Genetic variants of *Clock* transcription factor are associated with individual susceptibility to obesity[1–3]

Silvia Sookoian, Carolina Gemma, Tomas Fernández Gianotti, Adriana Burgueño, Gustavo Castaño, and Carlos Jose Pirola

ABSTRACT

Background: Altering circadian rhythmicity results in pathophysiologic changes resembling metabolic syndrome and fat accumulation.

Objective: We investigated the role of gene variants and derived haplotypes of the *CLOCK* transcription factor in obesity and related quantitative metabolic traits.

Design: Lean ($n = 715$) and overweight or obese ($n = 391$) unrelated subjects aged 34.4 ± 8.6 y were included in a population-based cross-sectional study. Six tag single-nucleotide polymorphisms (SNPs) with a minor (>10%) allele frequency (rs1554483 C/G; rs11932595 A/G; rs4580704 C/G; rs6843722 A/C; rs6850524 C/G, and rs4864548 A/G) encompassing 117 kb of chromosome 4 and representing 115 polymorphic sites ($r^2 > 0.8$) were genotyped. Association was tested by PLINK and WHAP software, and multiple testing was controlled by permutation test.

Results: The genotype frequencies of 4 tag SNPs—rs1554483, rs6843722, rs6850524, and rs4864548—had significant (empiric $P <$ 0.010, 0.021, 0.021, and 0.010, respectively) associations with overweight or obesity. Haplotype analysis showed that only paired haplotypes, including rs1554483 and rs4864548, had a significant effect on overweight or obesity. Combinations of these SNPs (haplotype block CG and GA) are responsible for the gene effect (GA frequencies: 47% in cases, 41% in controls; empiric $P < 0.011$). These findings were concurrently observed in a sample of persons from a hospital-based study, and the combined Mantel-Haenszel fixed effect was an odds ratio of 1.82 (95% CI: 1.31, 2.54; $P < 0.001$) for the paired haplotype, which included CG and GA for rs1554483 and rs4864548.

Conclusions: The present study suggests a putative role of the *CLOCK* polymorphism and related haplotypes in susceptibility to obesity. The haplotype of rs1554483G and rs4864548A was associated with a 1.8-fold risk of overweight or obesity. *Am J Clin Nutr* 2008;87:1606–15.

INTRODUCTION

Obesity is a complex, multifactorial chronic disorder that develops from interactive influences of social, behavioral, physiologic, metabolic, cellular, and molecular factors (1). Whereas the exact pathogenesis of the disorder is unknown, body weight is strongly influenced by biological and behavioral factors (2, 3). In fact, regulation of appetite and food intake is influenced by sleep duration, and sleep restriction may favor the development of obesity (4–6). Common genetic effects were observed between insomnia and both sleepiness and obesity, which suggests shared genetic contributions to these conditions (7).

The circadian variation in metabolic response has also implications for obesity. For instance, the timed seasonal development of obesity in animals may be induced by the biological clock (8). A report by Turek et al (9) showed that mutant mice that are homozygous for circadian locomotor output cycles protein kaput (*CLOCK*) have a greatly altered diurnal feeding rhythm, are hyperphagic and obese, and develop a metabolic syndrome with hyperleptinemia, hyperlipidemia, hepatic steatosis, hyperglycemia, and hypoinsulinemia.

Although the *CLOCK* transcription factor is a key component of the molecular circadian clock within pacemaker neurons of the hypothalamic suprachiasmatic nucleus (10), *CLOCK* also plays an important role in regulating fat and glucose metabolism in peripheral organs such as adipose tissue, muscle and liver (11). It it interesting that results from a whole-genome linkage scan using 380 microsatellite markers to identify genomic regions that may contain quantitative-trait loci for obesity showed that region 4q12 (the chromosome location of the *CLOCK* gene) may be linked to obesity (12).

Given the above evidence and the results of emerging studies showing that alteration in circadian rhythmicity results in pathophysiological changes resembling metabolic syndrome and fat accumulation, the objective of the present study was to investigate the role of gene variants and their predicted haplotypes of the linkage disequilibrium (LD) block of the gene *CLOCK* in human overweight or obesity and related quantitative metabolic traits. The present study was performed in samples obtained randomly

[1] From the Departamento de Genética y Biología Molecular de Enfermedades Complejas, Instituto de Investigaciones Medicas A Lanari, Universidad de Buenos Aires, Buenos Aires, Argentina (SS, CG, TFG, AB, and CJP); CONICET (SS, CG, AB, and CJP); and the Consejo de Investigación GCBA, Buenos Aires, Argentina (SS and GC).

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[3] Address reprint requests and correspondence to CJ Pirola, Instituto de Investigaciones Medicas, A Lanari, Departamento de Genética y Biología Molecular de Enfermedades Complejas, Combatiente de Malvinas 3150, 1427- Ciudad Autónoma de Buenos Aires, Argentina. E-mail: carlospirola@ciudad.com.ar or pirola.carlos@lanari.fmed.uba.ar.

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from the population and in an independent sample of persons ascertained a from clinic.

SUBJECTS AND METHODS

Subjects

Healthy subjects recruited from a factory in the Buenos Aires metropolitan area who underwent an annual health examination were included in a cross-sectional population-based study. Altogether, 1106 men aged 34.4 ± 8.6 y of self-reported European ancestry were included in the present study, in which 715 lean subjects were compared with 391 overweight subjects. A concurrent study was performed in a population of 200 persons aged 52.5 ± 12.6 y (64 healthy subjects and 136 cases) in a hospital-based study from the same geographical area. Subjects were diagnosed with metabolic syndrome according to the Adult Treatment Panel III (13). The subjects' medical history was investigated by using a self-administered questionnaire, and the answers were confirmed by individual interviews conducted by physicians.

