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## Investigation of 3111T/C polymorphism of the *CLOCK* gene in obese individuals with or without binge eating disorder: Association with higher body mass index

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### Abstract

Loss of circadian patterning of metabolism-related functions seems to play a role in the pathogenesis of obesity; therefore, it is reasonable to hypothesize that the functional 3111T/C single nucleotide polymorphism (SNP) of the (*Circadian locomotor output cycles kaput*) *CLOCK* gene may have a part in the genetic susceptibility to obesity. The aim of this study was to assess the frequencies of 3111T/C *CLOCK* gene SNP in overweight/obese subjects with or without binge eating disorder (BED) as compared to normal weight healthy controls. A total of 284 Caucasian subjects, including 92 normal weight healthy subjects and 192 overweight/obese patients (107 with BED) participated into the study. Genotype and allele frequencies did not significantly differ between normal weight controls and overweight/obese patients with and/or without BED. However, overweight/obese patients carrying the CC genotype had significantly higher values of body mass index (BMI) as compared to those carrying the CT and/or TT genotypes. Moreover, obese class III individuals had a significantly higher frequency of both the CC genotype and the C allele as compared to individuals with BMI < 40 kg/m<sup>2</sup>. Present findings show for the first time that the 3111T/C SNP of the *CLOCK* gene is not associated to human obesity and/or BED, but it seems to predispose obese individuals to a higher BMI.

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An exciting new study field in the etiopathogenesis of obesity is represented by the endogenous circadian clock system, which has been shown to have a part in the regulation of body weight (BW) and energy homeostasis. Indeed, BW fluctuations associated with changes in day length have been observed in rodents, which undergo significant weight gain by decreasing the length of the light phase [6]. Similarly, in the Siberian hamster, tightly controlled systems of energy balance have been identified that are coordinated by the length of the photoperiod [10]. Additional researches in humans suggest a link between disrupted sleep and obesity, since night-shift workers and sleep-restricted subjects have a higher incidence of obesity, diabetes and other metabolic or endocrine disturbances [7,15]. All these data suggest that

loss of circadian patterning of metabolism-related functions may play a role in the pathogenesis of obesity.

In mammals, the “master clock” driving circadian rhythms is located in the suprachiasmatic nucleus of the hypothalamus. The molecular mechanisms underlying circadian rhythmicity involve self-sustaining positive and negative transcriptional/translational feedback loops based on rhythmic expression of the mRNA and proteins of clock components [11]. The core oscillation is thought to be driven by several genes called “clock genes” for their crucial role in the clockwork.

The gene *Circadian locomotor output cycles kaput* (*CLOCK*) is part of the positive regulatory branch of the system, with its protein product driving transcription of other genetic components of the molecular oscillator [17]. A single nucleotide polymorphism (SNP) called 3111T/C (T to C nucleotide substitution in position 3111 of DNA sequence) in the 3'-flanking region of the human *CLOCK* gene has been identified [8]. It

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has been reported that individuals who were homozygous or heterozygous for the 3111C allele had an increased evening preference compared with those carrying the T/T genotype [8], which suggests a possible involvement of this polymorphism in human disorders characterized by alterations of endogenous circadian rhythms. Therefore, it is reasonable to hypothesize that the 3111T/C *CLOCK* gene SNP may have a part in the genetic susceptibility to obesity. In order to investigate this issue, we designed a case-control study aiming to assess the frequencies of 3111T/C *CLOCK* gene SNP in overweight/obese subjects as compared to normal weight healthy controls. Moreover, since in up to 30% of obese subjects seeking treatment obesity seems to be associated with binge eating disorder (BED) [16], we explored whether the 3111T/C *CLOCK* gene SNP was associated specifically to this phenotype.

Two hundred six overweight/obese Caucasian patients (30 men, 176 women) consecutively attending the outpatient unit of the Eating Disorder Center of the Department of Psychiatry of the University of Naples were enrolled into the study. According to the Diagnostic and Statistical Manual of Mental Disorders-IV edition (DSM-IV) criteria [1], 107 of them met the diagnosis of BED, as confirmed by the Structured Clinical Interview for DSM IV-Patient Edition (SCID-IP) [4]. Obese binge eaters who did not meet full criteria for BED (14 subjects) were excluded from the study. The final sample consisted of 192 overweight/obese subjects (28 men, 164 women).

