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ARTICLE

# Genetic variants in human CLOCK associate with total energy intake and cytokine sleep factors in overweight subjects (GOLDN population)

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**Despite the importance of total energy intake in circadian system regulation, no study has related human *CLOCK* gene polymorphisms and food-intake measures. The aim of this study was to analyze the associations of *CLOCK* single-nucleotide polymorphisms (SNPs) with food intake and to explore the specific role of the cytokine system. A total of 1100 individual participants in the Genetics of Lipid Lowering Drugs and Diet Network (GOLDN) study were included. Dietary intake was estimated with a validated questionnaire. Interleukin-6 (IL-6), monocyte chemotactic protein 1 (MCP1), tumor necrosis factor-α (TNF-α), IL-2 soluble receptor-α (IL-2sR-α) and adiponectin plasma concentrations were measured. Our results showed that four of five *CLOCK* SNPs selected were significantly associated with total energy intake ($P<0.05$). For SNP rs3749474, the energy intake and total fat, protein and carbohydrate intakes were significantly higher in minor allele carriers than in non-carriers. Frequency of the minor allele was greater in subjects with high energy intake than in those with low intake. Subjects with the minor allele were 1.33 times more likely to have high energy intake than non-carriers (95% CI 1.09–1.72, $P=0.0350$). All *CLOCK* SNPs were associated with plasma cytokine values, in particular with those that were highly correlated with energy intake: MCP1, IL-6 and adiponectin. Interestingly, minor allele carriers with high energy intake showed decreased cytokine values, which could be related with a lower anorectic effect and decreased sleep in these subjects. In conclusion, we show a novel association of genetic variation at *CLOCK* with total energy intake, which was particularly relevant for SNP rs3749474. Associations could be mediated through the alteration of cytokine levels that may influence energy intake and sleep pattern.**
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## INTRODUCTION

During the past century, lifestyle has changed dramatically and coincident trends with significant effects on metabolic alterations have emerged: food has become abundant, snacking frequency has increased and feeding has shifted toward the end of the day.[1] The gradual reduction in sleep time together with increased irregularity in inter-daily sleep–wake cycles have also been correlated with alterations in food intake.[2] These observations have resulted in new paradigms regarding the involvement of the circadian system in the regulation of multiple physiological and molecular processes, including those related with energy metabolism.[3]

It is now known that circadian systems can themselves be entrained by various stimuli, with feeding being a dominant external synchronizer. When feeding is scheduled during the nocturnal time, or when the daily periodic feeding signal is suppressed by constant snacking or the intake of high-energy or high-fat foods, the temporal organization generated by the suprachiasmatic nuclei (SCN) and reset by the light–dark cycle can be disrupted.[4] Conversely, feeding is subject to circadian regulation, and changes in the components of the endogenous oscillator regulating circadian rhythms may be involved in the rhythm of eating behavior and ultimately in total energy intake.[5]

Over the past few years, a revolution in the understanding of the molecular basis of internal clocks has led to the identification of a number of core clock genes and their proteins, and the development of elegant feedback models to explain the molecular gears of circadian clocks.[6] *CLOCK* gene is an important positive enhancer of the circadian SCN system. Studies performed in animal models with *CLOCK* mutants have shown that such animals show an attenuated diurnal feeding rhythm and become hyperphagic.[7] Both disruption of the circadian cycle and sleep deprivation can affect energy balance and, over time, may bring about substantial changes in the body fat content.

There seems to be an appreciable overlap between the circadian pacemaker and cytokine systems, with respect to their influence on energy intake patterns and sleep–wake regulation.[8] There is growing evidence that interleukin-6 (IL-6) and several other proinflammatory cytokines are 'sleep factors'.[9] In addition, it has been shown that these

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cytokines also influence energy intake by enhancing insulin and leptin sensitivity.[10] However, any possible role that they might have in coordinating sleep/wakefulness with food-motivated behavior awaits clarification. Yet, the evidence is increasingly strong that the neurophysiologic and metabolic mechanisms responsible for the control of food-seeking behavior and the control of sleep and wakefulness are coordinated so that hunger and vigilance are paired during the daylight hours, and satiety and sleep are paired during darkness.[8] The study of cytokines offers the possibility to connect the showed interactions among energy intake, sleep behavior and circadian system with metabolic alterations.

