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UCP1 –3826 AG+GG genotypes, adiponectin, and leptin/adiponectin ratio in severe obesity

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ABSTRACT. ***Background and aims:*** **Non-alcoholic fatty liver disease (NAFLD) and metabolic syndrome (MS) are well-recognized complications of obesity. This study was designed to evaluate the role of the UCP1 –3826 A>G polymorphism, adiponectin levels, leptin/adiponectin ratio (L/A), and main biochemical parameters in 102 unrelated severely obese adults [61 females and 41 males, median body mass index (BMI) = 47.8 kg/m^2] with NAFLD, with (MS+) or without MS (MS–) from Southern Italy.** ***Subject and methods:*** **The UCP1 polymorphism was tested by the TaqMan method, main biochemical parameters by routinary methods, adiponectin, and leptin serum levels by enzyme-linked immunosorbent assay. MS was diagnosed according to the American Heart Association criteria, liver steatosis was detected by ultrasound.** ***Results:*** **MS was present in 53% male and 66% female obese patients. Only total cholesterol (p=0.04 males and p=0.002 females) and L/A ratio (p=0.03 males) differed between MS+ and MS– obese patients. At multivariate analysis, severe liver steatosis was significantly associated with: UCP1 (AG+GG) genotypes [odds ratio-confidence interval (OR-CI): 4.25; 1.12-16.13], MS (OR-CI: 8.47; 1.78-40.25), low adiponectin levels (OR-CI: 0.92; 0.87-0.98), high alanine aminotransferase levels (OR-CI: 1.03; 1.00-1.06), age (OR-CI: 1.08; 1.00-1.15), and male gender (OR-CI: 10.78; 1.61-71.96).** ***Conclusion:*** **In addition to traditional factors, total cholesterol and L/A ratio appear to contribute to MS characterization in severe obesity. Furthermore, the UCP1 (AG+GG) genotypes and low adiponectin levels could predispose to a more severe liver steatosis independently of MS presence. Based on our data, polymorphic UCP1 (AG+GG) obese patients with low adiponectin levels appear to be high-risk subjects for worsening of liver steatosis, a NAFLD, possibly requiring a second-step evaluation by liver biopsy.**
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INTRODUCTION

The prevalence of obesity [body mass index (BMI) ≥30 kg/m^2] is increasing worldwide and it is estimated that up to 9% and 30% of adults in Italy and in the United States respectively are obese (1-3). Severe obesity (i.e. BMI>40 kg/m^2) has also reached a dramatically high level and now affects about 1-2% of the adult European and 4% of the US population (2). Obesity, and in particular severe obesity, is associated with an increased risk of cardiovascular disease (CVD), sudden death, Type 2 diabetes, hypertension, liver steatohepatitis, and dysfunctions involving the endocrine and reproductive systems, bone metabolism, inflammation, immunity, and some types of cancer (4, 5). In 1998, the World Health Organization described the "metabolic syndrome" (MS) as a cluster of metabolic risk factors, namely, abdominal obesity, dyslipidemia (hypertriglyceridemia and low HDL-cholesterol concentrations), elevated blood pressure and hyperglycemia, to identify subjects at a higher risk of CVD (6). These criteria have been updated by the American Heart Association (AHA) (7).

Recently, leptin/adiponectin (L/A) ratio has also been reported as a useful index to evaluate insulin resistance in the absence of hyperglycemia (8) and as a predictor for carotid atherosclerosis in healthy males (9).

Non-alcoholic fatty liver disease (NAFLD) is a well-recognized complication of obesity, which is associated with MS, and with a risk of cirrhosis and liver cancer (10, 11). Liver biopsy is the only diagnostic test that can, within the NAFLD spectrum, reliably distinguish simple steatosis from steatosis with necroinflammatory changes and hepatocellular injury [i.e., non-alcoholic steatohepatitis (NASH)]. However, because this differentiation does not affect the management of obese patients, liver biopsy is not routinely performed, and the first-step evaluation of the liver is based on biochemical and imaging studies (11). The prevalence of NAFLD and MS is expected to increase with increasing excess body fat (10, 12). NAFLD is associated with decreased levels of adiponectin, a protective adipokine that inhibits such pro-inflammatory cytokines as tumor necrosis factor α and nuclear factor κβ (11). Furthermore, low adiponectin expression in intra-abdominal adipose tissue of morbidly obese patients may predispose to the progressive form of NAFLD, namely NASH (13).

