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Original article

Circadian clock gene variants and their link with chronotype, chrononutrition, sleeping patterns and obesity in the European prospective investigation into cancer and nutrition (EPIC) study

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SUMMARY

Background & aims: The circadian clock is involved in the control of daily rhythms and is related to the individual's chronotype, i.e., the morningness-eveneningness preference. Knowledge is limited on the relationship between circadian genes, chronotype, sleeping patterns, chronutrition and obesity. The aim was to explore these associations within the EPIC-Spain cohort study.

Methods: There were 3183 subjects with information on twelve genetic variants of six genes (PER1, PER2, PER3, CRY1, NR1D1, CLOCK). Their association was evaluated with: chronotype and sleeping duration/quality (assessed by questionnaires), chrononutrition (number of meals and timing of intake assessed by a diet history), and also anthropometric measures of obesity at early and late adulthood (in two points in time), such as weight and waist circumference (assessed by physical measurements). Multivariable logistic and linear regression as well as additive genetic models were applied. Odds ratios (ORs), β coefficients, and p-values corrected for multiple comparisons were estimated. Genetic risk scores (GRS) were built to test gene-outcome associations further.

Results: At nominal significance level, the variant rs2735611 (*PER1* gene) was associated with a 11.6% decrease in long-term weight gain (per-allele $\beta = -0.12$), whereas three *CLOCK* gene variants (rs12649507, rs3749474 and rs4864548), were associated with a ~20% decrease in waist circumference gain (per-allele $\beta \sim -0.19$). These and other associations with body measures did not hold after multiple

Abbreviations: EPIC, European prospective investigation into cancer and nutrition study; SNP, Single nucleotide polymorphisms; BMI, Body mass index; WC, Waist circumference; HC, Hip circumference; WHR, Waist to hip ratio; GRS, Genetic risk score; WE, Weekend days; WO, Working days.

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testing correction, except waist-to-hip ratio and rs1801260, rs2070062 and rs4580704 (*CLOCK* gene). Associations with chrononutrition variables, chronotype and sleep duration/quality failed to reach statistical significance. Conversely, a weighted GRS was associated with the evening/late chronotype and with all other outcomes ($p < 0.05$). The chronotype-GRS was associated with an increased overweight/obesity risk (*vs* normal weight) in both early and late adulthood (OR = 2.2; $p = 0.004$, and OR = 2.1; $p = 0.02$, respectively).

Conclusion: Genetic variants of some circadian clock genes could explain the link between genetic susceptibility to the individual's chronotype and obesity risk.

1. Introduction

Circadian rhythms are cyclical changes in cellular, molecular and biological processes that repeat approximately once every 24 h [1]. They run in the background of nearly all living organisms to carry out essential functions and processes and are driven by endogenous molecular oscillators called the circadian clock [2]. This circadian clock plays a central role in many aspects of human physiology, including the regulation of sleep, metabolism and the immune system [3]. These circadian cycles can be affected by synchronising factors *(zeitgebers)* such as light, hormones or food intake [4]. The intrinsic changes to modern societies such as stress, mismatch of sleep-awake and work phases, or of food intake timings, among others, can produce disruptions in the circadian system, which are supposed to impair human health and well-being [2]. In fact, in recent years, a growing body of evidence is supporting the link between chronodisruption and obesity [4,5].

Significant efforts have been devoted to researching potential risk factors of obesity. Some lifestyle, environmental and genetic factors have been identified, suggesting that the aetiology of obesity is multifactorial [6]. In addition to modifiable risk factors for obesity such as physical activity and diet, it has been argued that improving circadian rhythm could be an effective way of preventing obesity, too [7]. Indeed, behaviours that influence obesity such as appetite and excessive food intake could be influenced by sleeping patterns, or the other way around, sleeping could impact food timing and consumption [5]. Further, some studies suggest that there is an interaction between obesity and chronobiology, from the genetic and epidemiological point of view [4,8–13]. Thus, genetic variants could directly affect obesity development or through behaviours linked to this disease [5].

In line with the above, previous studies have found that the circadian clock is regulated through a complex array of genes and their encoded protein products [14,15]. To date, there have been identified several circadian genes, also known as clock genes, with a proven role in human physiology: *CLOCK, CRY1, Rev-erb-α*, *PER1, PER2, PER3*, amongst others [2]. Over 300 genetic variants (i.e., single nucleotide polymorphisms, SNPs, such as rs150812083 and rs139315125 in PER2, have been associated with chronotype in genome-wide association studies (GWAS) [1,16]. Regarding the association between clock genes and obesity, some studies point to a positive association between some genetic variants with the risk of developing obesity and metabolic syndrome [8]. In particular, genetic variants of the *CLOCK* gene (e.g., rs3749474 and rs1801260) have been associated with a higher body mass index (BMI), with an increased energy intake and with reduction in sleep duration [8]. Also, polymorphisms of the *PER2* gene (rs2304672 and rs4663302) and *Rev-erb-α* gene (rs2314339, rs2071427), have been associated with abdominal obesity, frequent snacking, and skipping breakfast [5]. The results of many of these studies, mostly cross-sectional and of limited size, suggest that individuals carrying certain gene variants eat more, sleep less, ingest more fat, and have greater abdominal obesity [17–19]. Furthermore, several studies argue that some of these genetic variants are associated with certain obesogenic chronotypes and sleep patterns [5,8]. However, not all studies show support for associations involving the same genes or genetic variants [8,20], and not all genetic variants in circadian genes have been investigated in relation to obesity risk, sleep disruption or improper dietary behaviours. Thus, the assessment of risk genotypes of circadian clock genes is an area of study, yet to be exploited, that could serve to shed light on the relationship between chronotype, chrononutrition and obesity, with potential relevance for the prevention and treatment of obesity [21,22].

In view of the above, our aim was to evaluate associations between twelve SNPs of some circadian-related genes with chronotype and sleeping patterns, chrononutrition, and anthropometric measurements of obesity, in the Spanish European Prospective Investigation into Cancer and Nutrition (EPIC) study.

2. Material and methods

2.1. Study design

This study was conducted within a sample of the Spanish EPIC cohort, "the EPIC-Spain chronodiet study". EPIC-Spain is part of the EPIC study, a 10-country multicenter study designed to explore the role of dietary, lifestyle, environmental and genetic factors in the development of cancer and other chronic diseases. The complete design and study methods of the EPIC study have been described previously [23–25].

2.2. Study population

In the EPIC-Spain study, a total of 41,440 individuals (15,632 men and 25,808 women; 38% and 62%, respectively) aged 29–69 years were recruited between 1992 and 1996 from 5 regions in Spain (Asturias, Gipuzkoa, Navarra, Granada and Murcia). Participants were recruited from different socioeconomic and educational levels, both from urban and rural areas, and were mostly active blood donors [25,26]. All enrolled in the study voluntarily, and the study was approved by the ethical review boards of the International Agency for Research on Cancer and the Ethics Committee of the Bellvitge University Hospital.

For the "EPIC-Spain chronodiet study" involving a sub-sample, participants signed an informed consent. The study was also approved by the Ethics Committees of the participating centers and the Bellvitge University Hospital. This sub-sample comprised participants selected among men and women who were under 70 years old by December 2015. Age restrictions were applied due to evidence suggesting that the influence of chronotype or social jet-lag diminishes at older ages [27], and that the presence of chronic conditions and medication intake increases with age [28], thus interfering with the variables of interest. Exclusion criteria were being on a diet for health reasons at the time of the interview, having

significantly changed diet in the last year, and having a diagnosis of cancer or any other disease that may have provoked significant pathological weight changes. Those with an implausible caloric intake at recruitment of the cohort (above and below 1% of the ratio of total energy intake:energy requirement) were also excluded. Although bariatric surgery is known to affect weight loss [29], we did not consider this as an exclusion criterion. It is unlikely that there were subjects who had undergone this intervention given the low prevalence of morbid obesity (0.8%) in our study population at recruitment. The sub-sample was sampled by strata of sex and age groups. Of those selected participants, 4225 (75,5%) agreed to take part in the study, of which 3484 provided biological samples. As described below, due to low quality genotypic data, 301 participants were further removed. Thus, the final study population consisted of 3183 participants with data on diet, chronotype and lifestyle habits, and genetic data. Of these, a total of 2505 participants underwent a second anthropometric reassessment.