A group of 92 normal weight Caucasian healthy subjects (14 men, 78 women) were also recruited. They were mentally healthy as assessed by the SCID-I non-patient edition [5] and were specifically matched to overweight/obese patients on the basis of geographical place of origin. Their eating habits were carefully assessed by a clinical interview in order to exclude those subjects who had aberrant eating attitudes either in the past or at the time of the study.

In each subject, the body mass index (BMI) was calculated as the ratio between BW (kg) and height (m<sup>2</sup>). According to the World Health Organization BMI ranges [19], subjects were categorized as normal weight (18.5–24.9 kg/m<sup>2</sup>), overweight (25.0–29.9 kg/m<sup>2</sup>) or obese (30.0 kg/m<sup>2</sup> or above). Seventeen of the patients were overweight the remaining ones were obese. All of the subjects provided written informed consent to participate into the study after a complete description of the experimental

procedures. The study was approved by the local ethics committee. Experiments were conducted in accordance with the Declaration of Helsinki.

From all subjects, 20 ml of venous blood was drawn into EDTA vacuum tubes and immediately frozen at –20 °C till the extraction of genomic DNA from nucleated white blood cells. The polymorphic region of *CLOCK* gene was amplified by polymerase chain reaction (PCR) using primers and conditions as described by Katzenberg et al. [8]. Amplified fragments were digested by use Bsp 1286I restriction enzyme (New England Biolabs, England, UK). The unrestricted PCR product (T/T genotype) had a size of 221 bp; complete restriction (C/C genotype) produced bands of 125 and 96 bp.

Statistical analyses were performed using the BMDP statistic software package [3]. The Pearson  $\chi^2$  test was performed to compare genotype and allele frequencies among groups. Moreover, in order to test whether differences in phenotypic variables among the groups were influenced by 3111T/C polymorphism of the *CLOCK* gene, two-way, one-way analyses of variance (ANOVA) and the post-hoc Tukey's test were performed where appropriate.

The distribution of the 3111T/C genotypes did not differ significantly from that expected according to the Hardy–Weinberg equilibrium in both normal weight healthy controls ( $\chi^2 = 0.103$ ; d.f. = 1,  $P = \text{NS}$ ) and overweight/obese subjects ( $\chi^2 = 3.429$ ; d.f. = 1,  $P = \text{NS}$ ). Genotype and allele frequencies did not significantly differ between normal weight controls and overweight/obese patients ( $\chi^2 = 1.66$ ; d.f. = 2,  $P = 0.4$  for genotypes;  $\chi^2 = 0.008$ ; d.f. = 1,  $P = 0.9$  for alleles) (Table 1). Similarly, when data were reanalyzed after stratifying overweight/obese patients according to the presence of BED, the genotype and allele frequencies did not significantly differ among diagnostic groups ( $\chi^2 = 2.997$ ; d.f. = 4,  $P = 0.5$  for genotypes;  $\chi^2 = 0.136$ ; d.f. = 2,  $P = 0.9$  for alleles).

Clinical and anthropometrical characteristics of the study groups according to 3111T/C genotype are shown in Table 2. Statistical comparisons of BMI, maximum past BW and minimum past BW values among the 2 subject groups by means of two-way ANOVA revealed main effects of diagnosis on BMI ( $F_{1,278} = 284.62$ ,  $P < 0.000001$ ), maximum past BW ( $F_{1,251} = 178.8$ ,  $P < 0.00001$ ) and minimum past BW ( $F_{1,251} = 60.25$ ,  $P < 0.00001$ ) with a trend toward a significant

Table 1

Genotype and allele frequencies of 3111T/C *CLOCK* gene polymorphism in overweight/obese patients with or without binge eating disorder (BED) and in control subjects

	Genotypes			$P^a$	Alleles		$P^a$
	CC	CT	TT		C	T	
Control subjects ( $N = 92$ )	7 (7.6%)	39 (42.4%)	46 (50.0%)		53 (28.8%)	131 (71.2%)	
Overweight/obese subjects with or without BED ( $N = 192$ )	21 (10.9%)	68 (35.4%)	103 (53.6%)	0.4	112 (29.1%)	272 (70.8%)	0.9
Overweight/obese subjects with BED ( $N = 107$ )	14 (13.0%)	36 (33.6%)	57 (53.2%)	0.2	64 (29.9%)	150 (70.0%)	0.8
Overweight/obese subjects without BED ( $N = 85$ )	7 (8.2%)	32 (37.6%)	46 (54.1%)	0.8	48 (28.2%)	122 (71.7%)	0.8

<sup>a</sup> As compared to control subjects (Pearson  $\chi^2$  test).