A number of genes have been associated with human obesity phenotypes, including those encoding the thermogenic uncoupling proteins (UCP) (3). The reduced

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thermogenesis caused by UCP1 genetic variants has been implicated in increased susceptibility to obesity particularly when associated with aging and a high fat diet (14, 15). UCP1 is expressed only in mitochondria from brown adipose tissue where it uncouples respiration from ATP synthesis and dissipates energy as heat (14). The human UCP1 gene has been mapped to the long arm of chromosome 4 (16). An A>G point mutation at –3826 bp upstream from the UCP1 TATA box promoter has been related to changes in mRNA expression in intraperitoneal fat (16). Although the association between this UCP1 polymorphism and human obesity is controversial, it is clear that the minor variant allele is related to an increased propensity to gain weight over time (16).
The aim of this study was to evaluate, in a large population of severely obese adults from Southern Italy, the role of the UCP1 –3826 A>G gene polymorphism, adiponectin, and L/A ratio as risk factors in the onset of the obesity-associated complications, namely MS and liver steatosis.

MATERIALS AND METHODS

Study population

We studied 102 unrelated Caucasian adult patients [61 females (F) and 41 males (M), aged ≥18 yr] with severe obesity (median BMI =47.9 kg/m^2 males and 47.7 kg/m^2 females) from Southern Italy. The population was recruited at the obesity outpatient clinic of the Department of Clinical and Experimental Medicine, University of Naples "Federico II", Italy, from 2005 to 2006. Clinical and biochemical data were obtained from each patient at the first admission. All patients underwent screening for known obesity-related complications and CVD in a Day Hospital. Patients with previous CVD or cerebrovascular events, and alcohol abusers (i.e. alcohol consumption >20 g/day) were excluded from the study. Over 90% of patients had a family history of: obesity plus hypertension plus diabetes (52%), obesity (20%), hypertension (11%), diabetes (6%), hyperlipidemia (1%) or neoplasia (1%). We measured the following parameters in each individual: BMI [weight/height^2 (kg/m^2)], blood pressure and heart rate (following 5-min sitting). The general and biochemical characteristics of the population studied are reported in Table 1. As liver steatosis is 5-fold more frequent in obese patients than in lean individuals (17), we performed ultrasound liver examination in all patients. An Esaote Biomedica Apparatus (Firenze, Italy) equipped with a convex 3-5 MHz probe was used and the test imaging was read by two operators unaware of the laboratory data of the patients. Liver steatosis was also graded semiquantitatively on a scale of 0-3 (0= absent; 1= mild; 2= moderate; 3= severe) according to Saverymuttu et al. (18) on the basis of abnormally intense, high-level echoes arising from the hepatic parenchyma, liver-kidney difference in echo amplitude, echo penetration into the deep portion of the liver and clarity of liver blood vessel structure (19, 20). Healthy Caucasian normal-weight controls (M=29, F=66, BMI>20 and <25 kg/m^2) from the same geographic area were also recruited at the Ambulatory Medicine Service of the "Federico II" University Hospital. A venous blood sample was collected from each patient and control subject in the morning at 8.00 h after an overnight fast.

Laboratory investigations

Main biochemical and hormonal parameters [total cholesterol, HDL-cholesterol, triacylglycerols, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl-transferase (GGT), glucose, total proteins and insulin] were measured by routine laboratory methods. Insulin resistance was estimated according to the homeostasis model assessment (HOMA) and the formula: fasting insulin (mIU/l)/[22.5 × $e^{-\ln(\text{mmol/l glucose})}$].