Previous studies have examined the relationship between *CLOCK* gene variants and sleep behavior,[11] mental disorders[12–14] and obesity.[15,16] However, and despite the relevance of energy intake in the regulation of the circadian system, none of these studies have related variation at the *CLOCK* gene with food intake. Thus, the purpose of this work was to study the association of *CLOCK* gene polymorphisms with food intake and to examine whether the cytokine system has a significant role in this connection.

## MATERIALS AND METHODS

### Study participants and study design

The study sample consisted of 540 men and 560 women who participated in the Genetics of Lipid Lowering Drugs and Diet Network (GOLDN) study. GOLDN is part of the Program for Genetic Interactions Network and is funded by the NIH through the University of Alabama at Birmingham and in collaboration with the University of Utah, Washington University, Tufts University, University of Texas, University of Michigan, University of Minnesota and Fairview University of Minnesota Medical Center. The majority of participants were re-recruited from three-generational pedigrees from two National Heart, Lung, and Blood Institute Family Heart Study field centers (Minneapolis, Minnesota, and Salt Lake City, Utah).[17] Nearly all individuals were of European descent. The details of the study are available at https://dsgweb.wustl.edu/goldn/. The protocol was approved by the institutional review boards of the University of Alabama, the University of Minnesota, the University of Utah and the Tufts University. To avoid using data from under-reporters of total energy intake, all subjects who reported less than 600 kcal/day of energy intake were excluded from the study, resulting in a final sample size of 1100 individuals.

### Dietary intake

Dietary intake was estimated using the DHQ, a food-frequency questionnaire developed by the National Cancer Institute. It consists of 124 food items and includes both portion size and dietary supplement questions. Two studies have favorably assessed its validity.[18,19] The food list and nutrient database used with the DHQ are based on national dietary data (US Department of Agriculture (USDA) 1994–1996 Continuing Survey of Food Intakes by Individuals, available from the USDA Food Surveys Research Group). Discretionary fat was defined in the food guide pyramid[20,21] as 'all excess fat beyond amounts that would be consumed if only the lowest fat forms were eaten, and fats added to foods in preparation or at the table.' To derive discretionary fat intake, we included the fat from foods including cream, butter, margarine, cream cheese, oils, salad dressings, chocolate, whole milk, sour cream, ice cream, mayonnaise, coffee whitener, and baked goods and potato chips. The grams of fat contained in a serving were then separately calculated (eg, two tablespoons of cream at 2.9 g fat/tablespoon accounts for 5.8 g discretionary fat) and summed across all foods to derive total discretionary fat consumption.

### Biochemical analyses

We drew venous blood after the study participants had fasted overnight. Plasma samples were stored on ice and analyzed promptly. Blood collection, plasma separation and processing, and biochemical analyses for inflammatory markers, including IL-6, monocyte chemotactic protein 1 (MCP1), tumor necrosis factor-α (TNF-α) and IL-2 soluble receptor-α (IL-2sR-α, IL2RA), have been described previously.[22] Plasma adiponectin was measured using competitive RIA (Linco Research, St Charles, MO, USA).