After the subjects rested for 5 min in a quiet room, arterial systolic and diastolic blood pressures were measured on the right arm with a standard mercury sphygmomanometer while the subjects were in a sitting position. Health examinations included anthropometric measurements, a questionnaire on health-related behaviors, and biochemical determinations.

Body mass index (BMI; in kg/m^2) was calculated and was used as the index for relative weight. In addition, trained staff assessed the waist circumference of subjects in the standing position by measuring midway between the highest point of the iliac crest and the lowest point of the costal margin in the midaxillary line. Hip circumference was measured at the level of the femoral greater trochanter by the same observer. Classification of overweight and obesity was based on the BMI; those with a BMI ≥ 27 were classified as overweight or obese. It has been shown that

TABLE 1
Clinical and biochemical characteristics of the subjects in the population-based study and in the hospital-based study[1]

Variable	Lean subjects	Overweight or obese subjects[2]	*P*[3]
Population-based study			
Subjects (*n*)	715	391	
Age (y)	32.66 ± 0.29[4]	37.55 ± 0.45	0.001
BMI (kg/m^2)	24.28 ± 0.07	30.97 ± 0.29	0.001
Waist circumference (cm)	86.80 ± 0.36	103.00 ± 0.52	0.001
Waist-hip ratio	0.91 ± 0.003	0.96 ± 0.003	0.001
SBP (mm Hg)	118.96 ± 0.50	126.52 ± 0.80	0.001
DBP (mm Hg)	74.41 ± 0.34	79.35 ± 0.56	0.001
Leukocyte count (cells/μL)	6746.32 ± 67.14	7070.99 ± 87.91	0.001
Erythrocyte sedimentation rate (mm/1 h)	6.88 ± 0.12	7.74 ± 0.19	0.001
Fasting plasma glucose (mmol/L)	4.99 ± 0.02	5.10 ± 0.04	0.01
Fasting plasma insulin (pmol/L)	47.6 ± 1.7	78.5 ± 3.8	0.001
HOMA index	1.52 ± 0.05	2.66 ± 0.15	0.001
Total cholesterol (mmol/L)	4.86 ± 0.03	5.21 ± 0.05	0.001
HDL cholesterol (mmol/L)	1.20 ± 0.01	1.18 ± 0.02	NS
LDL cholesterol (mmol/L)	3.06 ± 0.03	3.25 ± 0.05	NS
Uric acid (mmol/L)	272 ± 2	306 ± 3	0.001
Triglycerides (mmol/L)	1.32 ± 0.03	1.72 ± 0.06	0.001
Hospital-based study			
Subjects (*n*)	64	136	
Male/Female	22/42	36/100	NS
Age (y)	46.14 ± 1.64	55.58 ± 1.06	0.001
BMI (kg/m^2)	25.61 ± 0.65	36.02 ± 3.11	0.001
Waist circumference (cm)	84.13 ± 2.14	103.07 ± 1.42	0.001
Waist-hip ratio	0.84 ± 0.01	0.91 ± 0.007	0.001
SBP (mm Hg)	121.64 ± 1.87	124.05 ± 2.11	NS
DBP (mm Hg)	75.86 ± 1.32	78.38 ± 1.49	NS
Fasting plasma glucose (mg/dL)	4.73 ± 0.07	5.74 ± 0.22	0.003
Fasting plasma insulin (pmol/L)	46.1 ± 3.5	94.5 ± 6.9	0.001
HOMA index	1.42 ± 0.12	3.44 ± 0.30	0.001
Total cholesterol (mmol/L)	5.59 ± 0.17	5.44 ± 0.15	NS
HDL cholesterol (mmol/L)	1.27 ± 0.09	1.20 ± 0.05	NS
LDL cholesterol (mmol/L)	3.22 ± 0.29	3.11 ± 0.14	NS
Uric acid (mmol/L)	220 ± 51	244 ± 27	NS
Triglycerides (mmol/L)	1.74 ± 0.13	2.02 ± 0.13	NS

[1] SBP and DBP, systolic and diastolic blood pressure, respectively; HOMA, homeostatic model assessment; NS, not significant.
[2] BMI ≥27 classified as overweight or obese.
[3] Statistical significance using Student's *t* test (log variable transformation was performed when variables showed nonnormal distribution).
[4] $\bar{x}$ ± SE (all such values).

TABLE 2
Tag single-nucleotide polymorphisms (SNPs) of the *CLOCK* gene genotyped in the study[1]

Location in the *CLOCK* gene	NCBI SNP reference[2]	Heterozygosity	Minor allele	MB position[3]	dsSNP alleles	MAF
Intron 12	rs1554483	0.494	G	56 162 745	C/G	0.433
Intron 10	rs11932595	0.478	G	56 164 525	A/G	0.360
Intron 8	rs4580704	0.395	G	56 167 635	C/G	0.298
Intron 7	rs6843722	0.483	C	56 172 260	A/C	0.407
Intron 1	rs6850524	0.49	C	56 222 925	G/C	0.346
Upstream	rs4864548	0.49	A	56 254 731	G/A	0.433

[1] MAF, minor allele frequency (within the study); *CLOCK,* circadian locomotor output cycles protein kaput; NCBI, National Center for Biotechnology Information; ds, double strand.
[2] SNPs on NCBI Reference Assembly (Internet: http://www.ncbi.nlm.nih.gov/SNP/).
[3] Mapped chromosome position (International HapMap Project).