2.3. Data collection

Due the prospective nature of the EPIC study, different levels of data from various time points were available in the EPIC-Spain chronodiet study (Supplemental Fig. 1). At recruitment (T0), from 1992 to 1996, food consumption measured through a diet history, anthropometric measurements, and lifestyle and medical history data were collected, along with biological samples for analyses of biomarkers and genetic studies. Participants were at this time young adults (mean age: 42.6 years). Approximately three years later (T1), the participants provided information on changes in some lifestyle exposures including weight. In 2017 (T2), the chronodiet study was set up and information was collected on lifestyle factors, medical history, some anthropometric measures, and, for the first time, on meal timing (i.e., chrononutrition), sleeping patterns and chronotype. Blood samples were also collected. Participants were at this time older adults (mean age: 65.3 years). After around 18–36 months, during 2018–2020 (T3), only anthropometric measurements were reassessed. The latter two assessments (T2 and T3) were part of the aforementioned chronodiet study, and were, thus, restricted to the study sample. For this project, this information was collected in every center through direct in-person interviews, and using standardized questionnaires, methods and protocols, similar to those applied in the recruitment of the cohort [25].

2.4. Assessment of chronotype, sleep patterns, and chrononutrition

Within the chronodiet study (at T2), chronotype data was collected by means of the Munich Chronotype Questionnaire (MCTQ) [30]. In brief, this questionnaire accounted for sleeping duration, timing of food intake and physical activity (type, time-of-day, and age at start and end of activity). In addition, information on sleeping patterns was collected using the Pittsburgh sleep quality index (PSQI), a questionnaire of 19 items (within 7 components) that allows to assess sleep quality over the previous month [31]. Based on the global score (0–21 points), we considered a score greater than 5 to assess poor sleep quality. The information on usual sleep times collected with the MCTQ was used to calculate mid-sleep time corrected for sleeping on working and weekend days (MSFc). Further details of the chronotype calculation procedure are described elsewhere [32,33]. The MSFc was used to classify the participants into five different chronotypes considering sex-specific cut-off points (percentiles 2.5%, 10%, 90% and 97.5%): extreme early type, slight early type, normal or moderate type, slight late type, and extreme late type [33].

Information on dietary intake was retrieved through a validated diet history, which served to derive data on food consumption in g/d, number of meals/day (breakfast, lunch. dinner, and midmorning and afternoon snack) and timing intake. Number of meals was categorized as less than 3, 4 to 5, and more than 5 when there were even more eating occasions. Timing of the main meals (breakfast, lunch and dinner) was defined as follows: time to breakfast as time from getting up to the first dietary intake (<30 min, 30 min − 1 h, 1 h−1½h, > 1 ½h), early mealtime for breakfast, lunch and dinner as before 9 am, 2 pm and 9 pm, respectively; late mealtime was considered otherwise. In addition, breakfast skippers were regarded as those who reported to have breakfast after 9 am.

2.5. Anthropometric measurements and body composition

Participants of the chronodiet study underwent anthropometric measurements of weight and height, waist and hip circumference. In a similar way with regard to the first recruitment, these measurements were taken in a standardized manner [25]. In brief, height was measured from head to toe, and weight was assessed with subjects in light underwear using a digital scale with a precision of 0.1 kg. Waist circumference (WC) was measured at the narrowest torso circumference, or midpoint between the lower ribs and the iliac crest if the natural waist could not be identified. Hip circumference (HC) at the widest diameter of the buttocks, and circumference around neck were also registered. Height, waist, hip and neck circumferences were measured to the nearest 1 cm.

Furthermore, body composition was evaluated by biological impedance (Tanita BIA MC-180MA) to determine fat percentage. All anthropometric measurements were reassessed within the chronodiet study after 18–36 months of follow-up (T3). Several indexes were calculated to assess obesity and weight changes from baseline measurements. BMI was calculated as weight in kilograms divided by the square of height in metres. Participants were classified as non-obese (BM < 30 kg/m2) and obese (BMI≥30 kg/m2), and as normal weight (BMI<25 kg/m2) and overweight/obese (BMI≥25 kg/m2). According to WC, the participants were classified into normal and moderately increased (<102 cm and <88 cm) and abdominal obese (≥102 cm and ≥88 cm), in men and women respectively. Waist to hip ratio (WHR) was also calculated. Thus, both overall and abdominal obesity measures were available at recruitment (in early adulthood: at T0 and T1) and during the chronodiet study (late adulthood; at T2 and T3). To account for changes in overall and abdominal obesity, we calculated change in weight from the recruitment to the first follow-up at T1 (short-term weight gain in early adulthood in kg), from recruitment to the second follow-up at T2 (long-term weight gain in kg), and further from the second to the third follow-up at T3 (short-term weight gain in late adulthood in kg). Likewise, for WC gain over time, we also calculated short- and long-term WC change in cm. Then, annual weight and WC changes were estimated as the rate of their change divided by the years of follow-up (i.e., change/years).

2.6. Circadian genes

The genetic analyses included 6 key circadian genes (*CLOCK:* clock circadian regulator, *CRY1:* Cryptochrome Circadian Regulator 1 or *MTERF2*, *NR1D1*: Nuclear Receptor Subfamily 1 Group D or Reverb-α, *PER1, PER2 and PER3*: Period Circadian Regulator 1.2 and 3), of which 12 genetic variants (i.e., single nucleotide polymorphisms, SNPs) were selected based on previous chronobiology and obesity-related (chronotype, sleeping duration, chronodiet, obesity, metabolic syndrome, etc.) studies [5,6,12,17,18,34–37]. In particular, the *CLOCK* gene comprised 6 genetic variants. Characteristics of these variants are summarized in Supplemental Table 1. All were involved

in molecular pathways related to circadian rhythm and regulation, according to functional information of SNPs in available data repositories (Supplemental Fig. 2) [38].

2.7. Genotyping and quality control

In addition to the questionnaire survey, venous blood samples were collected from the participants and stored at −80 °C until analyses in public biobanks. Genomic DNA was extracted from buffy coat samples (peripheral lymphocytes) of 3484 participants by using a commercial DNA kits (for example, Qiagen and a Maxwell 16 automated system Promega). Genotyping was performed *at the Spanish National Cancer Research Centre, in the Human Genotyping lab* (CeGen), for the 12 aforementioned genetic variants using the TaqMan® OpenArray™ Genotyping System (Thermo Fisher Scientific, Waltham, MA, USA) at Centro Nacional de Genotipado (CeGen-CNIO). Raw data were analyzed with the TaqMan® Genotyper v1.2 software (Thermo Fisher Scientific). About 5.5% of the samples were analyzed (N = 220 plus 11 control samples) in duplicate with a genotype concordance of 99.6% and 100%. Samples with weak amplification were removed (N = 301). Furthermore, quality control filters were applied to both SNPs and individual data. We tested whether any SNP had a missing genotype rate higher than 10% (SNP call rate), as well as departure from the Hardy–Weinberg equilibrium ($p < 0.01$). Participants with genotypic data showing a missing rate >10% (sample call rate) were also excluded. Minor allele frequency (MAF) was also checked (threshold >0,05) for all SNPs in the study sample, overall, by regions and by BMI strata. After these quality control procedures, 12 SNPs and 3183 individuals were finally included in the analyses. Missing genotypes were imputed through the *k-nearest neighbors method* (knni), whereby imputation is based on the mean of the k-nearest SNP (based on the Euclidean distance between SNPs). This method has a relatively high accuracy for low missing rates and tagged SNP studies [39]. Results of the genotype and allele frequencies of those SNPs, for unimputed and imputed data, are shown in Supplemental Tables 2–4. Allele frequencies in our study population matched those reported for the European population (Supplemental Table 1). Genotypes were recoded to "0", major homozygotes (reference), indicating absence of the risk allele, risk allele homozygotes were coded as genotype "2" and, heterozygotes as genotype "1".

2.8. Statistical analysis

Descriptive statistics by chronotype and obesity measures included median and interquartile range (IQR) for continuous variables, and absolute and relative frequencies for the categorical ones. Mann–Whitney U tests or $\chi 2$ test, respectively, were applied to evaluate differences in the distribution of the data by these strata, as appropriate. A $\chi 2$ test was used to test genotype frequencies in all subjects for Hardy–Weinberg equilibrium (HWE) by comparing the observed genotype frequencies with those expected under HWE. The differences in genotype frequencies and HWE by regions and BMI categories were also tested via $\chi 2$ tests.

Principal component analysis (PCA) was applied to infer highly correlated groups of SNPs and to evaluate genetic variation in our data by center, array or batch, sex and other variables [40]. The first three principal components explained over 60% of the total variance, but any of those components were related to these variables (Supplemental Fig. 3). Therefore, population stratification or genotyping batch effects were considered unlikely. Indeed, the EPIC-Spain study population could be considered genetically homogenous [41]. Nonetheless, any influence of potential genetic ancestry in our analyses was minimized by adjustment for center.

