *vs.* CT+TT genotypes.

Table 2: Biochemical parameters (mean±SD).

	Total group (n=1004)		**Male (n=271)**		**Female (n=733)**	
Parameters	CC n=623	CT+TT n=381	CC n=163	CT+TT n=108	CC n=460	CT+TT n=273
Resistin (µg/dl)	5.3±1.3	5.2±1.1	5.1±1.1	5.3±1.7	5.5±1.2	5.2±1.3
Adiponectin (µg/dl)	41.3±8.0	21.2±5.9*	31.1±7.1	11.2±4.1*	43.1±4.7	24.4±3.9*
Leptin (µg/dl)	64.1±12.9	60.2±11.3	31.2±10.2	35.8±10.4	76.7±8.1$	71.8±12.4$
Ratio adiponectin/leptin	0.63±0.3	0.35±0.1*	0.97±0.2	0.31±0.3*	0.56±0.2	0.35±0.1*
CRP (µg/dl)	5.6±1.2	5.7±1.5	5.4±1.9	5.7±1.8	5.7±1.2	5.6±1.1

CRP: C reactive protein. p<0.05, in CC *vs.* CT+TT genotypes.$ p<0.05 between genders

Table 3: Serum adipokine levels and C reactive protein (mean±SD).

C reactive protein (CRP) and plasma adipokine levels are shown in table 3. In both genotypes, leptin levels were higher in females than males. Again, we detected a relation between the minor T allele of rs822393 polymorphism and lower adiponectin levels under the dominant model (CC *vs.* CT+TT: delta: 19.9±3.2 mg/ml;*p*=0.02). In males, adiponectin levels (CC *vs.* CT+TT: delta: 18.8±3.9 µg/ml;*p*=0.03) and in females (delta: 20.1±3.8 µg/ml;p=0.01) were lower in T allele carriers than non T allele carriers, too. The adiponectin/leptin ratio also showed the same differences by genotypes. T allele carriers showed lower ratio (CC *vs.* CT+TT: delta: 0.29±0.1 mg/ml;*p*=0.02) than non T allele carriers. This statistic difference was maintained in males (CC *vs.* CT+TT: delta: 0.53±0.4 mg/ml;*p*=0.01) and females (CC *vs.* CT+TT: delta: 0.28±0.2 mg/ml;*p*=0.03), too.

	Total group (n=1004)		**Male (n=271)**		**Female (n=733)**	
Parameters	CC n=623	CT+TT n=381	CC n=163	CT+TT n=108	CC n=460	CT+TT n=273
Percentage of MetS	53.8%	53.9%	58.9%	59.4%	43.7%$	37.7% $
Percentage of central obesity	81.5%	79.6%	95.5%	97.1%	76.7%$	72.7%$
Percentage of Hypertriglyceridemia	12.6%	9.7%	15.2%	10.5%	11.8%	9.4%
Low HDL cholesterol	9.5%	22.1%*	10.4%	22.2%*	9.8%	22.7%*
Percentage of Hypertension	44.3%	43.5%	50.6%	54.5%	42.1%	39.8%
Percentage of hyperglycemia	23.2%	22.6%	28.5%	31.1%	21.5%$	29.5%
Diabetes Mellitus	9.4%	10.4%	9.6%	12.1%	9.3%	9.7%

The cutoff points for the criteria of; central obesity (waist circumference >88 cm in female and >102 in male), hypertension (systolic BP>130 mmHg or diastolic BP>85 mmHg or specific treatment), hypertriglyceridemia (triglycerides >150 mg/dl or specific treatment), low HDL cholesterol < 40 mg/dl (males) or <50 mg/dl (females) or hyperglycemia (fasting plasma glucose >110 mg/dl or drug treatment for elevated blood glucose). Diabetes mellitus by American diabetes Association (ref 10)*p<0.05, in CC *vs.* CT+TT genotypes.$ *p*<0.05 between genders.

Table 4: Metabolic syndrome, components of MetS and diabetes mellitus.

Table 4 shows the percentages of metabolic syndrome (MS), different components of MS (central obesity, hypertriglyceridemia, hypertension, hyperglycemia and low HDL-levels) and diabetes mellitus type 2. In the all group, the percentage of individuals who had metabolic syndrome (MS) was 46.2% (n=463) and 53.8% patients without MS (n=541). In both genotypes, metabolic syndrome rate and percentage of central obesity were higher in males than females. T allele carriers had a higher percentage of low HDL cholesterol.

Logistic regression showed that the T allele increased the risk of low HDL cholesterol (OR=2.75, 95% CI=1.87-4.02, *p*=0.03), after adjusting by BMI and age. This association remained in males; risk of low HDL-cholesterol (OR=2.44, 95% CI=1.66-3.57, *p*=0.03). The analysis in female group showed the same results; risk of low HDL-cholesterol (OR=2.92, 95% CI=1.99-4.26, *p*=0.02), after adjusting by BMI and age, too.