Table 1 - *Main general and biochemical characteristics (median value and 2.5th-97.5th percentiles) of 102 severely adult obese patients (males=41; females=61).*

	Males		Females	
Age (yr)	34.5	18.0-57.0	31.0	18.4-67.0
BMI (kg/m^2)	47.9	38.7-93.4	47.7	40.0-76.0
Systolic blood pressure (mmHg)[a]	130.0	105.2-179.5	120.0	94.0-160.0
Diastolic blood pressure (mmHg)[b]	85.0	55.6-110.0	80.0	60.0-100.0
Heart rate (b/min)	80.0	56.4-108.0	76.0	57.9-100.0
Adiponectin (μg/ml)	17.2	3.3-57.1	20.2	3.3-48.3
Leptin (ng/ml)[c]	56.8	3.7-212.8	138.9	40.4-240.2
L/A ratio	3.3	0.02-50.0	5.8	0.9-50.0
Glucose (mmol/l)	5.2	3.2-10.6	4.9	3.6-7.7
Insulin (mIU/l)[a]	27.9	9.3-75.0	19.2	7.1-55.5
HOMA[d]	6.3	1.6-18.6	4.2	1.4-12.7
Total cholesterol (mmol/l)[e]	4.5	3.1-6.1	4.8	2.9-6.7
HDL cholesterol (mmol/l)[c]	1.0	0.6-1.5	1.2	0.9-1.9
Triacylglycerols (mmol/l)	1.3	0.4-3.5	1.4	0.6-3.2
AST (U/l)[a]	28.0	13.0-93.8	20.0	11.0-67.5
ALT (U/l)[c]	44.0	17.2-176.2	24.0	9.1-111.0
GGT (U/l)[c]	38.0	16.0-333.0	22.0	7.3-154.3
Total proteins (g/dl)	7.5	6.9-8.3	7.4	6.7-8.3

Statistically significant differences at Mann-Whitney test: [a]p=0.001; [b]p=0.004; [c]p<0.0001; [d]p=0.002; [e]p=0.011. BMI: body mass index; L/A: leptin/adiponectin; HOMA: homeostasis model assessement; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: γ-glutamyl transferase.

Total serum adiponectin and leptin concentrations were measured in duplicate in obese and control subjects by an enzyme-linked immunosorbent assay (LINCO Research, Mo, USA), using monoclonal anti-human adiponectin and leptin antibodies. We also calculated the L/A ratio.

Genomic DNA was extracted from whole blood (Nucleon BACC-II; Amersham Science Europe). The UCP1 –3826 A>G gene polymorphism was assayed with the Real Time TaqMan method. We used two fluorescent probes: one specific for the wild-type allele (VIC-CAGTTTGATCAAGTGCAT-Q-MGB, where VIC is the fluorescent reporter dye, Q the quencher molecule and MGB an enhancer of stabilization of the DNA-probe duplex), and one specific for the mutant allele (FAM-CAGTTTGATCGAGTGCAT-Q-MGB, where FAM is the fluorescent marker). We used the Primer Express software (Applied Biosystems, Foster City, CA) to design the PCR primers (forward: 5'-CTTGGGTAGTGACAAAGTAT-3'; reverse: 5'-CTTAAGGGTCAGATTTCTAC-3'). Reaction mixtures were assembled in a 384-well plate using a Biomek 2000 Workstation (Beckman Instruments, Fullerton, CA). Each well contained 40 ng genomic DNA, 36 nM primers, 8 nM probes, and 2.5 µl TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA) in a total reaction volume of 5 µl. We also tested negative and positive controls (i.e. no DNA sample and homozygote and heterozygote samples for the UCP1 –3826 A>G polymorphism previously typed by sequence analysis on an ABI Prism 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA). Real Time PCR was run on an ABI Prism 7900HT instrument and data were analyzed with the Sequence Detection System (SDS 2.1) and the SDS Enterprise Database (Applied Biosystems). The amplification protocol consisted of: 50 C for 2 min; 95 C for 10 min; 92 C for 15 sec and 60 C for 1 min for 40 cycles; final extension at 60 C for 1 min; final soak at 25 C.

MS was diagnosed according to the recently defined AHA criteria. Namely, the syndrome was diagnosed if 3 out of 5 criteria were present (7). All patients and controls gave their informed consent to the study, which was carried out according to the Helsinki II Declaration.

Statistics

For each investigated parameter we calculated the median value and the percentile (2.5^{th}-97.5^{th}) range. The Mann-Whitney test and/or χ^2, when necessary, were used for between-group comparison. Differences were considered significant at p level <0.05. Binomial logistic analysis was used to investigate the association between MS or liver steatosis severity (severe *vs* moderate steatosis) and the UCP1 –3826 A>G polymorphism, and in relation to biochemical and clinical characteristics. Allele frequencies were estimated by gene counting. Agreement with Hardy-Weinberg equilibrium was tested with a χ^2 goodness-of-fit test. Statistical analyses were carried out with the SPSS package for Windows (Ver.15; SPSS Inc. Headquarters, Chicago, Ill).