The sum score and chronotype groups were used either as a continuous (continuous MCTQ score or chronotype scale, respectively) or as a binary variable (extreme/slight evening type, *vs* moderate type & extreme/slight morning type). Binary variables for meal timing and anthropometric measures were also created by collapsing some categories. For example, obese and overweight, normal weight and overweight, and mid and late hour mealtimes, were combined in the analyses. Normal weight, or normal weight and overweight, or early hour mealtimes, where appropriate, were considered as reference categories.

Non-normally distributed outcome variables according to Kolmogorov–Smirnov test (all continuous anthropometric measures, chrononutrition, sleeping and chronotype variables) were logarithmically transformed. Linear regression analyses were performed to test for associations between every SNP and with the continuous outcomes (e.g., weight, BMI, WC, HC, % of fat, weight or WC change, MCTQ score, sleeping duration, number of meals, etc.), whereas logistic regression models were fitted for binary outcome variables (e.g., overall or abdominal obese *vs* non-obese, or overweight/obese *vs* normal weight, extreme late or early chronotype *vs* others, bad vs good sleeping quality, less than five meals *vs* more, late *vs* early mealtimes, skipping breakfast *vs* not, etc.). Beta (β) coefficients, odds ratios (OR) and corresponding 95% Confidence Intervals (CI), were derived from these analyses. Additive genetic models of inheritance were considered. Thus, for every one-unit increase in risk alleles in each SNP we expect our continuous outcomes to change by "100*(exp(β)-1)" percent, or by the OR associated with this allele increase in the case of binary outcomes.

Several multivariable-adjusted models were fitted to control for potential confounders. The first model (M1) was adjusted for sex, age in years, and center. The second model (M2) was further adjusted for obesity and/or related variables, namely educational level (primary school, technical school, secondary school, or university degree), physical activity (MET-hours/week, as the sum of recreational and vigorous activities), smoking status (never, former, and current smoker), and total energy intake (Kcal/d). In association analyses with obesity measures, we fitted an additional multivariable model adjusted for BMI or WC in early or late adulthood, where appropriate, except when associations with annual weight or waist gain were explored in the analyses. Models were also adjusted for BMI in association analyses with sleeping and eating patterns. Analyses were performed overall, and separately for men and women.

SNP-based Genetic Risk Sore (GRS): An unweighted and weighted genetic risk score (uwGRS and wGRS, respectively) was generated based on the selected genetic variants. The number of risk alleles (0, 1 or 2) of each genetic variant was summed for every individual in the uwGRS. For the wGRS, risk alleles were weighted by their effect sizes (i.e., estimates: per allele odds in logistic regression models, or per allele β coefficients in linear regression models, all adjusted for age, sex and center) on the outcomes (chronotype, sleeping patterns, anthropometric measures, and chrononutrition variables, in multivariate regression models) [42]. Absolute values of the coefficients were considered as weights. Two approaches were applied in association analyses of the weighted GRS:

1) The GRSs derived from estimates of the association between the SNPs and the outcomes, e.g., the obesity-like GRS, were associated further with their corresponding outcome (in this example, with obesity) so as to explore circadian clock gene effects on these outcomes: SNPs → outcome; then, outcome-GRS → outcome
2) The GRS resulting from estimates of the association between the SNPs and the person's chronotype, the chronotype-like GRS, was tested for its association with the other outcomes in order to assess whether genetic susceptibility to chronotype could

subsequently lead to the other outcomes: SNPs → chronotype; then, chronotype-GRS → outcome

In addition, a GRS restricted to the SNPs of the CLOCK gene was built to test its association with the outcomes. SNPs in linkage disequilibrium (LD) according to LDLink (D > 0.8: rs1801260 and rs2070062, rs12649507 and rs3749474, rs4864548 and rs3749474, rs4864548 and rs12649507) [43], were included in these analyses (Supplemental Fig. 4). Potential effect modification by sex was evaluated via Wald test for the interaction term GRS*sex.

In sensitivity analyses, results were compared with individual SNP analyses in participants with genotype data for that particular SNP of interest, and GRS association analyses in participants with complete genotyped data (N = 2778). Prunning of SNPs in LD to minimize multi-collinearity effects in regression models was also considered to build the GRS.

All p-values were two-sided, with $p < 0.05$ indicating statistical significance. P-values were also corrected for multiple comparisons to reduce the instance of false associations by the Benjamini-Hochberg method [44]. Statistical analyses were performed with the R program version 4.0.1 (The R Foundation for Statistical Computing) [45]. We used the *snpReady* package for the genetic data processing and imputation (together with *impute* package) of missing genotypes [46], *PredictABLE* for the GRS [47], and *SNPassoc* for the association analyses [48].

3. Results

3.1. Demographic and risk factors by chronotype and other variables

Tables 1 and 2 summarize the demographics and risk factor distributions by the participant's chronotype. There were significant differences in chronotypes by regions, with Asturias showing more frequently the late/evening chronotypes, and Murcia the early/morning chronotypes ($p < 0.001$). Several anthropometric measures were highest in participants belonging to the late chronotype (e.g., WHR, $p = 0.005$), or tended to increase across the chronotype strata (e.g., BMI, $p = 0.07$). Compared to participants of the early chronotypes, those of the late chronotypes showed a higher increase of weight ($p = 0.004$) and WC ($p = 0.008$) over time. Sleeping duration was also higher among participants of the late chronotypes ($p < 0.001$). The latter were also more prevalent among the youngest participants ($p < 0.001$) and ever smokers ($p = 0.04$). To have breakfast, lunch and dinner at later hours was also more common for late chronotype participants ($p < 0.001$). These participants were more likely to skip this meal ($p < 0.001$). The genotype frequencies did also not differ across chronotype groups (Supplemental Tables 5 and 6). No significant differences were noted in genotype frequencies by BMI categories, by meal and sleeping patterns (data not shown).

3.2. Associations with chronotype, sleeping patterns and anthropometric measures of obesity

No genetic variant was significantly associated with the individual's chronotype when considering a continuous classification scale of the chronotype. Associations were also non-significant with regard to MCTQ scores or after collapsing slight and extreme groups of the early and late type (Supplemental Table 7). Only variant rs2304672 tended to be associated with the extreme late ($p = 0.07$) or early ($p = 0.09$) chronotype as compared to all other groups (Table 3). Additional adjustment for BMI or sleeping patterns did not alter these associations (data not shown).

Associations between the SNPs and the sleep parameters failed to reach the significance threshold (Supplemental Table 8), despite some variants of the *CLOCK* gene (rs12649507, rs1801260, rs2070062) and of the NR1D1 gene (rs2314339) showed a nominally significant association with weekend sleep duration. BMI adjustment did not affect these associations either (data not shown).

Fig. 1 shows associations between the SNPs and several obesity measures in early and late adulthood. We observed significant inverse associations between variant rs2735611 and weight gain (per-allele $\beta = -0.124$; i.e., weight decreases by 11.6%), and between variants rs12649507, rs3749474 and rs4864548 and WC gain (per-allele $\beta = -0.197$, -0.204 and -0.187, respectively; i.e., ~20% decrease in WC). These associations emerged when long-term changes of weight and WC were considered (i.e., ~20 years of follow-up), though not for changes of either two in the short term (data not shown). Mutual adjustment for baseline BMI or WC did not change the results (data not shown). Considering obesity measures as binary outcomes, there were no significant associations with abdominal obesity, or overall obesity (and overweight) during early and late adulthood compared to non-obese or normal weight subjects (Supplemental Tables 9 and 10). Of note, variant rs2304672 seemed to increase the risk of overweight and obesity in early adulthood compared to normal weight (OR = 1.26; 95% CI:1.03–1.54), but this association did not hold after multiple test correction (Supplemental Table 9). This variant tended to increase susceptibility to present a higher BMI and WC in early adulthood (Fig. 1). Other variants, while pointing to increased or decreased obesity indicators (for example, rs4580704 with decreasing WC), did not attain multiple-testing statistical significance either.

Interestingly, several SNPs of the CLOCK gene were associated with modestly increased (rs1801260 and rs2070062) or decreased (rs4580704) WHR in early adulthood, even after multiple test correction. Two of them were associated with WHR in late adulthood as well, though none of them survived the multiple hypothesis correction in multivariate models (Supplemental Table 11).