Table 2  
Clinical characteristics of healthy controls and patients with overweight/obesity according to 3111T/C CLOCK genotypes

	Healthy controls			Patients with overweight/obesity		
	CC	CT	TT	CC	CT	TT
Age (years)	25.1 ± 4.1	27.5 ± 5.3	25.7 ± 4.4	40.0 ± 9.8	35.1 ± 10.6	40.1 ± 12.3
Body mass index (kg/m <sup>2</sup> )	21.8 ± 3.4	22.2 ± 5.2	21.4 ± 2.0	43.2 ± 8.0	40.9 ± 7.5	38.3 ± 7.4*
Minimum past body weight (kg)	56.5 ± 6.9	50.8 ± 6.5	54.5 ± 5.1	75.6 ± 15.7	71.3 ± 14.5	68.7 ± 14.1
Maximum past body weight (kg)	66.3 ± 8.1	58.8 ± 6.5	62.8 ± 7.5	116.5 ± 21.2	113.5 ± 21.1	109.5 ± 27.0

\*  $P < 0.05$  as compared to CC genotype (post-hoc Tukey's test).

effect of genotype on BMI ( $F_{2, 278} = 2.66$ ,  $P = 0.07$ ) but not on maximum past BW ( $F_{2, 251} = 0.54$ ,  $P = 0.5$ ) and minimum past BW ( $F_{2, 251} = 0.99$ ,  $P = 0.3$ ), and no significant diagnosis X genotype interactions ( $F_{2, 278} = 1.29$ ,  $P = 0.2$  for BMI;  $F_{2, 251} = 0.91$ ,  $P = 0.4$  for maximum past BW, and  $F_{2, 251} = 1.57$ ,  $P = 0.2$  for minimum past BW). Because of these results, one-way ANOVA was performed to assess the effects of genotypes on BMI in each diagnostic group. This analysis showed that genotype significantly affected BMI in the overweight/obese individuals ( $F_{2, 189} = 4.91$ ,  $P = 0.008$ ), but not in normal weight healthy controls ( $F_{2, 89} = 0.44$ ,  $P = 0.6$ ). Indeed overweight/obese patients carrying the CC genotype had significantly higher values of mean BMI as compared to TT individuals (Table 2).

When we stratified overweight/obese subjects in those with BMI  $\geq 40$  kg/m<sup>2</sup> (obese class III, according to WHO criteria) or with BMI  $< 40$  kg/m<sup>2</sup>, both CC and CT genotypes as well as the C allele were significantly more frequent in the obese class III group ( $\chi^2 = 12.492$ ; d.f. = 2,  $P = 0.001$  for genotypes;  $\chi^2 = 11.177$ ; d.f. = 1,  $P = 0.0008$  for alleles) (Table 3). The C allele was associated with a significant risk to develop a class III obesity (OR = 2.138; CI = 1.352–3.380).

There is substantial evidence that major components of energy homeostasis, including the sleep–wake cycle, thermogenesis, feeding, and glucose and lipid metabolism, are subjected to circadian regulation that synchronizes energy intake and expenditure with changes in the external environment [9]. Therefore, a role for endogenous molecular mechanisms underlying altered circadian rhythmicity in obesity seemed reasonable, and prompted us to investigate the frequency of the 3111T/C SNP of the CLOCK gene in patients with overweight/obesity. We could not find any significant association between this SNP and overweight/obesity. Moreover, when we stratified overweight/obese patients according to the presence or not of BED, no signif-

icant difference emerged in genotype distribution between the groups, suggesting that BED was not a phenotypic variable associated with the 3111T/C CLOCK gene SNP in overweight/obese patients. However, overweight/obese patients carrying the CC genotype had significantly higher values of BMI as compared to those carrying the TT genotype, suggesting that the C allele of the 3111T/C SNP of the CLOCK gene may confer to obese individuals a susceptibility to reach a higher BMI. This was corroborated by the demonstration that both the CC genotype and the C allele were significantly more frequent in obese class III individuals as compared to obese subjects with BMI  $< 40$  kg/m<sup>2</sup>.