BMI ≥ 27 can predispose a person to a greater risk of cardiovascular disease and that the risk of death rises more steeply (by 60%) in those with a BMI of >27 than in those with a BMI below this cutoff. Moreover, a BMI > 27 is associated with a greater risk of heart disease, blood pressure, diabetes, hypertension, and elevated cholesterol concentrations in addition to other health risks (14–16). A recent study also showed that a BMI of 27 is associated with the highest sensitivity (68%) and specificity (90%) for a waist circumference of ≥40 inches, which is the measurement used to define the metabolic syndrome according to the National Cholesterol Education Program guidelines (17).

All participants were asked to fast for ≥8 h, and blood was drawn from subjects who had lain in a supine resting position for ≥30 min. Serum insulin, total cholesterol, HDL and LDL cholesterol, triacylglycerols, and plasma glucose were measured by standard clinical laboratory techniques. Homeostatic model assessment (HOMA), calculated as fasting serum insulin (μU/mL) × fasting plasma glucose (mmol/L)/22.5, was used to evaluate an insulin resistance index.

Written informed consent was obtained from all participants in accordance with the procedures approved by the Ethics Committee of our institution. All of the investigations performed in this study were conducted in accordance with the guidelines of the Declaration of Helsinki.

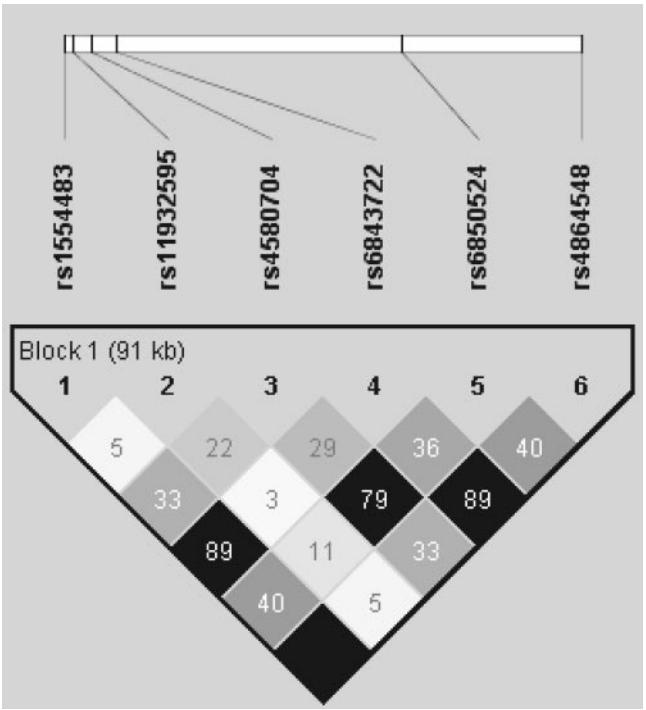

FIGURE 1. Linkage disequilibrium (LD) plot across the *CLOCK* gene. SNP, single-nucleotide polymorphism. The horizontal white bar depicts the 117-kb DNA segment of chromosome 4q12 analyzed in the sample. The 6 tagSNP locations are indicated by hatch marks. An LD plot is depicted in the bottom part of the figure: each diamond represents the magnitude of LD for a single pair of markers. Black indicates strong LD ($r^2 = 1.0$); white indicates no LD ($r^2 = 0$); and the gray tones indicate intermediate LD. The numbers inside the diamonds stand for r^2 values × 100.

Genotype and haplotype analysis

The genetic analyses were done on genomic DNA extracted from white blood cells by using a standard method as previously described (18). To assess the contribution of *CLOCK* gene variants to obesity and related quantitative metabolic traits, we selected tag SNPs (tSNPs) and multimarker predictors as effective

TABLE 3
Association of *CLOCK* tag single-nucleotide polymorphisms (SNPs) with overweight and obesity defined by the currently used BMI cutoffs (25 and 30, respectively) in a population-based study[1]

NCBI SNP reference[2]	Allelic comparison[3]	OR (95% CI)	Empirical *P*[4]
BMI 25			
rs1554483	G vs C	1.20 (1.01, 1.43)	0.045
rs11932595	A vs G	1.05 (0.88, 1.26)	0.576
rs4580704	C vs G	1.33 (1.11, 1.64)	0.001
rs6843722	C vs A	1.18 (0.99, 1.40)	0.071
rs6850524	G vs C	1.26 (1.05, 1.52)	0.012
rs4864548	A vs G	1.20 (1.01, 1.43)	0.045
BMI 30			
rs1554483	G vs C	1.36 (1.04, 1.77)	0.024
rs11932595	A vs G	1.25 (0.93, 1.67)	0.133
rs4580704	C vs G	1.20 (0.89, 1.61)	0.232
rs6843722	C vs A	1.23 (0.95, 1.61)	0.123
rs6850524	G vs C	1.27 (0.95, 1.69)	0.108
rs4864548	A vs G	1.36 (1.04, 1.77)	0.024

[1] $n = 1106$. OR, odds ratio; NCBI, National Center for Biotechnology Information. BMI was measured in kg/m^2.
[2] SNPs on NCBI.
[3] The risk allele versus the other allele as a reference.
[4] Empirical *P* value after the multiple comparison correction by permutation tests by using 10 000 permutations. PLINK software was used for assessing allelic associations (basic allelic chi-square test, 1df).