### DNA isolation and genotype analysis

We selected tag single-nucleotide polymorphisms (tSNPs) as effective proxies for untyped SNPs in strong linkage disequilibrium (LD) by using the Tagger[23] based on HapMap Caucasian European Utah data (www.hapmap.org) with a minor allele frequency (MAF) $\geqslant 0.10$ and a minimum $r^2$ of 0.8. Tagger uses an algorithm that selects tagSNPs to construct single- and multi-marker tests to capture alleles of interest based on the computed correlation $r^2$ between them. The pair-wise LD among SNPs was estimated as correlation coefficient ($R$) and Lewontin's $D'$ using Haploview (Cambridge, MA, USA).[24] The LD plot of the five SNPs is shown in Figure 1. SNPs were selected considering the LD structure by choosing tSNPs for the three large LD block. The literature reports of genetic associations or biological function of interest, and bioinformatics functional assessment, were also considered. SNP rs1464490 was selected because it was the tSNP for a large LD block (LD1); rs3749474, also from LD1, was selected after bioinformatics functional assessment suggested important changes to mRNA structure; and rs4864584 was selected because it was previously associated with overweight/obesity.[15,16] From the different SNPs comprising LD2, selection of rs4580704 was based on its previous relationship with BMI.[16] From LD3, 3111T/C caught our attention because a literature report showed associations with sleep alterations[11] and binge eating disorders.[5] Their locations in the *CLOCK* gene, Hardy–Weinberg equilibrium and MAF are listed in Table 1. *CLOCK* genotype frequencies did not deviate from Hardy–Weinberg equilibrium. Because SNPs rs3749474, rs1464490 and rs4864548 were almost in complete LD and showed a similar pattern of phenotypic associations, only the results for rs3749474 were analyzed.

DNA was isolated from blood samples using routine DNA isolation sets (Qiagen, Valencia, CA, USA). We performed genotyping of *CLOCK* gene polymorphisms using a TaqMan assay with allele-specific probes on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the standardized laboratory protocols.[25]

### Bioinformatics analysis

We performed bioinformatics analysis of the genomic DNA sequence encompassing the different SNPs to ascertain putative biological consequences of the different alleles. SNPs mapping to regions upstream of the transcription start site or within introns were studied with MAPPER[24] to identify potential allele-specific transcription factor binding sites. No intronic SNPs altered splice acceptor or donor sites or other signals recognized by the splicing machinery,

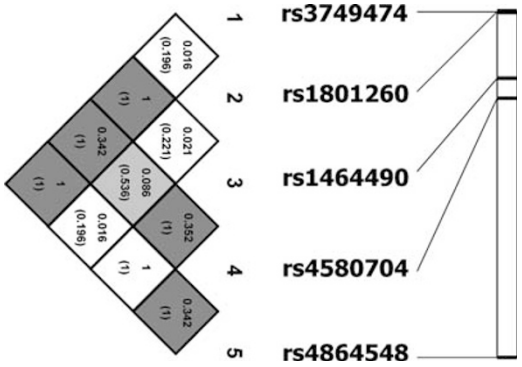


**Figure 1** Linkage disequilibrium (LD) plot across the *CLOCK* gene. The horizontal white bar depicts the 113-kb DNA segment of chromosome 4q12 analyzed in the sample. The five tagSNP locations are indicated by hatch marks. An LD plot is depicted in the bottom part of the figure: each diamond represents 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 indicate the $r^2$ (D') value.

such as the poly-pyrimidine tract. Polymorphisms within the 3′-UTR of the mRNA can exert an effect on the folding of the mRNA with concomitant changes in mRNA stability. Potential effects of the SNP on mRNA were tested with RNAfold[26] within the Vienna RNA package (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