Fat percentage decreased by 3% for every one-unit increase in risk alleles of the variant rs4580704 only at the nominal significance level ($\beta = -0.026$; $p = 0.009$, corrected-p: 0.108). No other genetic variants were associated with this measure (Supplemental Table 12). Other anthropometric variables (neck and HC) did not show statistically significant associations with any genetic variant (data not shown).

3.3. Associations with chrononutrition variables

Fig. 2 shows associations between the SNPs and the chrononutrition variables. No genetic variant was significantly associated with mealtimes, number of meals or time from waking up to breakfast. Nominal significant associations were only observed for lunch at later afternoon ($OR_{rs12649507} = 1.14$, $p = 0.031$, corrected-$p = 0.368$), and for eating breakfast right after waking up ($OR_{rs228697} = 1.22$, $p = 0.047$, corrected-$p = 0.569$). Adjustment for BMI did not influence any of these associations (data not shown).

3.4. SNP-based GRS

Both, the uwGRS and wGRS were normally distributed in the study population (Supplemental Fig. 7). Table 4 shows estimates on the association between the wGRS with continuous outcomes of chronotype, sleeping patterns and anthropometry measures. The wGRS was not significantly associated with chronotype or sleeping duration but with some anthropometric measures. For each additional risk allele, we found that BMI increased significantly along with WC in early adulthood.

Table 1
Socio-demographic and anthropometric characteristics of the study population (3183 individuals of the EPIC-Spain chronodiet study) by chronotype.[a]

	1: Extreme early type; N = 81		2: Slight early type; N = 222		3: Moderate type; N = 2505		4: Slight late type; N = 234		5: Extreme late type; N = 79		p-value[b]
	N/MED	%/IQR	N/MED	%/IQR	N/MED	%/IQR	N/MED	%/IQR	N/MED	%/IQR	
Center											<0.001
Asturias	21	25.90%	45	20.30%	655	26.10%	94	40.20%	35	44.30%	
Granada	8	9.88%	51	23.00%	419	16.70%	29	12.40%	10	12.70%	
Murcia	24	29.60%	40	18.00%	527	21.00%	41	17.50%	19	24.10%	
Navarra	9	11.10%	31	14.00%	439	17.50%	37	15.80%	11	13.90%	
Gipuzkoa	19	23.50%	55	24.80%	465	18.60%	33	14.10%	4	5.06%	
Sex											0.995
Men	35	43.20%	93	41.90%	1031	41.20%	96	41.00%	32	40.50%	
Women	46	56.80%	129	58.10%	1474	58.80%	138	59.00%	47	59.50%	
Age (y)	65	[63.0; 68.0]	66	[62.0; 69.0]	65	[61.0; 68.0]	64	[60.2; 67.0]	64	[60.5; 66.4]	<0.001
Education											0.386
Higher	14	17.70%	39	17.60%	514	20.60%	40	17.20%	9	11.40%	
None	16	20.30%	44	19.90%	419	16.80%	35	15.00%	14	17.70%	
Primary	30	38.00%	81	36.70%	897	35.90%	105	45.10%	34	43.00%	
Secondary	7	8.86%	29	13.10%	310	12.40%	25	10.70%	13	16.50%	
Technical	12	15.20%	28	12.70%	356	14.30%	28	12.00%	9	11.40%	
Smoking[a]	.										0.037
Fomer	27	33.30%	87	39.20%	971	38.80%	104	44.40%	23	29.10%	
Smoker	15	18.50%	29	13.10%	301	12.00%	35	15.00%	20	25.30%	
Never	39	48.10%	106	47.70%	1230	49.10%	95	40.60%	36	45.60%	
WO sleep (ms)[b]	24.9	[21.3; 27.0]	25.1	[21.7; 28.2]	26.1	[23.3; 28.5]	26.7	[23.4; 28.8]	27.9	[25.1; 28.8]	<0.001
WE sleep (ms)[b]	25.2	[22.5; 28.7]	25.9	[23.1; 28.8]	27.0	[24.0; 2937]	25.8	[23.4; 28.5]	26.5	[24.1; 28.2]	<0.001
BMI (kg/m^2)	28.2	[25.8; 31.7]	28.7	[25.0; 31.4]	27.8	[25.2; 30.8]	28.6	[25.8; 31.6]	28.5	[25.7; 31.1]	0.070
WC (cm)	96.2	[87.1; 104]	94.2	[87.0; 103]	93.5	[85.0; 102]	95	[87.2; 103]	97	[87.5; 101]	0.128
HC (cm)	105	[98.9; 111]	104	[97.5; 110]	103	[97.5; 109]	102	[97.0; 109]	102	[97.5; 110]	0.360
Fat %	32.2	[25.4; 38.9]	31.3	[25.2; 37.2]	31.5	[25.4; 37.2]	31.8	[26.0; 37.8]	31.9	[26.4; 37.2]	0.741
Mets/h	77	[60.3; 106]	78.8	[60.5; 103]	79.6	[56.6; 103]	74.9	[54.6; 97.0]	79.2	[49.2; 100]	0.407
BMI class[a]	.										0.099
normal	13	16.00%	54	24.30%	563	22.50%	41	17.50%	15	19.00%	
overweight	39	48.10%	81	36.50%	1135	45.30%	104	44.40%	35	44.30%	
obese	28	34.60%	85	38.30%	792	31.60%	88	37.60%	28	35.40%	
WC class[a]	.										0.359
normal	39	48.10%	112	50.50%	1366	54.50%	116	49.60%	40	50.60%	
obese	41	50.60%	110	49.50%	1138	45.40%	118	50.40%	39	49.40%	
WHR	0.91	[0.85; 0.96]	0.9	[0.84; 0.96]	0.9	[0.84; 0.96]	0.92	[0.86; 0.98]	0.93	[0.87; 0.98]	0.005
BMI (kg/m^2)[c]	26	[24.4; 28.5]	27	[24.6; 30.2]	26.4	[24.1; 28.9]	26.6	[24.1; 29.6]	26.1	[24.5; 28.0]	0.125
WC (cm)[c]	87	[80.0; 95.9]	90	[79.0; 99.0]	88	[79.0; 96.0]	87.5	[78.8; 97.0]	87	[79.0; 97.5]	0.623
HC (cm) [c]	102	[99.0; 108]	104	[98.1; 110]	103	[98.0; 107]	102	[98.0; 107]	103	[99.0; 109]	0.297
W Gain/Year	0.13	[0.14; 0.38]	0.06	[0.14; 0.31]	0.1	[0.10; 0.33]	0.17	[0.05; 0.41]	0.2	[0.03; 0.43]	0.004
WC Gain/Year	0.28	[0.03; 0.63]	0.23	[0.04; 0.50]	0.23	[0.00; 0.50]	0.3	[0.04; 0.61]	0.35	[0.18; 0.61]	0.008

Abbreviations: Body mass index (BMI), waist circumference (WC), hip circumference (HC), weight (W), waist to hip ratio (WHR), metabolic equivalents (Mets), weekend (WE), workdays (WO), median (MED), number (N), intercuartil range (IQR), milliseconds (ms).

[a] Numbers do not sum up due to missing data (N = 2 in smoking, N = 20 in BMI, N = 2 in WC). Also, there were 62 subjects with missing data on chronotype.

[b] p-values derived from $\chi 2$ and Kruskal–Wallis tests, where appropriate, accounting for pairwise comparison correction.

[c] Refers to anthropometric measures in early adulthood. All others are measures in late adulthood.

The most striking increase was observed for weight gain, independently of baseline WC and other possible confounders ($\beta = 0.81$, $p = 0.009$), even keeping statistical significance after adjusting for multiple testing. The wGRS was, however, not associated with WC gain. There was also a positive association with WHR in late adulthood ($\beta = 0.05$, corrected-p<0.05), regardless of BMI. There were no associations between the uwGRS with the studied outcomes (data not shown). For binary outcomes (Table 5 and Fig. 2, b), we observed that the wGRS was significantly associated with every outcome. Considering the chronotype-GRS, for which risk of evening/late chronotype (*vs* morning and moderate chronotype) increased by 2.62 (95%CI: 1.16–5.99), we found a higher risk of overweight and obesity (*vs* normal weight) in those participants carrying susceptibility alleles of the evening/late chronotype. Moreover, the impact of the chronotype-GRS on this risk was alike in early and late adulthood ($OR_{\text{per risk allele}} = 2.22$; 95%CI:1.28–3.86, and 2.10; 95%CI:1.14–3.88, respectively). There were no associations with any other anthropometric outcome. With respect to meal timing variables (Fig. 2, b), no associations were observed with the weighted or unweighted chronotype-GRS. All estimates were in similar range when the score was restricted to variants of the *CLOCK* gene (Supplemental Table 13).