TABLE 4
Genotype distribution of *CLOCK* tag single-nucleotide polymorphisms (SNPs) according to overweight or obesity in population-based and hospital-based studies[1]

NCBI SNP reference and genotype	Lean subjects	Obese or overweight subjects	OR (95% CI)[2]	Cumulative OR (95% CI)[3]	P^4
	n	*n*			
Population-based study (n = 1106)					
rs1554483					
CC	251	111	—	—	
CG	337	192	1.29 (0.96, 1.73)	1.36 (1.079, 1.72)	0.005
GG	123	86	1.58 (1.09, 2.29)	—	
rs11932595					
GG	93	48	—	—	
AG	323	173	1.04 (0.69, 1.58)	1.07 (0.85, 1.36)	0.277
AA	294	168	1.11 (0.73, 1.69)	—	
rs4580704					
GG	72	35	—	—	
CG	303	146	0.99 (0.62, 1.61)	1.24 (0.97, 1.58)	0.043
CC	333	205	1.27 (0.80, 2.03)	—	
rs6843722					
AA	273	123	—	—	
AC	322	192	1.32 (0.99, 1.77)	1.31 (1.03, 1.65)	0.013
CC	112	73	1.45 (0.99, 2.11)	—	
rs6850524					
CC	100	47	—	—	
CG	306	146	1.02 (0.67, 1.55)	1.33 (1.05, 1.70)	0.010
GG	280	186	1.41 (0.94, 2.14)	—	
rs4864548					
GG	248	111	—	—	
AG	336	191	1.27 (0.95, 1.71)	1.33 (1.06, 1.69)	0.009
AA	121	83	1.53 (1.05, 2.23)	—	
Hospital-based study (n = 200)					
rs1554483					
CC	25	26	—	—	
CG	29	65	2.16 (1.00, 4.61)	2.69 (1.51, 4.81)	0.001
GG	10	45	4.33 (1.67, 11.64)	—	
rs11932595					
GG	6	12	—	—	
AG	32	46	0.72 (0.20, 2.34)	1.68 (0.94, 3.00)	0.041
AA	26	78	1.50 (0.42, 4.85)	—	
rs4580704					
GG	9	8	—	—	
CG	29	59	2.29 (0.70, 7.55)	1.73 (0.97, 3.08)	0.030
CC	26	69	2.99 (0.90, 9.86)	—	
rs6843722					
AA	28	25	—	—	
AC	26	71	3.06 (1.43, 6.55)	2.83 (1.57, 5.11)	0.001
CC	10	40	4.48 (1.73, 12.05)	—	
rs6850524					
CC	15	12			
CG	28	57	2.55 (0.96–6.79)	2.33 (1.31–4.14)	0.002
GG	21	67	3.99 (1.46–10.86)		
rs4864548					
GG	27	25			
AG	28	68	2.63 (1.23–5.60)	3.04 (1.69–5.49)	0.001
AA	9	43	5.16 (2.09–14.35)		
Combined studies (n = 1306)					
rs1554483					
CC					
CG			1.38 (1.06–1.80)	1.51 (1.21–1.86)	0.001
GG			1.84 (1.33–2.54)		
rs11932595					
GG					
AG			0.99 (0.69–1.44)	1.14 (0.97–1.35)	0.232
AA			1.15 (0.79–1.66)		

(*Continued*)

TABLE 4 (*Continued*)

NCBI SNP reference and genotype	Lean subjects	Obese or overweight subjects	OR (95% CI)[2]	Cumulative OR (95% CI)[3]	P^4
	n	*n*			
rs4580704					
GG					
CG			1.13 (0.74–1.70)	1.30 (1.04–1.62)	0.021
CC			1.43 (0.95–2.14)		
rs6843722					
AA					
AC			1.48 (1.14–1.92)	1.46 (1.18–1.81)	0.001
CC			1.73 (1.24–2.41)		
rs6850524					
CC					
CG			1.19 (0.82–1.70)	1.45 (1.16–1.81)	0.001
GG			1.65 (1.15–2.37)		
rs4864548					
GG					
AG			1.41 (1.08–1.83)	1.5 (1.22–1.86)	0.001
AA			1.83 (1.32–2.54)		

[1] Subjects with a BMI ≥ 27 (in kg/m^2) were classified as overweight or obese. NCBI, National Center for Biotechnology Information.

[2] Odds ratios (OR) and 95% CIs toward the first genotype for the other 2 genotypes are indicated. The reference genotype was the homozygous genotype for the protective allele from the association test analysis using HAPLOVIEW software. The assignment was performed whether the genotype is the more common or the less common one.

[3] Cumulative ORs using proportional odds model (Liu-Agresti method; 27) are indicated. Cumulative OR stands for the cumulative effect of the 2 genotypes (heterozygous and homozygous) for the risk allele (and then risk allele copy) in comparison with homozygous genotype for the nonrisk allele.

[4] One-sided alternative (cases > controls) significance from the extended Mantel-Haenszel (MH) test for trend.