### Statistical analysis

Plasma cytokines concentrations were log transformed to achieve approximate normality. We used Pearson's $\chi^2$ and Fisher's tests to test differences in frequencies. We applied ANOVA and the Student's *t*-test to compare crude means across genotype groups. We tested different genetic inheritance models and a dominant model was applied in the final analyses for all SNPs selected except for rs1801260 (3111T/C), which followed a recessive model. We performed multivariate adjustments of the associations by analysis of covariance and estimated adjusted means. We adjusted analyses for sex, age and family relationships. The familial relations within the population were adjusted using a generalized linear model implemented in the GENMOD procedure in SAS (SAS Institute, Inc., Cary, NC, USA) assuming an exchangeable correlation structure within pedigree.[27,28] We also tested the statistical homogeneity of the effects by sex in the corresponding regression model with interaction terms. The *a priori* analysis plan was to analyze the association between genotypes and energy intake, although several dietary intake variables, such as fat, protein, carbohydrate intake and so on, were also tested. A second objective was to analyze associations between genotypes and interleukins to evaluate potential mechanisms for the observed associations. We fitted logistic regression models to estimate the odds ratio (OR) and 95% CI of total energy intake associated with the *CLOCK* polymorphism and to control for the effect of covariates and family relationships. We used routine regression diagnostic procedures to ensure the appropriateness of the models. Statistical analyses were performed using SPSS 15.0 software (SPSS, Barcelona, Spain). A two-tailed *P*-value of $<0.05$ was considered statistically significant.

**Table 1 Description of *Clock* SNPs**

| *Name* | *Location* | *HW P-value* | *MAF* | *Alleles* | *Minor allele* |
|---|---|---|---|---|---|
| rs3749474 | 3′-UTR | 0.9618 | 0.406 | T/C | T |
| rs1801260 | 3′-UTR | 0.1816 | 0.372 | A/G | G |
| rs1464490 | Intron 11 | 1 | 0.415 | T/C | T |
| rs4580704 | Intron 9 | 0.8464 | 0.332 | C/G | G |
| rs4864548 | Promoter | 0.9618 | 0.406 | A/G | A |

HW *P*-value, Hardy–Weinberg equilibrium expectations *P*-values; MAF, minor allele frequency.

## RESULTS

### Clinical and food intake characteristics of the population

The general characteristics of the population studied, including dietary intake and cytokines measured, are shown in Supplementary Table 1. Significant inverse correlations were found in the total population between different cytokines and energy intake, as estimated using partial Pearson's correlation after controlling for sex and BMI, for each cytokine measured except for IL2RA. Cytokines for which we found significant correlations with energy intake are IL-6 ($r=-0.08$; $P=0.016$), MCP1 ($r=-0.13$; $P=0.00001$), TNF-α ($r=-0.23$; $P=0.0001$) and adiponectin ($r=-0.12$; $P=0.0001$).

### CLOCK SNPs and energy intake and interleukins

We first examined the association between the different *CLOCK* polymorphisms and food intake variables (Supplementary Table 2). Men and women were analyzed together because no heterogeneity of the effect was observed by sex. We found significant and novel associations between dietary intake and genotypes for every *CLOCK* SNP except for rs1801260 (3111T/C) in which data showed a similar trend, but did not achieve statistical significance. For rs3749474, carriers of the minor allele T reported significantly higher energy intake than non-carrier subjects, whereas for rs4580704 the carriers of the minor allele C reported the lowest values for energy intake. These differences remained statistically significant after additional adjustment for sex, age, family relationships and BMI, suggesting that data were independent of body size. When analyzing dietary habits of the population, strong associations were found between genetic variant and total and discretionary fat intake (Supplementary Table 2). We used a logistic regression model to test the association of the *CLOCK* SNPs with total energy intake, which was dichotomized according to the corresponding median values for each sex (2246 kcal/day for men and 1658 kcal/day for women; Table 2). For rs3749474, the frequency of the minor allele T was significantly greater in subjects with high energy intake than in those with low energy intake. Moreover, minor allele carriers had a greater probability of having a high energy intake than non-carriers. However, for rs4580704 the minor allele C frequency was greater in subjects with low energy intake. These data remained significant after adjusting for BMI.