3.5. Stratified analyses by sex

Mixed results were observed in association analyses between the genetic variants and the anthropometric measures in both men and women (Supplemental Figs. 5 and 6). For instance, in men, there were positive and significant associations between variants rs1801260 and rs2070063 with early and late adulthood BMI, while variant rs4580704 was inversely associated with early adulthood WC. In men, there were several variants promoting (rs4580704) WC gain or preventing weight (rs2071427 and rs2314339) and WC (rs12649507, rs3749474 and rs4864548) gain, whereas in women, only variant rs228697 was related to a lower WC gain over time. Associations between weight and WC gain in men were statistically significant after multiple test correction (rs12649507, rs4580704 and rs3749474). In women, these associations were only nominally statistically significant. Concerning the GRS, there was no interaction by sex ($p > 0.05$) in relation to any of these measures (data not shown).

Table 2
Characteristics of the study population (3183 individuals of the EPIC-Spain chronodiet study) by chronotype and chrononutrition variables.

	1: Extreme early type; N = 81		2: Slight early type; N = 222		3: Moderate type; N = 2505		4: Slight late type; N = 234		5: Extreme late type; N = 79		p-value[b]
	N/MED	%/IQR	N/MED	%/IQR	N/MED	%/IQR	N/MED	%/IQR	N/MED	%/IQR	
Breakfast											<0.001
≤9:00 am	74	91.40%	200	90.10%	1976	78.90%	121	51.70%	34	43.00%	
>9:00 am	7	8.64%	22	9.91%	528	21.10%	113	48.30%	45	57.00%	
Time to breakfast[a]											<0.001
<30 min	10	12.80%	35	16.20%	500	20.80%	65	31.00%	21	35.60%	
30 min-1h	13	16.70%	37	17.10%	660	27.50%	78	37.10%	18	30.50%	
<1 h 30min	22	28.20%	49	22.70%	747	31.10%	46	21.90%	16	27.10%	
>1 h 30min	33	42.30%	95	44.00%	492	20.50%	21	10.00%	4	6.78%	
Lunch[a]											<0.001
≤2:00 pm	59	72.80%	130	58.60%	1242	49.60%	84	35.90%	25	31.60%	
2–3:00 pm	13	16.00%	69	31.10%	953	38.00%	116	49.60%	35	44.30%	
>3:00 pm	1	1.23%	16	7.21%	177	7.07%	20	8.55%	5	6.33%	
Dinner[a]											<0.001
≤9:00 pm	49	60.50%	108	48.60%	812	32.40%	39	16.70%	15	19.00%	
9:00 pm	12	14.80%	53	23.90%	694	27.70%	57	24.40%	13	16.50%	
>9:00 pm	10	12.30%	42	18.90%	793	31.70%	107	45.70%	30	38.00%	
N meals	.										0.262
≤5	29	35.80%	104	46.80%	1206	48.10%	113	48.30%	35	44.30%	
>5	52	64.20%	118	53.20%	1299	51.90%	121	51.70%	44	55.70%	

[a] Numbers do not sum up due to missing data. For dinner:292. For Lunch: 177 For time to breakfast: 171. Also, there were 62 subjects with missing data on chronotype.
[b] p-values derived from $\chi 2$ and Kruskal–Wallis tests, where appropriate, accounting for pairwise comparison correction.

As for the chrononutrition and sleep pattern variables, results did not appreciably differ between men and women (data not shown).

3.6. Sensitivity analyses

Overall, results of the current study did not change when restricting the analyses to genotype SNPs without missing alleles on an individual basis (for example, Supplemental Tables 14 and 15), and in aggregate form via the GRS (Supplemental Table 16). For the latter, results remained the same when SNPs in LD were removed from the analyses (Supplemental Table 17).

4. Discussion

In this study we analysed the association of twelve genetic variants of clock genes with chronotype, sleeping patterns, chrononutrition variables, and obesity risk, within the Spanish EPIC-chronodiet study. Ours is the first study exploring associations between these genetic variants, individually and collectively, with a wide range of chronobiology-related outcomes. Several associations at the nominal significance level were observed between some genetic variants and a few anthropometric measures (for example, the *CLOCK* variants: rs12649507, rs3749474 and rs4864548), suggesting that there is genetic predisposition to obesity in carriers of certain minor frequency alleles, and circadian genes in general. In fact, higher scores in the SNP-based GRS were associated with weight gain, and with the evening-like chronotype, and in turn with overweight and obesity in both early and late adulthood. Furthermore, we found some nominally significant associations between variants rs2070062 and rs12649507 with shorter sleep duration, and variant rs228697 with short time lag between waking up and breakfast.

4.1. Associations with chronotype

Several GWAS studies have identified genetic variants associated with a self-reported chronotype [1,16,49]. To date, the most comprehensive GWAS, conducted within 697,828 individuals from the UK-BIOBANK and 23andMe studies [16], identified over 300 genetic variants associated with the individual's chronotype. Our study considered tagged SNPs of some circadian genes and possibly lacked statistical power to find an association between the genetic markers and chronotype. Indeed, in a Finnish study of 8433 participants that also focused on candidate SNPs, a single variant (rs4131403 in gene *NR1D2*) out of 20 variants of key clock genes (CLOCK, NR1D2, PER1, PER2, PER3, etc.) was reported to be associated with chronotype [50]. Moreover, in our study, the individual's chronotype relied on a self-reported measure, whereas the aforementioned GWAS considered a more reliable assessment by monitoring sleep timing and duration [16]. However, our findings support an association between these SNPs, when combined in a GRS, with an evening chronotype (*vs* morning/moderate chronotype), and confirms that there is genetic predisposition to present a certain chronotype. Other studies, including a secondary analysis of the Finnish study, have also proposed GRS of chronotype [16,20,50]. For instance, a GRS comprised of 15 chronotype-related SNPs was associated with the individual's chronotype in a Spanish study of 1693 participants [20]. Importantly, the fact that our GRS considered various clock genes associated with chronobiological aspects of obesity, has made it possible to establish associations not only with chronotype but with other factors.

4.2. Associations with obesity measures

A number of previous studies have also assessed associations between circadian gene variants with obesity risk [8]. Most previous studies on the association between circadian gene variants with obesity risk have considered variants of a single gene in relation to this outcome (CLOCK, *Rev-erb-α*, etc) [13,18,37,51]. In our study, we have covered more cronobiological-related genes. Also, unlike previous studies, we have taken into account changes in body anthropometrics from early to late adulthood, allowing us to identify novel associations, such as for example, the negative (nominal) association between variants rs12649507 and rs3749474 with WC gain over time. In addition, we confirm, though only at the nominal level, the following previously reported associations: minor allele carriers of variant rs1801260 (A/G) tended to have lower BMI values as in an intervention study of overweight and obese subjects from Spain (N = 447) [18], minor allele carriers of variant rs2071427 (C/T) showed a higher BMI as in the MONICA Lille,

Table 3
Association between the 12 SNPs and the individual's chronotype (extreme groups and MCTQ score; Munich Chronotype Questionnaire score) in the EPIC-Spain chronodiet study.