[5] An MH test for trend, MH ORs (95% CI) to the reference genotype and MH cumulative ORs (95% CI) for the combined studies are shown.

surrogates for single untyped SNPs by using the web-based service of the Tagger computer program (aggressive tagging approach) (19) for whites from the Caucasian European Utah dataset (Internet: www.hapmap.org) with a minor allele frequency ≥ 0.10 and a minimum r^2 of 0.8. The algorithm used in the Tagger program (Internet: http://www.broad.mit.edu/mpg/tagger/) selects tSNPs to construct single-marker and multimarker tests to capture alleles of interest based on the computed correlation r^2 between them (19); multimarker analysis was performed by using HAPLOVIEW software [version 3.32; Whitehead Institute for Biomedical Research, Cambridge, MA; Internet: http://www.broad.mit.edu/mpg/haploview/ (20)].

Genotyping was performed by using a high-throughput genotyping method involving polymerase chain reaction amplification of genomic DNA with 2-tailed allele-specific primers that introduce priming sites for universal energy transfer–labeled primers (PreventionGenetics, Marshfield, WI) as previously described (21). To ensure genotyping quality, we included DNA samples as internal controls, hidden samples of known genotype, and negative controls (water). No genotype with a signal below a negative control was scored. The error analysis was performed by replicating 8 times a blinded sample (that always belongs to the same person) across the templates of the project. On 216 genotypes for the "blinded sample," we had only 1 unmatched genotype (0.46% error).

Haplotype frequencies and LD measures were estimated by using Haploview software (20). PLINK software [version 0.99p; Internet: http://pngu.mgh.harvard.edu/purcell/plink/ (22)] was used for assessing associations between SNPs and affection status and quantitative traits and for testing Hardy-Weinberg equilibrium. SNP haplotype analysis was performed by using both WHAP software [version 2.09; Internet: http://pngu.mgh.harvard.edu/purcell//whap/) (23)] and HAPLOVIEW (20). Control for multiple testing was done by using the maximum of the test statistics permutation testing of individual label to obtain an empirical *P* value using 10 000 permutations.

A power estimation for the sample of 391 cases and 715 controls was performed for single-point allelic effects, with an odds ratio (OR) of 1.5, at a nominal significance level of 0.008 that corresponds to an empiric *P* value of 0.05 for HapMap-predicted minor allele frequency of 0.29 (rs4580704) to 0.45 (rs4864548) of a potential susceptibility marker and a prevalence of the disease of 26% (24). This analysis gave us an estimated power of 100% under the additive model.

In addition, because subdivision or recent admixture of populations in case-control association studies may lead to spurious associations between phenotypes and truly unlinked loci, we used a collection of 13 SNPs randomly selected at different loci (located in chromosomes 4, 15, 17, 13, 1, and 3). Next we analyzed the data with STRUCTURE software (version 2.0; Internet: http://pritsch.bsd.chicago.edu/structure.html) (25) and computed the sum of chi-square tests from each locus with the number of df equal to the sum of the number of individual loci (26) to explore a possible stratification in both populations.

Statistical analysis

Quantitative data were expressed as means ± SEs. For univariate analysis, differences between groups were assessed by analysis of variance or Student's *t* test on log-transformed variables when variable variance was homogenous as assessed by Levene's test. Otherwise, we used the nonparametric Kruskal-Wallis test by ranks. Logistic regression was used for testing of multivariate associations between overweight or obesity and genotypes and haplotypes after adjustment for covariates such as

age, sex, and HOMA index after log transformation of the variables. We used CSS/STATISTICA software (version 6.0; StatSoft, Tulsa, OK) to perform these analyses. Results from the different populations were combined by Mantel-Haenszel (MH) meta-analysis. Heterogeneity was evaluated with the Q statistic and the I^2 statistic, a transformation of Q that estimates the percentage of the variation in effect sizes that is due to heterogeneity. For the sake of simplicity, P values were rounded to 3 decimals.

RESULTS

Clinical features, anthropometric variables, and laboratory findings of the participants according to overweight or obesity status are shown in **Table 1** for both population-based and hospital-based studies. Overweight or obese subjects are older and had most of the risk factors of the metabolic syndrome: elevated waist circumference and waist-hip ratio, systolic and diastolic blood pressures, fasting glucose and insulin, HOMA index, total cholesterol, triacylglycerols, and uric acid. They also had some features of low-grade inflammation, such as elevated leukocyte count and erythrocyte sedimentation rate.

Clock gene variants

To minimize the burden of genotyping the multiple variants present in the candidate gene *CLOCK,* we selected 6 tagSNPs showing a minor allele frequency > 10% (ie, rs1554483 C/G, rs11932595 A/G, rs4580704 C/G, rs6843722 A/C, rs6850524 G/C, and rs4864548 G/A) encompassing 117 kb in chromosome 4 (56 139 000–56 256 000). The 6 tagSNPs represent 115 polymorphic sites with an r^2 >0.8 considering the HapMap project data (**Table 2**). The basic LD plot between the studied SNPs is shown in **Figure 1**. The Tagger algorithm showed the previously mentioned single-marker tags and 8 multimarker tags.

No marker showed a departure from Hardy-Weinberg equilibrium ($P > 0.1$), which indicated robust genotyping performance (data not shown). The genotyping success rate was 99.5% for rs1554483, 99.4% for rs11932595, 99% for rs4580704, 99% for rs6843722, 96.3% for rs6850524, and 98.6% for rs4864548.