**Table 2 Genotype distribution of *CLOCK* SNPs according to low ($<2246$ kcal/day for men and $<1658$ kcal/day for women) and high ($>2246$ kcal/day for men and $>1658$ kcal/day for women) energy intake in the GOLDN population**

| | *Low energy intake* | | *High energy intake* | | | *Linear regression* | |
|---|---|---|---|---|---|---|---|
| *NCBI SNP reference genotype* | n | *%* | n | *%* | P-*value*[e] | *OR (95% CI)* | P-*value*[e] |
| *rs3749474* | | | | | | | |
| CC | 232 | 42.57 | 192 | 34.5 | 0.013 | 1.33 (1.09–1.72)[a] | 0.035 |
| TT+TC[c] | 313 | 57.43 | 353 | 63.5 | | | |
| *rs4580704* | | | | | | | |
| CC | 226 | 40.8 | 257 | 46.2 | 0.045 | 1.23 (0.95–1.58) | 0.117 |
| GG+CG[c] | 327 | 59.0 | 292 | 52.5 | | | |
| *rs1801260* | | | | | | | |
| AG+AA | 458 | 82.7 | 456 | 82 | 0.716 | 1.11(0.78–1.55)[b] | 0.572 |
| GG[d] | 89 | 16.1 | 94 | 16.9 | | | |

[a]Cumulative OR stands for the cumulative effect of the two genotypes (heterozygous and homozygous) for the risk allele in comparison with homozygous for the non-risk allele.
[b]OR stands for the effect for the risk allele in comparison with the two genotypes (heterozygous and homozygous) for the non-risk allele.
[c]Dominant model (minor allele).
[d]Recessive model (minor allele).
[e]Adjusted for sex, age and family relationships.

**Table 3 Associations of *CLOCK* SNPs with different cytokines**

| | | *rs 3749474* | | | | *rs4580704* | | | | *rs1801260* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Cytokines (pg/ml)* | *Genotype* | *Mean* | *EEM* | P-*value*[c] | *Genotype* | *Mean* | *EEM* | P-*value*[c] | *Genotype* | *Mean* | *EEM* | P-*value*[c] |
| IL-6 | CC | 2.14 | 0.15 | 0.008 | CC | 1.93 | 0.141 | 0.092 | AG+AA | 2.02 | 0.23 | 0.731 |
| | TT+TC[a] | 1.89 | 0.12 | | GG+CG[a] | 2.03 | 0.124 | | GG[b] | 1.77 | 0.10 | |
| MCP1 | CC | 215.46 | 2.95 | <0.0001 | CC | 204.18 | 2.76 | 0.026 | AG+AA | 213.16 | 4.44 | 0.532 |
| | TT+TC | 203.99 | 2.35 | | GG+CG[a] | 211.96 | 2.44 | | GG[b] | 184.91 | 1.99 | |
| TNF-$\alpha$ | CC | 3.26 | 0.25 | 0.352 | CC | 3.58 | 0.21 | 0.994 | AG+AA | 3.50 | 0.38 | 0.127 |
| | TT+TC | 3.56 | 0.20 | | GG+CG[a] | 3.32 | 0.24 | | GG[b] | 3.09 | 0.17 | |
| IL2RA | CC | 1025.58 | 17.70 | 0.825 | CC | 1005.8 | 16.54 | 0.507 | AG+AA | 1030.84 | 26.86 | 0.079 |
| | TT+TC | 1020.56 | 14.10 | | GG+CG[a] | 1033.3 | 14.62 | | GG[b] | 976.86 | 12.03 | |
| Adiponectin | CC | 8600.91 | 209.34 | 0.061 | CC | 8130.3 | 195.3 | 0.050 | AG+AA | 8461.70 | 316.30 | 0.024 |
| | TT+TC | 8124.39 | 166.72 | | GG+CG[a] | 8428.60 | 172.6 | | GG[b] | 7352.37 | 141.64 | |

[a]Dominant model (minor allele).
[b]Recessive model (minor allele).
[c]Adjusted for sex, age and family relationships.

There were significant associations between *CLOCK* gene variants and cytokine measures, in particular with those highly correlated with energy intake, such as MCP1, IL-6 and adiponectin (Table 3). For rs3749474, the minor allele was associated with a decreased mean value of these cytokines, whereas for rs4580704 the minor allele was associated with the highest plasma concentrations of MCP1 and adiponectin. It is important to highlight that the associations between interleukins and genotypes were not altered after adjusting for energy intake. Similarly, significant relationships between genotypes and food intake were not altered when adjustment for cytokine levels was added to the model.