4. Discussion

The primary findings of this study are the detected significant associations between the rs822393 and low levels of HDL cholesterol, adiponectin and adiponectin/leptin ratio. To our knowledge, this is the first study investigating the association between this *ADIPOQ* gene polymorphism and Metabolic Syndrome in Caucasian adults obese subjects. According to literature, more than 500 SNPs map within the adiponectin locus. One of these common genetic variants is rs822393. However, the data in the literature on the role of this SNP in the metabolism of obese patients are scarce and contradictory. For example, He et al [18] did not detect any significant associations between this SNP and neither adiponectin levels nor metabolic parameters. In contrast, Salazar et al [7] reported strong associations between rs822393 polymorphism and HDL-cholesterol levels as our results. Salazar's study was carried out in an adolescent and Hispanic population [7], this population is very different of our Caucasian adult obese population. Therefore, these concordant results are not population-dependent. Our findings are of potential importance because of the strong evidence of the inverse association between HDL-cholesterol levels and cardiovascular disease [19].

Adiponectin regulates lipid and glucose metabolism, modulating the development of multiple disorders including diabetes mellitus and obesity. A possible mechanism that may explain the detected associations in our study is that this SNP of ADIPOQ could alter adiponectin functions and therefore modify lipid levels. Adiponectin increases serum HDL cholesterol [20] through the following mechanisms; via the activation of lipoprotein lipase and ATP-binding cassette trasporter A1 and inhibition of hepatic lipase [21] and via the increase of the hepatic production of ApoA1, the main apolipoprotein of HDL-cholesterol [22]. Our results are in line with this hypothesis, with a significant low adiponectin levels in T allele carriers. This decrease could be explained by the fact that this SNP is an intronic variant with the potential capacity to modify the alternative-splicing pattern [23].

In our study, we did not find any relationship of rs822393 with obesity-related parameters. Moreover, Zayani et al [24] reported that this SNP and others in *ADIPOQ* gene were related with BMI, subjects with T allele showed higher BMI than non-T allele carriers. The results in the literature are contradictory, for

example, Peralta et al [25] did not find any relationship of this SNP with different anthropometric parameters related to obesity. Different populations studied and their different genetic background may explain these differences.

On the other hand, some studies [26] have shown a relationship between SNP rs822393 and insulin resistance in a Caucasian population. The association was stronger in parents than the offspring. This could indicate that the influence of variants in adiponectin on insulin sensitivity may take many years to manifest. However, in a study carried out in southern India [8], no relationship of this polymorphism with diabetes mellitus or metabolic syndrome was detected, only a slight relationship was detected when analyzing a haplotype with 8 different variants of the *ADIPOQ* gene. These results being in line with those obtained in our work. The association of the T allele with lower levels of adiponectin has been described more frequently in the literature [26], and this could explain, as we have previously commented, our findings in HDL cholesterol levels. In the CARDIA study (Coronary artery development in young adults) a very strong relationship was demonstrated in a young Caucasian population [27] between this polymorphism and low levels of adiponectin. An additional finding in our work was the relationship of this SNP with the adiponectin/leptin ratio. Leptin, another adipocyte-derived factor, parallels the degree of adiposity [28]. The adiponectin/leptin ratio is a marker of adipose tissue disfunction and inflammation [29]. For example, an adiponectine/leptin ratio higher than 1 can be considered as normal whereas a ratio below or near to 0.5 may indicate an increase in the metabolic risk [30], as shown by the carriers of the T allele.

Some limitations of this study should also be acknowledged when interpreting our findings. First limitation is the cross-sectional design of this study, so causality associations could not be determined. Second, the associations between this SNP and metabolic parameters could be modified by gene-gene and gene-environmental interactions. Third, unfortunately we did not measure APOA-1 concentration in our study, an interesting molecule in the metabolism of HDL cholesterol. Fourth, the study has been realized in obese subjects, so the data are not generalizable to the entire population. Finally, we have evaluated only Caucasian subjects, ethnic differences in genetic background and the environment they live in would play a crucial role in genetic effects.

In conclusion, we observed an association between rs822393 polymorphism with HDL-cholesterol, adiponectin and adiponectin/leptin ratio in Caucasian adult obese subjects. With future prospects, these data could be helpful to program clinical and health strategies, using this SNP to support the individual classical risk factor determination in this high-risk population of overweight subjects. Of note, is however, that further research is needed to assess the link between this SNP and others with adiponectin levels, metabolic syndrome and promising index such as ratio adiponectin/leptin [31-32].

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (HVUVA committee 4/2017) and with the

1964 Helsinki declaration and its later amendments or comparable ethical standards.

Funding Sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of Interests

All authors have no conflicts of interest.

Informed Consent

Informed consent was obtained from all individual participants included in the study.

Authors Contribution

Daniel Antonio de Luis designed the study an wrote the article

Olatz Izaola realized nutritional evaluation

R Aller realized laboratory analysis

D Primo realized nutritional evaluation

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