In univariate analysis, after the multiple comparison correction by permutation tests, the genotype frequencies of 4 tSNPs in lean and overweight or obese persons showed significant differences. For rs1554483, rs6843722, rs6850524, and rs4864548, the empiric P values were <0.010, 0.021, 0.021, and 0.010, respectively. It is interesting that we were able to show that the results remained significant when currently recommended cutoffs for overweight (BMI ≥ 25) and obesity (BMI ≥ 30) were used, at least for rs1554483 and rs4864548 (**Table 3**).

Genotype counts in lean and obese persons for each tSNP are shown in **Table 4**. No significant association was observed between any quantitative metabolic trait (total cholesterol, HDL and LDL cholesterol, triacylglycerols, HOMA, fasting glucose, and fasting insulin) and the tSNPs analyzed (data not shown). Logistic regression analysis, adjusted for age and HOMA index, showed a still significant effect of 3 tSNPs (ie, rs1554483, $P < 0.02$; rs6850524, $P < 0.01$; and rs4864548, $P < 0.04$) on the risk of overweight or obesity.

Using the WHAP software, we further evaluated the LD pattern for the previously mentioned tSNPs. The estimation of haplotypes from genotype data in our population showed 6 possible combinations covering 99.1% of the common haplotypes with a frequency of >0.01 (**Table 5**). Among all of the haplotypes, the GACCGA haplotype alone explains much of the overall association at the locus (empiric $P < 0.045$).

TABLE 5
Estimated haplotype frequency distributions of *CLOCK* gene single-nucleotide polymorphisms (SNPs) in lean and overweight or obese subjects[1]

Haplotype	Lean subjects (n = 1430)	Overweight or obese subjects (n = 782)	Empiric P[2]
GACCGA	0.29	0.34	0.044
CAGACG	0.31	0.27	0.051
CGCAGG	0.22	0.21	0.623
GGCCGA	0.09	0.09	0.511
CGCACG	0.04	0.03	0.247
GACAGA	0.02	0.03	0.188

[1] Subjects with a BMI ≥ 27 (in kg/m^2) were classified as overweight or obese. Haplotypes are composed of variants of rs1554483, rs11932595, rs4580704, rs6843722, rs6850524, and rs4864548 in that order. n = the number of chromosomes examined (2N haplotypes).

[2] Overall comparison by chi-square test.

In an attempt to dissect the association signal, we performed the analysis removing individual markers one by one, and we observed that *CLOCK* gene variant haplotype frequencies of rs1554483C/rs6843722A/rs4864548G and rs1554483G/rs6843722C/rs4864548A in obese persons differed significantly from those in lean subjects (cases, 53% versus controls, 59%; empiric $P < 0.009$; cases, 43% versus controls, 39%; empiric $P < 0.026$, respectively). Dropping out rs6843722 showed that this SNP has no independent effect. Haplotype analysis consistently showed that only paired haplotypes (all possible haplotypes of SNPs pairs), which included rs1554483 and rs4864548, showed a significant effect on obesity or overweight (data not shown).

It is not surprising that further analysis indicated that only combinations of rs1554483 and rs4864548 (haplotype block CG and GA) are mostly responsible for the gene effect (CG frequencies: cases, 53% versus controls, 59%; GA frequencies: cases, 47% versus controls, 41%; empiric $P = 0.0102$). In addition, we found that subjects carrying the haplotype of rs1554483-G and rs4864548-A are 1.5 times as likely to be overweight or obese (OR: 1.50; 95% CI: 1.03, 2.18; $P < 0.033$, after adjustment for age and HOMA index) as are those with other haplotypes.

Finally, we were able to validate the results of the population-based study in the concurrent sample of persons ascertained from a hospital-based study, because we found a significant effect on overweight or obesity for rs1554483 (empiric $P < 0.005$), rs6843722 (empiric $P < 0.005$), rs6850524 (empiric $P < 0.015$), and rs4864548 (empiric $P = 0.005$). These associations persisted after control for age and sex by logistic regression (rs1554483, $P < 0.001$; rs6843722, $P < 0.001$; rs6850524, $P < 0.002$; and rs4864548, $P < 0.001$).

Genotype counts in cases and controls for each tSNP are shown in Table 4. Moreover, the haplotype block CG and GA for rs1554483 and rs4864548, respectively, showed a significant effect (empiric $P < 0.001$) on obesity as compared with the lean control group (CG frequencies: cases, 44% and controls, 63%; GA frequencies: cases, 56% and controls, 37%). The combined MH fixed effect for both populations was an OR of 1.82 (95% CI: 1.31, 2.54; $P < 0.001$). The test for heterogeneity in combined

TABLE 6
Association study of single-tag single-nucleotide polymorphism (SNP) and multimarker tagSNP tests (haplotypes) in *CLOCK* with overweight or obesity in the combined group[1]

	Risk allele	Haplotype	Frequency	OR (95% CI)	*P*
Single-tagSNP					
rs1554483	*G*		0.433	1.361 (1.156, 1.603)	0.001
rs11932595	*A*		0.640	1.105 (0.931, 1.311)	0.252
rs4580704	*C*		0.702	1.223 (1.023, 1.462)	0.028
rs6843722	*C*		0.407	1.330 (1.128, 1.569)	0.001
rs6850524	*G*		0.645	1.386 (1.166, 1.649)	0.001
rs4864548	*A*		0.433	1.344 (1.140, 1.548)	0.001
Multimarker tagSNP test					
rs11932595, rs6850524		AC	0.305	0.804 (0.674–0.960)	0.016
		GC	0.046	0.649 (0.431–0.977)	0.038
rs4580704, rs4864548		CA	0.429	1.349 (1.147–1.588)	0.001
		GG	0.304	0.812 (0.680–0.970)	0.022
		CG	0.267	0.856 (0.711–1.031)	0.108
rs4580704, rs6843722		CA	0.293	0.890 (0.743–0.1.06)	0.206
rs4580704, rs6850524		GC	0.304	0.795 (0.665–0.950)	0.011
rs6850524, rs4864548		GG	0.221	0.952 (0.782–1.160)	0.626

[1] $n = 1306$. Results from the 2 studies were combined by Mantel-Haenszel meta-analysis of individual odds ratios (ORs) calculated from counts obtained by using HAPLOVIEW software in each study.