To the best of our knowledge, no study so far investigated the role of the 3111T/C CLOCK gene SNP in human obesity with or without BED; hence, our results are not comparable to literature data. However, animal data support the idea that a dysfunction in the CLOCK gene may lead to aberrant eating behaviour and obesity. In fact, Turek et al. [18] reported that mutant mice expressing a dysfunctional splice variant of the CLOCK gene, despite only mild disruption of their locomotor activity rhythm, had a near total loss of selective nocturnal feeding, consuming close to 50% of their daily food intake during the light phase. Moreover, these mice were hyperphagic and developed obesity associated with metabolic abnormalities.

The 3111T/C CLOCK gene SNP seems to have functional consequences, since it has been shown to affect mRNA stability and half-life [12] with possible significant effects on the level of protein finally being translated; hence, this polymorphism may be responsible of alterations in endogenous circadian rhythms. Actually, the 3111T/C SNP has been associated to some circadian rhythm disorders occurring in certain human disorders such as the wake-sleep cycle dysregulation in major depression and bipolar disorders [13], the long-term recurrence of affective episodes in bipolar patients [2] and the time course of insomnia during antidepressant treatments [14]. Therefore,

Table 3  
Genotype and allele frequencies of 3111T/C CLOCK gene polymorphism in obese patients with body mass index  $\geq 40$  kg/m<sup>2</sup> or  $< 40$  kg/m<sup>2</sup>

	Genotypes			P	Alleles		P
	CC	CT	TT		C	T	
Obese subjects with BMI $\geq 40$ kg/m <sup>2</sup> (N = 86)	12(13.9%)	40(46.5%)	34(39.5%)		64(37.2%)	108(62.8%)	
Overweight/obese subjects with BMI $< 40$ kg/m <sup>2</sup> (N = 106)	9(8.5%)	28(26.4%)	69(65.1%)	0.001	46(21.6%)	166(78.4%)	0.0008

a role for the 3111T/C *CLOCK* gene SNP in human obesity seemed reasonable. Our findings suggest that, although no significant association was found between this polymorphism and overweight/obesity with or without BED, the C allele may predispose obese individuals to reach a higher BMI value.

The major limitation of this study is the lack of circadian measures that could allow to characterize an intermediate phenotype, which might be more strictly associated to the *CLOCK* 3111T/C SNP. To this regard, subjects with night eating syndrome would be of particular interest; hence, this area is worth of future investigations. The second study limitation is the fact that the normal control group was younger than the obese sample; so, we cannot exclude that some of our normal weight controls could develop overweight/obesity at an older age and this could have been responsible for present negative results. The last study limitation is the relatively low number of subjects included in the groups. However, although the number of subjects was relatively low for a genetic association study, the power analysis showed that the present sample size had a power of 0.89, 0.84 and 0.80 to detect a small effect size ( $w = 0.15$ ) at an alpha value of 0.05 to find significant allelic associations between control women and all overweight/obese patients, overweight/obese BED patients and overweight/obese individuals without BED, respectively. Therefore, although our results need to be replicated in larger samples, the present total sample size can be considered to be large enough statistically. Moreover, our findings are strengthened by at least two factors. First, all subjects came from the same locality and ethnic background; second, the control subjects were carefully assessed to exclude both eating disorders and other psychiatric conditions, which could have occurred at a clinical or subclinical level and thereby confound the results.

In conclusion, present findings show for the first time that the 3111T/C SNP of the *CLOCK* gene is not associated to human obesity and/or BED, but it could be a biological vulnerability factor predisposing obese individuals to reach a higher BMI value. Further studies need to elucidate whether polymorphisms of other genes implicated in the endogenous circadian oscillator system may play a role in the vulnerability to obesity and/or BED.