Extreme Late (N = 79) *vs* all other	Ref/minor	Gene	Model 1				Model 2			
			OR	LCI	UCI	p-value	OR	LCI	UCI	p-value
rs12649507	**G/A**	**CLOCK**	0.728	0.503	1.055	0.094	0.722	0.497	1.049	0.088
rs1801260	**A/G**	**CLOCK**	0.976	0.680	1.400	0.895	0.979	0.682	1.407	0.910
rs2070062	**A/C**	**CLOCK**	0.930	0.646	1.340	0.697	0.929	0.644	1.341	0.694
rs2071427	**C/T**	**NR1D1**	0.974	0.676	1.403	0.885	0.958	0.663	1.383	0.818
rs228697	**C/G**	**PER3**	1.020	0.608	1.710	0.940	1.027	0.611	1.726	0.920
rs2287161	**G/C**	**MTERF2/CRY1**	1.302	0.947	1.792	0.104	1.312	0.953	1.807	0.096
rs2304672	**G/C**	**PER2**	1.539	0.948	2.496	0.081	1.582	0.971	2.576	0.065
rs2314339	**C/T**	**NR1D1**	1.135	0.705	1.828	0.601	1.138	0.704	1.839	0.597
rs2735611	**A/G**	**PER1**	0.950	0.630	1.431	0.805	0.997	0.657	1.513	0.987
rs3749474	**C/T**	**CLOCK**	0.812	0.573	1.151	0.241	0.813	0.572	1.154	0.246
rs4580704	**C/G**	**CLOCK**	1.257	0.914	1.730	0.159	1.257	0.912	1.731	0.162
rs4864548	**G/A**	**CLOCK**	0.843	0.601	1.183	0.324	0.846	0.602	1.189	0.336
Extreme Early (N = 81) *vs* all other	Ref/minor	Gene	OR	LCI	UCI	p-value	OR	LCI	UCI	p-value
rs12649507	**G/A**	**CLOCK**	0.958	0.676	1.358	0.811	0.955	0.670	1.360	0.797
rs1801260	**A/G**	**CLOCK**	1.278	0.911	1.792	0.155	1.265	0.896	1.786	0.182
rs2070062	**A/C**	**CLOCK**	1.268	0.904	1.778	0.169	1.249	0.884	1.763	0.207
rs2071427	**C/T**	**NR1D1**	0.990	0.690	1.422	0.957	0.995	0.690	1.435	0.978
rs228697	**C/G**	**PER3**	0.816	0.456	1.459	0.492	0.851	0.476	1.521	0.586
rs2287161	**G/C**	**MTERF2/CRY1**	1.192	0.871	1.631	0.273	1.192	0.868	1.637	0.279
rs2304672	**G/C**	**PER2**	0.531	0.260	1.084	0.082	0.539	0.263	1.104	0.091
rs2314339	**C/T**	**NR1D1**	1.118	0.689	1.815	0.651	1.170	0.719	1.906	0.527
rs2735611	**A/G**	**PER1**	0.892	0.588	1.353	0.591	0.867	0.563	1.334	0.516
rs3749474	**C/T**	**CLOCK**	0.945	0.674	1.324	0.742	0.943	0.670	1.328	0.737
rs4580704	**C/G**	**CLOCK**	0.802	0.576	1.116	0.190	0.807	0.577	1.129	0.211
rs4864548	**G/A**	**CLOCK**	1.021	0.736	1.416	0.901	1.029	0.739	1.434	0.864
Log MCTQ score	Ref/minor	Gene	β	LCI	UCI	p-value	β	LCI	UCI	p-value
rs12649507	**G/A**	**CLOCK**	0.001	−0.013	0.015	0.887	0.001	−0.013	0.015	0.927
rs1801260	**A/G**	**CLOCK**	−0.001	−0.016	0.014	0.906	0.000	−0.015	0.014	0.959
rs2070062	**A/C**	**CLOCK**	−0.003	−0.018	0.012	0.672	−0.003	−0.017	0.012	0.726
rs2071427	**C/T**	**NR1D1**	−0.002	−0.017	0.013	0.798	−0.002	−0.017	0.013	0.812
rs228697	**C/G**	**PER3**	−0.010	−0.032	0.013	0.396	−0.011	−0.033	0.011	0.328
rs2287161	**G/C**	**MTERF2/CRY1**	0.000	−0.013	0.013	0.967	0.000	−0.013	0.013	0.968
rs2304672	**G/C**	**PER2**	0.017	−0.005	0.040	0.129	0.017	−0.005	0.040	0.136
rs2314339	**C/T**	**NR1D1**	0.005	−0.016	0.026	0.625	0.005	−0.016	0.025	0.656
rs2735611	**A/G**	**PER1**	−0.002	−0.018	0.015	0.858	−0.001	−0.017	0.016	0.949
rs3749474	**C/T**	**CLOCK**	−0.002	−0.015	0.012	0.829	−0.001	−0.015	0.012	0.841
rs4580704	**C/G**	**CLOCK**	0.003	−0.011	0.016	0.692	0.003	−0.011	0.016	0.706
rs4864548	**G/A**	**CLOCK**	0.000	−0.014	0.013	0.979	0.000	−0.014	0.013	0.966

Model 1: Adjusted for age in years, sex (male, female) and center (Asturias, Gipuzkoa, Navarra, Granada and Murcia).
Model 2: Model 1, additionally adjusted for energy intake (kcal/d), physical activity (Mets/h), educational level (none, primary, secondary, technical studies, higher education), smoking habit (never, former and smoker).
Significant p-values are highlighted in bold. Corrected p-values for multiple testing (Benjamini-Hochberg method) are not shown. All were >0.05. Upper (UCI) and lower (LCI) 95% confidence intervals are shown.

MONA LISA and HELENA studies (N = 3480 adults) [37], and minor allele carriers of variants rs2314339 (C/T in our study) presented lower obesity parameters as in a Spanish and North American study population (N = 2212) [51]. Also, in agreement with previous studies, we observed that genetic susceptibility to a later chronotype was associated with a higher prevalence of overweight and obesity [20,52].

The biological mechanisms underlying the potential link between clock gene variants and obesity could be through the connection between the central nervous system (CNS) and peripheral tissues in night and day circadian cycles. An imbalance between these phases affect peripheral tissues signalling pathways in the CNS, and may provoke appetite and satiety dysregulation, thereby leading to weight gain and obesity [53]. Also, the CNS is a controller of hormonal homeostasis; thus, another potential mechanism involved in the development of overweight and obesity throughout life could go through hormonal impairment [54]. Clock genes are also known to regulate eating behaviour and energy intake. In particular, the genetic variant rs1801260 has been related to emotional eating [5], and other CLOCK gene variants including the above seem to predispose to an overall increased energy intake, and in turn, to obesity development [8]. An interaction between clock gene variants and environmental factors is also possible, whereby methylation of these genes could cause the silencing of their expression, and eventually obesity [5,14].

4.3. Associations with sleeping patterns

The variant rs2070062 (T/G) was found to be significantly associated with sleep duration (β = −0.027, p = 0.003) in the study by Riestra et al. [13] This study, conducted among 2962 African Americas from the Jackson Heart Study, focused on 23 SNPs of the *CLOCK* gene. This variant (A/C) was also nominally associated with sleep duration in our study. In addition, as previously reported in an European study of 77,000 participants [12], we also observed a shorter sleep duration in carriers of the A allele of the rs12649507 SNP, although only at nominal level. Other genetic variants of the clock genes that showed associations with sleep patterns in these studies were not considered in ours. Thus, despite we could not replicate associations reported in earlier studies, our results also