MH was significant for rs1554483, rs6843722, and rs4864548. The MH effects for the individual SNPs according to genotypes are shown in Table 4.

Association results for the 6 single-marker and 8 multimarker tests from the 2 populations were combined by MH fixed effect and are presented in **Table 6**. The probabilistic estimate of each multimarker haplotype was compared individually against the others by using the HAPLOVIEW software.

We also analyzed BMI values as a continuous trait in both populations, and we observed that mean BMI values for each genotype were significantly different across genotypic groups—in particular, for rs1554483 and rs4864548—in both studies (**Table 7**). In addition, rs4580704 genotypes showed significant differences in the population-based study and rs6843722 genotypes showed significant differences in the hospital-based study. Information about waist circumference in both populations according to genotypes is shown in Table 7. We observed that mean waist circumference values for each genotype showed significant differences across genotypic groups—in particular, for rs1554483 and rs4864548 in the population-based sample.

We found no evidence of stratification in our samples because cases and controls had similar Q values and were assigned with a similar distance to clusters by the STRUCTURE software program (data not shown) with no further improvement in the fitting model when adding up to 4 clusters (the ln of likelihood was maximum for K = 1). Moreover, the sum of the chi-square test of individual loci indicated a nonsignificant difference in allele distributions corresponding to 13 loci in cases and controls (sum chi-square test, 14.35; df, 13; $P = 0.35$).

DISCUSSION

We examined the association of obesity with gene variants and haplotypes of the LD block of the *CLOCK* transcription factor in a sample of 1106 unrelated men of self-reported European ancestry. In this study, 4 of 6 tSNPs (ie, rs1554483, rs6843722, rs6850524 and rs4864548) were significantly associated with overweight or obesity after control for multiple testing by permutation test. In addition, we were able to show significant differences in BMIs across genotypic groups. When, trying to determine which of the associated variants contains the true signal, we observed that haplotype frequencies of rs1554483C/rs6843722A/rs48645448G and rs1554484G/rs6843722C/rs48645448A in obese subjects differed significantly from those in lean subjects. The haplotype of rs1554483-G and rs4864548-A was associated with a 1.50-fold risk of overweight or obesity. We observed comparable results in an independent group of subjects from a second sample.

Combining both studies by the MH approach ($n = 1306$), we observed an even stronger association with overweight or obesity (fixed-effect model, OR: 1.82; 95% CI: 1.31, 2.54; $P < 0.001$). A note of caution should be added, because we observed heterogeneity for some SNPs (3 of 6). However, it is worth mentioning that we may expect some degree of biological variability between populations, particularly because of natural heterogeneity among persons, owing to differences in their physiologic stages and sex, among other factors. Nevertheless, the effects remain in the same direction in both populations.

A limitation of our study should be noted, especially with respect to the partial dependence of the results on the definition of obesity. Nevertheless, we were able to show that the results remained significant even when currently used cutoffs for overweight and obesity are applied, at least for rs1554483 and rs48644. Furthermore, these 2 SNPs are associated with different BMIs in both the population-based and hospital-based samples and with different waist circumferences in the population-based sample. Although population stratification can lead to false-positive association results, we examined the issue of population stratification by using multilocus genotype data. No evidence of stratification difference was observed in our populations.

TABLE 7
Analysis of the continuous traits BMI and waist circumference values for each genotype in both populations