RESULTS

The main biochemical values obtained in male and female obese patients are reported in Table 1. MS was more prevalent in severely obese females (66%) than in males (53%) and a sex-dependent contribution was observed for hypertension (73% M and 31% F, p<0.001), dyslipidemia (54% M and 69% F, p=0.01) and hyperglycemia (34% M and 23% F, p=0.04), apart from waist circumference, which was well above normal limits in all subjects.

Table 2 - *Genotypes and allele frequencies of UCP1 –3826 A>G in obese patients (no.=102) and controls (no.=95).*

UCP1 genotypes	no. (%)		UCP1 allele	(%)	
	Obese	Controls		Obese	Controls
AA	51 (50.0)	52 (54.8)	A	0.71	0.72
AG	42 (41.2)	33 (34.7)	G	0.29	0.28
GG	9 (8.8)	10 (10.5)			

Further, among other tested biochemical parameters, only total cholesterol and L/A levels differed significantly between patients MS+ and MS– [respectively, median total cholesterol: (M), 4.4 mmol/l *vs* 4.6 mmol/l, p=0.04; (F), 4.7 mmol/l *vs* 5.1 mmol/l, p=0.002; median L/A ratio: (M) 3.7 *vs* 1.5, p=0.03; (F), 5.5 *vs* 6.6, p=ns). Among MS– obese patients 68% were insulin resistant (HOMA>4).

Liver steatosis was investigated by ultrasound and was present in all obese patients, it was severe in a higher percentage of MS+ *vs* MS– subjects (39% *vs* 20%, p=0.005) and more frequent in males than in females (57% *vs* 29%, p=0.0001). In obese with severe liver steatosis at univariate analysis we observed significantly higher median concentrations of AST, ALT, GGT, insulin, glucose, L/A ratio, and significantly lower HDL-cholesterol, adiponectin concentrations and AST/ALT ratio than in obese with mild/moderate liver steatosis (respectively, 28.0 U/l *vs* 21.0 U/l, p<0.0001; 42.5 U/l *vs* 27.0 U/l, p=0.001; 35.0 U/l *vs* 24.0 U/l, p=0.004; 28.4 mmol/l *vs* 19.2 mmol/l, p<0.0001; 5.2 mmol/l *vs* 5.0 mmol/l, p=0.03; 7.7 *vs* 4.0, p=0.007; 1.06 mmol/l *vs* 1.13 mmol/l, p=0.006; 12.7 µg/ml *vs* 24.2 µg/ml, p=0.001; 0.7 *vs* 0.8, p=0.03).

Table 2 shows the genotype and allele frequencies of the UCP1 –3826 A>G polymorphism in our obese patients and control subjects; genotype frequencies were in Hardy-Weinberg equilibrium (p=0.9). UCP1 (AG+GG) genotypes were more frequent in patients with severe liver steatosis than in those with mild/moderate liver steatosis (21/31; 65% *vs* 30/70; 43%, p=0.0003). UCP1 (AG+GG) genotypes did not differ among MS+ and MS– obese patients (46% *vs* 56%; ns). Binomial logistic regression showed that severe liver steatosis in obese patients was associated with the UCP1 (AG+GG) genotypes, low adiponectin levels, high ALT levels, age, MS, and male sex (Table 3).

DISCUSSION

The prevalence of MS in our severely obese subjects (M: 53% and F: 66%) was comparable to those reported in the QUOVADIS (Quality of Life in Obesity: Evaluation and Disease Surveillance) study (53%), a multicenter evaluation carried out in Italy (21) and slightly higher than in the general populations of European and US Caucasians of a similar age range (22).