## DISCUSSION

This study provides novel evidence supporting the hypothesis that genetic variation at *CLOCK* is related with total energy intake. Specifically, four out of five *CLOCK* SNPs (rs3749474, rs4864548, rs1464490 and rs4580704) were significantly associated with energy intake. The genetic effect seems to be mainly attributed to effects on intake of all macronutrients, including total fat, carbohydrate and protein. Moreover, *CLOCK* genetic variants were significantly associated with plasma cytokine levels. These novel associations provide strong evidence in support of the important role of the circadian system in food intake and *vice versa*,[29] as suggested by molecular, experimental, clinical and epidemiological studies.[3]

It is important to highlight that *CLOCK* gene was subjected to positive selection pressures (iHS=2.47, $P$=0.0030) in African population during human evolution according to the positive selection map in the human genome (Haplotter; see http://hg-wen.uchicago.edu/selection/haplotter.htm).[30] Food intake and feeding time could be important environmental factors that have probably driven this positive selection. CLOCK proteins in conjunction with other proteins may act directly as sensors for feeding-related signals.[31] All these CLOCK proteins contain PAS domains, an ancient sensory domain conserved throughout eukaryotic organisms that detect redox state, hypoglycemia and oxygen balance.[32] PAS-containing clock proteins have the ability to sense the environment and adjust their transcriptional activity accordingly. We hypothesize that this ability is compromised in subjects with the minor allele at rs3749474. Furthermore, because CLOCK functions as a transcriptional regulator of different nuclear receptors that are known to respond to food, lipids, vitamins and fat-soluble hormones, this particular polymorphism could alter this regulation, thus influencing food intake.[31,33]

SNP rs3749474, situated in 3′-UTR, showed the strongest associations in the population studied. The mechanism by which this particular SNP was associated with energy intake could be related with the changes in *CLOCK* mRNA structure. Indeed, our analysis with the RNAfold program suggests that this one-base change (T/C) in the *CLOCK* 3′-UTR transforms the structure of this particular region. The 3′-UTR is characterized by sequences such as SECIS or AU-rich elements (AREs) that are recognized by particular proteins that exert an affect on mRNA stability or location in the cell. Although specific binding sites are not elucidated in the analysis presented here, the difference in the predicted allele-specific structures is consistent with the notion that significant effects on mRNA structure and stability are likely to occur. On the other hand, this SNP is in complete LD with rs4864548 (Figure 1), which is located in the promoter region. Variation in the promoter activity may also explain the results that we observed.

In the present study, significant associations were found not only between the *CLOCK* genotypes and total energy intake, but also for fat, protein and carbohydrate intakes (total amount, g) for rs3749474 and rs4580704. Of particular relevance were the associations found with total and discretionary fat intake. These data are of special interest because high-fat intake, which is one characteristic of modern societies, has been related with disturbances in the internal machinery of *CLOCK* genes.[3] In experimental models, high-fat diets cause metabolic alterations that ultimately disrupt the internal circadian system.[4] Significant associations were also present after adjusting for BMI, indicating that the relationships between *CLOCK* genotypes and energy intake were independent of obesity. Nevertheless, it is interesting to highlight that SNP rs4864548, which is in complete LD with rs3749474, has been previously associated with obesity. It has been shown that minor allele carriers, who showed greater energy intake in the present study, show a greater risk of being overweight and/or obese when compared with major allele carriers, and are also more likely to have metabolic syndrome, type II diabetes and cardiovascular disease.[15,16] Moreover, this particular SNP has been previously related with nonalcoholic fatty liver disease, an inflammatory condition that is a common consequence of obesity.[34]