## References

- [1] American Psychiatric Association, Diagnostic and Statistical Manual of Mental Disorders, 4th ed., American Psychiatric Press, Washington DC, 1990.
- [2] F. Benedetti, A. Serretti, C. Colombo, B. Barbini, C. Lorenzi, E. Campori, E. Smeraldi, Influence of *CLOCK* gene polymorphism on circadian mood fluctuation and illness recurrence in bipolar depression, *Am. J. Med. Gen., Part B (Neuropsychiat. Gen.)* 123 (2003) 23–26.
- [3] J. Dixon (Ed.), *BMDP Statistical Software*, University of California Press, Berkeley, 1985.
- [4] M.B. First, R.L. Spitzer, M. Gibbon, J.B. Williams (Eds.), *Structured Clinical Interview for DSM-IV Axis I disorders SCID-I*. New York State Psychiatric Institute, Biometrics Research, New York, 1995.
- [5] M.B. First, M. Gibbon, R.L. Spitzer, J.B. Williams (Eds.), *Structured Clinical Interview for Axis I DSM-IV Disorders-Non-Patient Ed – (SCID-I/NP, Version 2.0)*, New York State Psychiatric Institute, Biometrics Research, New York, 1996.
- [6] M.R. Gorman, Differential effects of multiple short day length on body weight of gonadectomized Siberian hamsters, *Physiol. Biochem. Zool.* 76 (2003) 398–405.
- [7] B. Karlson, A. Knutsson, B. Lindahl, Is there an association between shift work and having a metabolic syndrome? Results from a population based study of 27,485 people, *Occup. Environ. Med.* 58 (2001) 747–752.
- [8] D. Katzenberg, T. Young, L. Finn, L. Lin, D.P. King, J.S. Takahashi, E. Mignot, A *CLOCK* polymorphism associated with human diurnal preference, *Sleep* 21 (1998) 569–576.
- [9] S.E. la Fleur, Daily rhythms in glucose metabolism: suprachiasmatic nucleus output to peripheral tissue, *J. Neuroendocrinol.* 15 (2003) 315–322.
- [10] P.J. Morgan, A.W. Ross, J.C. Mercer, P. Barrett P, Photoperiodic programming of body weight through the neuroendocrine hypothalamus, *J. Endocrinol.* 177 (2003) 27–34.
- [11] S. Reppert, D. Weaver, Molecular analysis of mammalian circadian rhythms, *Annu. Rev. Physiol.* 63 (2001) 647–676.
- [12] J. Ross, Control of messenger RNA stability in higher eukaryote, *Trends Genet.* 12 (1996) 171–175.
- [13] A. Serretti, F. Benedetti, L. Mandelli, C. Lorenzi, A. Pirovano, C. Colombo, E. Smeraldi, Genetic dissection of psychopathological symptoms: insomnia in mood disorders and *CLOCK* gene polymorphism, *Am. J. Med. Gen., Part B (Neuropsychiat. Gen.)* 121 (2003) 35–38.
- [14] A. Serretti, C. Cusin, F. Benedetti, L. Mandelli, A. Pirovano, R. Zanardi, C. Colombo, E. Smeraldi, Insomnia improvement during antidepressant treatment and *CLOCK* gene polymorphism, *Am. J. Med. Gen., Part B (Neuropsychiat. Gen.)* 137 (2005) 36–39.
- [15] K. Spiegel, L. Leproult, E. Van Cauter, Impact of sleep debt on metabolic and endocrine function, *Lancet* 354 (1999) 1435–1439.
- [16] R.L. Spitzer, S. Yanowski, T. Wadden, R. Wing, M.D. Marcus, A. Stunkard, M. Devlin, J. Mitchell, D. Hasin, R.L. Horn, Binge eating disorder: its further validation in a multisite study, *Int. J. Eat. Disord.* 13 (1993) 137–153.
- [17] T.D. Steeves, D.P. King, Y. Zhao, A.M. Sangoram, F. Du, A.M. Bowcock, R.Y. Moore, J.S. Takahashi, Molecular cloning and characterization of the human clock gene: expression in the suprachiasmatic nuclei, *Genomics* 57 (1999) 198–200.
- [18] F.W. Turek, C. Joshu, A. Kohsaka, E. Lin, G. Ivanova, E. McDearmon, A. Laposky, S. Losee-Olson, A. Easton, D.R. Jensen, R.H. Eckel, J.S. Takahashi, J. Bass, Obesity and metabolic syndrome in circadian clock mutant mice, *Science* 308 (2005) 1043–1045.
- [19] WHO, Obesity: preventing and managing the global epidemic. Report of a WHO consultation. WHO Technical Report Series 894. Geneva: World Health Organization 2000.