NCBI SNP reference[2] and genotype	Subjects	BMI	P^3	Waist circumference	P^3
	n			*cm*	
Population-based study (n = 1106)					
rs1554483					
CC	362	26.31 ± 0.26[4]	0.0329	91.0 ± 0.7	0.020
CG	529	26.79 ± 0.21		93.7 ± 0.6	
GG	209	26.86 ± 0.34		93.0 ± 0.9	
rs11932595					
AA	462	26.67 ± 0.23	0.9616	93.0 ± 0.6	0.670
AG	496	26.71 ± 0.22		92.5 ± 0.6	
GG	141	26.35 ± 0.42		91.9 ± 1.1	
rs4580704					
CC	538	26.74 ± 0.21	0.0310	93.2 ± 0.6	0.220
CG	449	26.65 ± 0.23		92.2 ± 0.6	
GG	107	26.01 ± 0.48		91.6 ± 1.3	
rs6843722					
AA	396	26.49 ± 0.25	0.0837	91.5 ± 0.6	0.170
AC	514	26.73 ± 0.22		93.7 ± 0.6	0.170
CC	185	26.80 ± 0.36		92.6 ± 0.9	
rs6850524					
CC	147	25.97 ± 0.41	0.055	91.4 ± 1.1	0.210
CG	452	26.71 ± 0.23		92.4 ± 0.6	
GG	466	26.83 ± 0.23		93.4 ± 0.6	
rs4864548					
AA	204	26.84 ± 0.35	0.0464	93.1 ± 0.9	0.020
AG	527	26.79 ± 0.21		93.8 ± 0.6	0.020
GG	359	26.34 ± 0.26		91.2 ± 0.7	
Hospital-based study (n = 200)					
rs1554483					
CC	51	28.34 ± 4.14	0.022	94.6 ± 2.8	0.630
CG	94	35.45 ± 3.16		96.2 ± 2.1	0.630
GG	55	31.47 ± 4.23		99.2 ± 2.8	
rs11932595					
AA	104	35.46 ± 3.02	0.0682	96.8 ± 2.0	0.940
AG	78	29.42 ± 3.37	0.0682	96.7 ± 2.2	0.940
GG	18	29.26 ± 7.23		92.5 ± 4.8	
rs4580704					
CC	95	30.50 ± 3.22	0.2503	96.7 ± 2.1	0.180
CG	88	35.58 ± 3.35		96.6 ± 2.2	
GG	17	27.73 ± 7.34		91.5 ± 4.7	
rs6843722					
AA	53	27.87 ± 4.12	0.0059	93.7 ± 2.7	0.580
AC	97	35.88 ± 3.18		96.8 ± 2.0	0.580
CC	50	31.15 ± 4.47		98.9 ± 2.9	
rs6850524					
CC	27	28.00 ± 5.71	0.0977	93.0 ± 3.9	0.340
CG	85	35.42 ± 3.27		96.2 ± 2.2	
GG	88	30.91 ± 3.27		97.6 ± 2.1	
rs4864548					
AA	52	31.23 ± 4.60	0.0410	98.7 ± 2.9	0.860
AG	96	35.29 ± 3.27		95.7 ± 2.1	
GG	52	28.37 ± 4.28		96.0 ± 2.8	

[1] NCBI, National Center for Biotechnology Information.
[2] SNPs on NCBI Reference Assembly.
[3] Kruskal-Wallis by rank test.
[4] $\bar{x}$ ± SE (all such values).

Our study is an extension of an initial estimate of the association of the *CLOCK* variants with the previously mentioned phenotype (28). By the time the present report was undergoing review, another study was reported that replicated the association of the *CLOCK* variants with obesity, at least with a haplotype containing the rs4864548 in the promoter region of the gene, in

a smaller sample of adults from a family-based study (29). In addition, a recent report showed that several *CLOCK* genes, such as *Bmal1*, *Per2*, and *Cry1*, are expressed in both subcutaneous and visceral fat, and these *CLOCK* gene expressions were related to the features of the metabolic syndrome (30). The effects of circadian gene networks on obesity- and metabolic syndrome–associated phenotypes extend beyond the *CLOCK* gene; for instance, it was recently shown that Rev-erb alpha also regulates lipid metabolism, adipogenesis, and vascular inflammation and also cross-talks with several other nuclear receptors involved in energy homeostasis and circadian rhythm (31, 32).

Although the molecular mechanisms underlying the observed association are unknown, several lines of evidence support the connection between the *CLOCK* variants and overweight or obesity. For instance, obesity has been associated with dysregulated circadian expression profiles of leptin, adiponectin, and other fat-derived cytokines (33). In addition, it was recently shown that *Clock* is involved in obesity-induced disordered fibrinolysis in *ob/ob* mice by regulating plasminogen activator inhibitor type 1 gene expression in a tissue-dependent manner (34). Another mouse model has shown relations between circadian mechanism dysfunction and glucose homeostasis regulation because inactivation of the known clock components *Bmal1* and *CLOCK* suppresses the diurnal variation in glucose and triacylglycerols (35).

Finally, new evidence that *CLOCK* mutant mice are hyperphagic and obese (9) suggests a previously unrecognized link between molecular controls of circadian rhythm and energy homeostasis (10). In fact, *CLOCK* mutant mice fed either a regular or high-fat diet showed significant increases in energy intake and body weight (9) and had lower orexin and ghrelin transcription levels than did wild-type mice. The variants that we observed in association with obesity were all located in different introns of *CLOCK*. To further investigate whether these tSNPs—and also the ones that were tagged in high LD—may have potential functional significance, we used both PupaSNP (Spanish National Genotyping Center, Barcelona, Spain: Internet: http://www.cegen.org) and FASTSNP (Institute of Biomedical Sciences and Institute of Information Science, Academia Sinica, Taipei, Taiwan) programs (data not shown). The analysis retrieved showed that the previously mentioned tSNPs or their tagged SNPs could affect DNA triplexes in the gene sequences, which have been suggested to be regulatory regions for control of gene expression (36). Although possible functional implications of the overweight- or obesity-associated SNPs deserve further investigation, it is tempting to speculate that the variants we are showing may affect regulatory regions for controlling gene expression by playing a role as genetic modifiers. In conclusion, the present study suggests a potential role of the *CLOCK* polymorphisms and their derived haplotypes in greater susceptibility to overweight or obesity. We hope that the present study can serve as a primer; further research is needed to extend the current findings showing the intimate mechanism by which *CLOCK* variants may lead to the mentioned phenotype.

The authors' responsibilities were as follows—SS: study design, data collection, analysis and interpretation of data, and writing of the manuscript; CG: sample collection and preparation; TFG: sample preparation and biochemical determinations; AB: sample collection and preparation; GC: data collection and assisting patients; and CJP: study design, analysis and interpretation of data, writing of the manuscript, and leadership for the project. None of the authors had a personal or financial conflict of interest exist.

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