We detected higher L/A ratio in obese male MS+ than in obese male MS– patients (3.7 *vs* 1.5) as previously reported for MS+ and MS– non-obese male patients (0.79

Table 3 - *Association between liver steatosis severity and clinical, biochemical variables in obese patients.*

Variable[a]	β	SE	*p*	Odds ratio	95% CI
UCP1[b]	1.45	0.68	0.033	4.25	1.12-16.13
Adiponectin	–0.08	0.03	0.012	0.92	0.87-0.98
MS	2.13	0.79	0.007	8.47	1.78-40.25
ALT	0.03	0.01	0.020	1.03	1.00-1.06
Male gender	2.38	0.97	0.014	10.78	1.61-71.96
Age	0.07	0.04	0.050	1.08	1.00-1.15

[a]Included variables at binomial logistic regression analysis were age, aspartate aminotransferase, alanine aminotransferase (ALT), g-glutamyl transferase, CHE, adiponectin, leptin/adiponectin ratio, gender, UCP1, metabolic syndrome (MS); [b]UCP1 genotypes were included as AA and AG+GG. CI: confidence interval.

vs 0.52) (9). Increased fat content is associated with insulin resistance in Type 2 diabetic patients (23). In our severe obese group 68% of MS– patients had HOMA>4. These data agree with the lipotoxicity theory, namely that increased and prolonged exposure to excessive free fatty acids results in decreased insulin secretion (22). In a chronic context such as severe obesity, lipids accumulate in muscle, liver, and pancreatic islet cells, and this event has been implicated in impaired insulin signaling and insulin secretion (24). In fact, in Zucker diabetic fatty rats, islet lipid accumulation precedes the development of diabetes (24). Further, insulin resistance and systemic hypertension features of the MS are also independently associated with advanced forms of NAFLD (25, 26).

Steatosis is frequent in obesity (27), particularly in severe obesity (12). Ultrasound studies showed that all our patients were affected by NAFLD, which was more severe in MS+ than in MS– obese patients. As a rule, imaging studies cannot predict the severity of NAFLD, which ranges from simple steatosis to steatohepatitis. However, no guidelines recommend liver biopsy in obese patients, except in the setting of persistent hypertransaminasemia or if it is necessary to rule out a cause of NAFLD other than MS or insulin resistance (11). In our obese population, the lack of risk factors other than MS, insulin resistance or persistent hypertransaminasemia did not justify liver biopsy.

There is compelling evidence that decreased adiponectin levels are involved in the development of NAFLD (28) in close association with insulin resistance, independently of obesity (29, 30). In our adult severely obese patients, serum adiponectin values were lower in patients with severe than in those with mild/moderate liver steatosis, and severe liver steatosis was also associated with older age and higher ALT transaminases. Recently, data obtained in an experimental model showed that adiponectin is a key regulator for the progression of hepatic fibrosis toward steatohepatitis (31).

The frequencies of the UCP1 AG and GG genotypes in our obese patients (respectively 41.2% and 8.8%) were similar to those reported for other Caucasians, namely between 29% and 42% for UCP1 AG and between 4% and 15% for UCP1 GG (16, 32-37), but lower than those reported in Japanese and Korean populations (respectively, 45-54% for AG and 23-27.5% for GG) (16, 38-40). An interesting finding of our study is that liver steatosis was more severe in obese patients bearing UCP1 (AG+GG) genotypes compared to those bearing the UCP1 AA genotype (odds ratio=4.25). This finding may suggest a genetic association between liver steatosis in obese subjects and the G allele. Interestingly, intraperitoneal fat UCP1 mRNA expression was found to be lower in obese subjects bearing the –3826 G polymorphism than in subjects with two wild-type alleles (16). Moreover, in a murine model, hepatic UCP1 overexpression reduced fat in the liver and adipose tissue, thereby improving insulin resistance in mice with high-fat-diet-inducing diabetes and obesity (41). Recently, the UCP1 gene was found to be expressed in the visceral adipose tissue of adult lean and obese patients in which brown adipocytes were dispersed among white adipocytes in a ratio of 1 to 100-200 (42). Interestingly, after dieting and a BMI reduction, UCP1 mRNA levels remained lower in obese than in lean subjects, which supports a genetic predisposition in obesity to a low energy dispersion (42).

In conclusion, in addition to traditional factors, total cholesterol and L/A ratio appear to contribute to MS characterization in severe obesity. Further, the UCP1 (AG+GG) genotypes and low adiponectin levels could predispose to a more severe liver steatosis independently of MS presence. Based on our data, polymorphic UCP1 (AG+GG) obese patients with low adiponectin levels appear to be high-risk subjects for worsening of liver steatosis or NAFLD, possibly requiring liver biopsy aimed to promote preventive interventions.

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