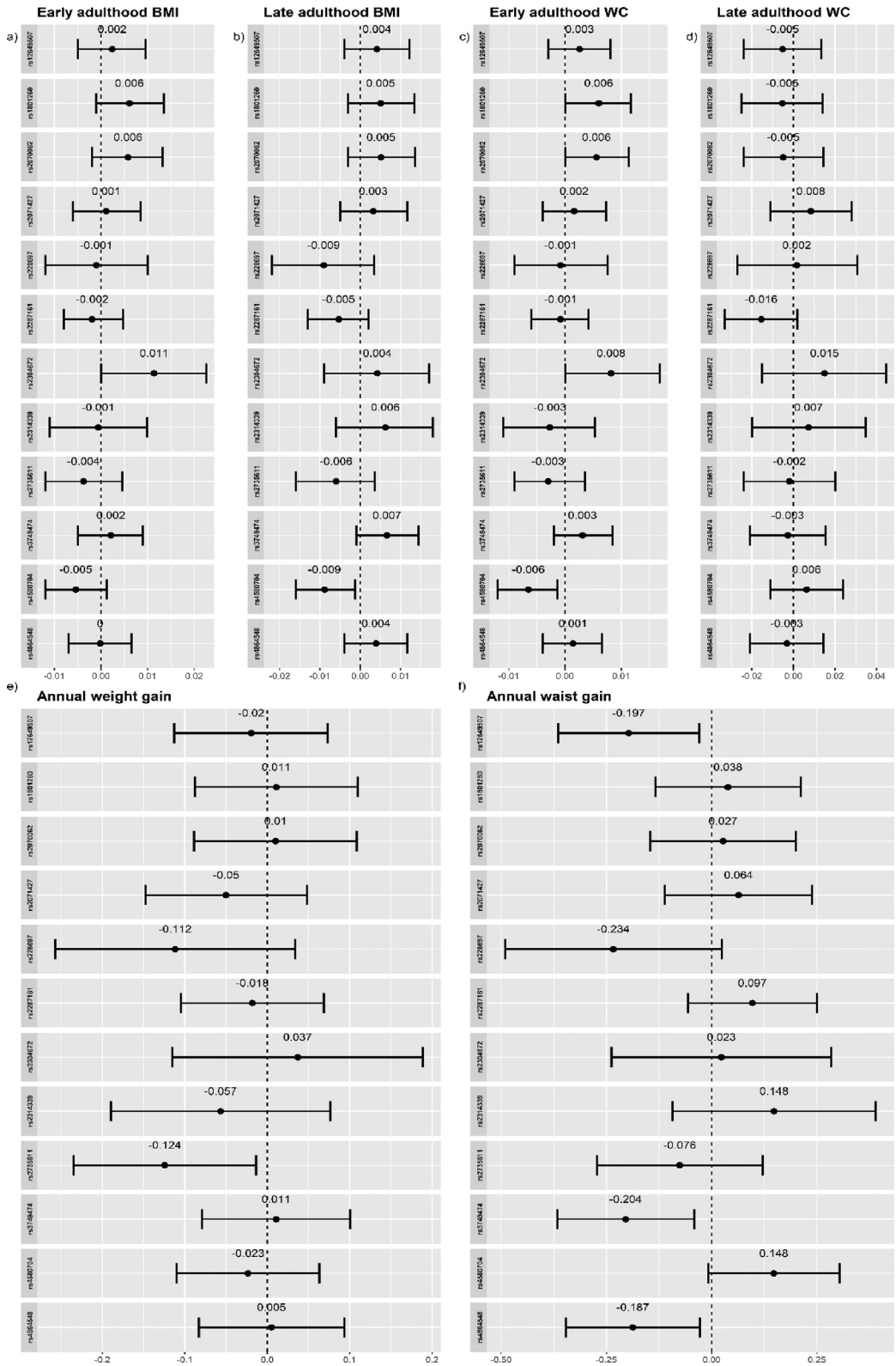

Fig. 1. Coefficient plots of the association between SNPs and obesity measures (BMI and WC in early and late adulthood, and annual weight and waist gain) among 3183 individuals of the EPIC-Spain chronodiet study. Point estimates for β coefficients and horizontal lines for 95% CI (x axis) are provided for every SNP (y axis). Multivariable linear regression adjusted for age in years, sex (male, female) and center (Asturias, Gipuzkoa, Navarra, Granada and Murcia), total energy intake (Kcal/d), physical activity (Mets-h/week), educational level (none, primary, secondary, technical studies, higher education), smoking habit (never, former and smoker). Additive genetic model. Anthropometric measures were log-transformed to approximate a normal distribution. Corrected p-values for multiple testing are not shown. All were >0.05.

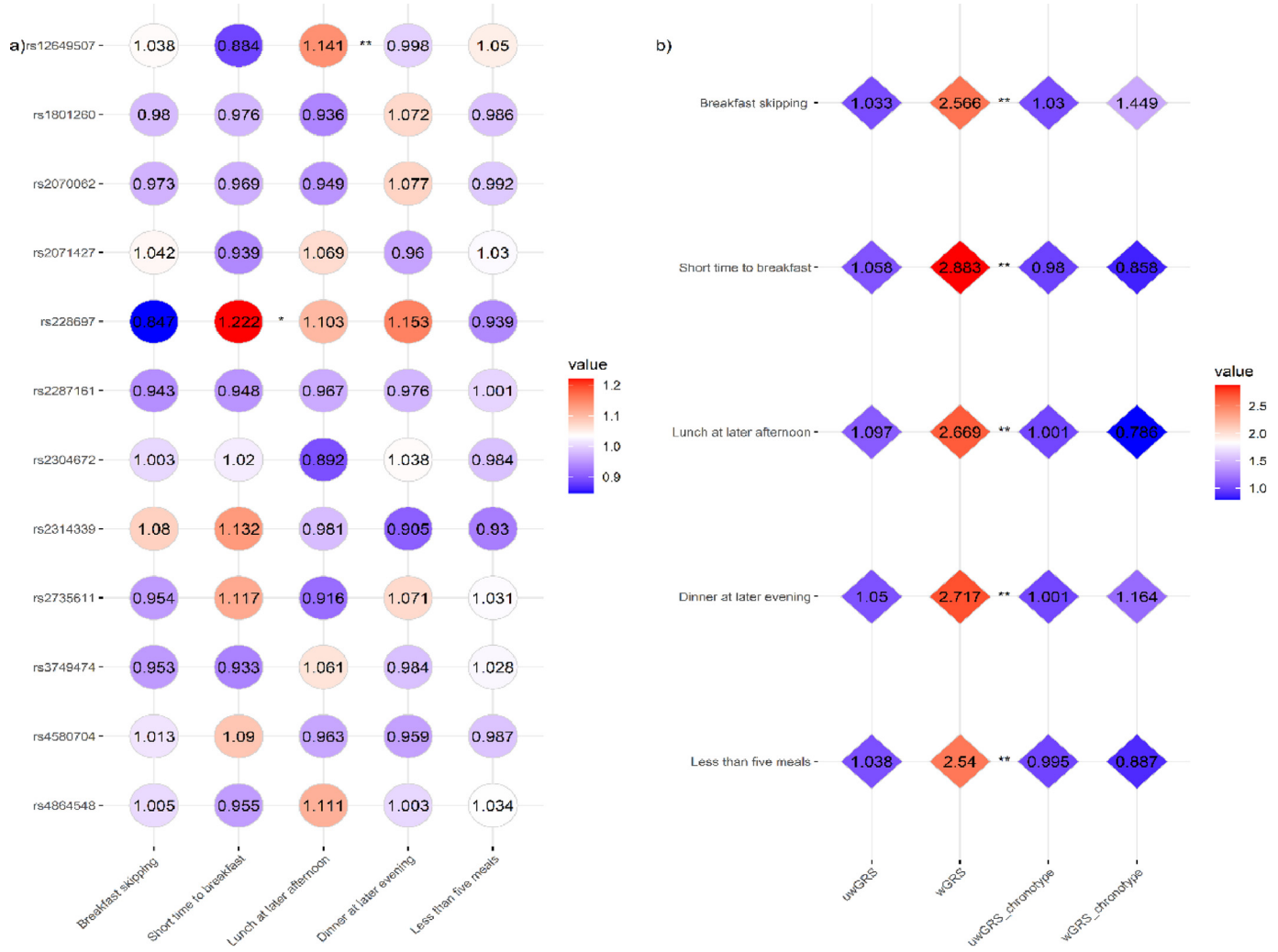

Fig. 2. Coefficient plots of the association between SNPs and meal timing, individually (a) and combined in the GRS (b), among 3183 individuals of the EPIC-Spain chronodiet study. Point estimates for OR are provided for every SNP (y axis in a) and the GRss (x axis in b). Multivariable logistic regression adjusted for age in years, sex (male, female) and center (Asturias, Gipuzkoa, Navarra, Granada and Murcia), total energy intake (Kcal/d), physical activity (Mets-h/week), educational level (none, primary, secondary, technical studies, higher education), smoking habit (never, former and smoker). Additive genetic model. Corrected p-values for multiple testing are not shown. All were >0.05 in individual SNP analyses. Nominal statistical significance is indicated as * ($p < 0.05$), and ** ($p < 0.001$).

support that longer sleep duration might protect against obesity in subjects carrying risk alleles in clock genes. Altered sleep patterns are known to cause chronodisruptions, affecting SNS and peripheral tissue, adipose tissue physiology and hormone secretion, and consequently, leading to altered feeding behaviour and obesity [13,52].

4.4. Associations with chrononutrition

Whether chronotype and its genetic background are determinants of meal timing or food intake remains controversial [4]. Some studies have suggested that adults with an evening chronotype are more likely to skip breakfast than those with a morning chronotype [55,56]. Evening chronotypes have been also associated with higher energy and macronutrient intakes in the evening while with lower intakes in the morning (breakfast) in a study carried out in Finland (N = 1854 adults) using 24-h food recalls [57]. Other studies have reported associations between evening chronotypes and intake of specific foods, showing highly variable results [4]. Results of recent studies also suggest that evening chronotype is associated with a lower adherence to healthy dietary patterns such as the Mediterranean diet [58,59]. In the current study, we did not analyse associations with intakes of specific food, nutrients or dietary patterns in order to keep the focus on meal timing. However, it is important to note that ours is the first study assessing whether genetic susceptibility variants of the circadian rhythm regulate the timing of eating. Our study yielded novel results in this regard. In particular, there were not any associations with the SNPs, except borderline significant associations for breakfast shortly after waking up.