Our results showed significant and inverse correlations between energy intake and different plasma cytokine values, not only in the

crude analysis, but also when adjusted for BMI, suggesting that these associations were independent of obesity. The anorexigenic effect of interleukins has been widely shown, especially in the mechanisms of sickness.[35] All the cytokines released by macrophages act on the hypothalamus to provoke alterations in the normal homeostatic condition. These include increased sleep, loss of appetite and major alterations in lipid and protein metabolism.[35] Cytokines also blunt gastric ghrelin secretion and ghrelin reciprocally suppresses production of cytokines (IL-1, IL-6 and TNF-$\alpha$). The appetite response to supraphysiologic doses of ghrelin might be the result of overcoming cytokine inhibition or downmodulation of cytokines by ghrelin.[35] These effects could also explain the positive association between body size and energy intake in the current study (data not shown) in spite of the high plasma levels of cytokines that characterize obesity. However, further studies are needed to confirm this hypothesis.

In our study, IL-6, MCP1 and adiponectin were significantly associated with *CLOCK* gene polymorphisms, particularly with rs3749474. It has been shown that some cytokines may regulate circadian clock genes by interfering with E-box-mediated transcription.[36]

*CLOCK* gene polymorphisms have been studied in relation with sleep pattern disorders.[11] A connection between sleep and food consumption has been previously described.[8] It is generally accepted that together with hormones and neuropeptides, which regulate appetite, different cytokines exert effects on sleep time.[37,38] In particular, plasma IL-6 has been considered to be a sleep factor because of its association with fatigue.[9]

Our data show strong associations with plasma IL-6 levels for SNP rs3749474. As has been previously discussed, changes in the *CLOCK* 3́-UTR structure could account for these results. Current data show that carriers of the minor allele, who reported high energy intake, also presented decreased plasma cytokine concentrations that could result, on the one hand, in a lower anorectic effect,[8] and on the other, in decreased sleep.[35] It has been previously reported that short sleepers, defined as those who sleep less than 6 h, have circadian system alterations with significantly reduced circulating levels of the anorectic hormone leptin and increased levels of the orexigenic hormone ghrelin.[39,40] These factors would compel these individuals to eat more and/or more often.

Rs3749474 and rs4560704 SNPs were associated with MCP1 concentrations. This cytokine functions to recruit monocytes to sites of injury and infection. MCP1 has been associated with depressive disorder[41] and with obesity,[42] both of which are strongly related with circadian system alterations.[3] However, so far, no studies have focused on the chronobiological aspects of MCP1. Whether a circadian rhythmicity for MCP1 exists and whether it is associated with energy intake or sleep disorders remain to be determined.

Associations between CLOCK SNPS and plasma adiponectin concentrations were also observed, especially for the 3111C/T variant. It has been reported that adiponectin could influence energy intake through activation of AMP-activated protein kinase that modulates activity in the hypothalamus to have a role in feeding.[43] Previous studies have shown that changes in the circadian rhythmicity of adiponectin influence appetite and total energy intake.[44]

It remains to be elucidated, in the present work, whether differences in energy intake between carriers and non-carriers, as described above, result from the direct effects of cytokines on energy intake by their anorectic effect or from their particular influence on sleep patterns (or both). Although cytokines are good sleep markers, further studies on chronobiological aspects should include not only classical dietary questionnaires and measurement of cytokine plasma concentrations, but also additional questionnaires about the timing of daily activities and sleep,[45] temporal food intakes,[46] seasonal changes in mood behavior[47] and annual variations in weight. In addition, our results are derived from multivariate analyses and are hypothesis generating rather than conclusive, and hence future replication studies in independent populations are needed to confirm these results.

In summary, we report differences in total energy intake that are associated with different *CLOCK* gene variants. These data were supported by corresponding plasma concentrations of specific cytokines that have been previously identified as good markers of energy intake and sleep patterns. On the basis of these results, and to improve future dietary advice in preventive and clinical interventions, future recommendations should include preferred meal times and minimum night time sleeping requirements.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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