4.5. Genetic risk scores

We combined all genetic variants in a polygenic risk score of circadian genes to prove the existence of a genetic relationship with the circadian clock and obesity-related factors. Indeed, the GRS was associated with obesity, with either categorical or continuous level data, and with all studied outcomes including evening/late chronotype, suggesting that there is a common genetic regulation of sleep, the timing of eating and body weight. Our study also highlights an association between genetically predicted chronotype (the chronotype-GRS) and overweight/obesity risk in early and late

Table 4
Association between the weighted genetic score (wGRS) with continuous outcomes (log-scale) of chronotype, sleeping and anthropometry in the EPIC-Spain chronodiet study.

wGRS	Model 1				Model 2				Model 3			
	β	LCI	UCI	p-value	β	LCI	UCI	p-value	β	LCI	UCI	p-value
Chronotype and sleep												
MCTQ score	0.059	−0.05	0.167	0.289	0.052	−0.056	0.160	0.348	0.053	−0.054	0.16	0.331
Chronotype	0.052	−0.057	0.161	0.347	0.046	−0.063	0.154	0.410	0.045	−0.063	0.154	0.413
WO sleep	−0.03	−0.108	0.049	0.458	−0.031	−0.109	0.047	0.437	−0.021	−0.099	0.056	0.589
WE sleep	0.012	−0.023	0.048	0.496	0.022	−0.05	0.095	0.546	0.023	−0.049	0.096	0.532
Anthropometric measures												
Early BMI	0.075	0.022	0.127	**0.005**	0.058	0.008	0.109	**0.024**	0.014	−0.016	0.045	0.359
Late BMI	0.072	0.01	0.133	**0.022**	0.062	0.004	0.12	**0.037**	0.008	−0.023	0.039	0.613
Early WC	0.057	0.016	0.097	**0.006**	0.048	0.009	0.087	**0.015**	0.013	−0.012	0.038	0.308
Late WC	0.03	−0.113	0.173	0.681	0.025	−0.117	0.168	0.728	0.016	−0.122	0.154	0.821
Early HC	0.026	−0.001	0.053	0.063	0.022	−0.004	0.048	0.099	−0.001	−0.018	0.017	0.95
Late HC	−0.031	−0.194	0.132	0.713	−0.031	−0.195	0.132	0.706	−0.046	−0.206	0.115	0.577
Early WHR	0.032	0.007	0.057	**0.013**	0.028	0.003	0.053	**0.029**	0.016	−0.007	0.039	0.168
Late WHR	0.055	0.028	0.082	**5.46E-05**	0.052	0.026	0.079	**9.71E-05**	0.05	0.025	0.074	**5.95E-05**
Fat %	0.134	−0.014	0.283	0.076	0.132	−0.016	0.28	0.08	0.096	−0.04	0.233	0.166
Neck C	0.020	−0.106	0.145	0.759	0.018	−0.108	0.144	0.779	0.014	−0.111	0.139	0.828
W gain/Y	0.81	0.198	1.422	**0.01**	0.811	0.204	1.418	**0.009**	0.807	0.199	1.415	**0.009**
WC gain/Y	0.614	−0.174	1.402	0.127	0.643	−0.144	1.431	0.109	0.653	−0.134	1.441	0.104

Model 1: Adjusted for age in years, sex (male, female) and center (Asturias, Gipuzkoa, Navarra, Granada and Murcia).
Model 2: Model 1, additionally adjusted for energy intake (kcal/d), physical activity (Mets/h), educational level (none, primary, secondary, technical studies, higher education), smoking habit (never, former and smoker).
Model 3: Model 2, additionally adjusted for baseline BMI (except in WC analyses) or WC (except in weight, BMI, chronotype and sleep analyses) in the chronodiet study.
Significant p-values are highlighted in bold. Corrected p-values for multiple testing are not shown. Those <0.05 are underlined. Upper (UCI) and lower (LCI) 95% confidence intervals are shown.
Abbreviations: Body mass index (BMI), waist circumference (WC), hip circumference (HC), weight (W), waist to hip ratio (WHR), weekend (WE), workdays (WO), median (MED), Munich Chronotype Questionnaire (MCTQ) score.

Table 5
Association between the weighted genetic risk score (wGRS) and the chronotype-GRS with binary outcomes of chronotype, sleeping and anthropometry in the EPIC-Spain chronodiet study.

	N	Unweighted chronotype-GRS			Weighted chronotype-GRS			Unweighted outcome-GRS			Weighted outcome GRS		
		Late chronotype-GRS→outcome						Outcome-GRS→outcome					
		OR	LCI	UCI	OR	LCI	UCI	OR	LCI	UCI	OR	LCI	UCI
Chronotype													
Early and moderate	2808	1.00			1.00			1.00			1.00		
Late	313	1.056	0.973	1.148	**2.619**	**1.156**	**5.985**	1.056	0.973	1.148	**2.619**	**1.156**	**5.985**
Sleep quality													
Good	3100	1.00			1.00			1.00			1.00		
Bad	83	1.029	0.883	1.201	1.104	0.240	5.202	**1.275**	**1.082**	**1.505**	**2.637**	**1.564**	**4.428**
Anthropometric measures													
Early adulthood													
Abdominal obese													
Non-obese	2441	1.00			1.00			1.00			1.00		
Obese	772	0.975	0.920	1.035	0.914	0.515	1.626	**1.054**	**1.000**	**1.110**	**2.989**	**1.469**	**6.116**
Obese													
Normal/overweight	2592	1.00			1.00			1.00			1.00		
Obese	591	0.965	0.905	1.029	0.771	0.414	1.441	**1.068**	**1.016**	**1.124**	**2.703**	**1.591**	**4.636**
Overweight/obese													
Normal weight	1052	1.00			1.00			1.00			1.00		
Obese/overweight	2131	1.031	0.975	1.090	**2.224**	**1.282**	**3.865**	**1.071**	**1.019**	**1.127**	2.741	**1.705**	**4.423**
Late adulthood													
Abdominal obese													
Non-obese	1700	1.00			1.00			1.00			1.00		
Obese	1481	1.006	0.955	1.060	1.653	0.992	2.762	1.030	0.993	1.068	**2.533**	**1.322**	**4.870**
Obese													
Normal/overweight	2129	1.00			1.00			1.00			1.00		
Obese	1034	0.969	0.918	1.022	1.036	0.614	1.750	**1.088**	**1.029**	**1.152**	**2.683**	**1.509**	**4.794**
Overweight/obese													
Normal weight	698	1.00			1.00			1.00			1.00		
Obese/overweight	2465	1.029	0.967	1.096	**2.104**	**1.142**	**3.878**	1.057	0.992	1.128	**2.797**	**1.328**	**5.924**

ORs adjusted for age in years, sex (male, female) and center (Asturias, Gipuzkoa, Navarra, Granada and Murcia), energy intake (kcal/d), physical activity (Mets/h), educational level (none, primary, secondary, technical studies, higher education), smoking habit (never, former and smoker).
Significant p-values are highlighted in bold.

adulthood. The GWAS conducted by Jones et al. showed that chronotype is not causally linked to BMI [16]. However, this study did not account for body composition changes with age, which take place even in the absence of changes in body weight [60]. It is important to highlight that we have assessed associations between a broader range of anthropometric measures collected at different stages of adulthood. Also, our score accounted for genetic variants of multiple chronobiological outcomes.

Among the strengths of this study is the consideration of major covariates (age, sex, educational level, smoking habit and physical activity) that can modify susceptibility to the studied outcomes (obesity, chronotype, etc.). GRS allowed us to evaluate the cumulative effect of all genetic variants, which individually showed a small effect on these outcomes [42]. In addition, we used physical measures for anthropometric assessments, enabling us to provide more reliable results on the association between the genetic variants and the measures of obesity.

Among the limitations, we did not consider the exposure to epigenetic factors, gene–environmental and gene–gene interactions, all of which are known to modify genetic risk. Our study included participants from European ancestry in a Mediterranean population, which might hinder extrapolation of our findings to other populations. A diet history method to gather information on meal timing and to derive food intakes was used. While dietary measurement error cannot be neglected, this method provides high quality dietary data [24]. Self-reported bias is also likely in the assessment of sleep parameters and the individual's chronotype. We did not choose effect sizes from previous GWAS to build the GRS as this information was lacking for the majority of the studied outcomes; indeed, our selected SNPs were shown to be associated with a variety of chronobiological factors of obesity in previous, mostly candidate-gene, studies [5,6,12,17,18,34–37]. In addition, effect sizes of the GRS were derived from our own study population. Therefore, we cannot discard overfitting in our GRS models. Also, including a larger number of SNPs with larger effect sizes might improve the power of the GRS model. Nonetheless, while sample size and effect sizes might be an issue, this approach has allowed us to establish associations with some chronobiology-related outcomes. We considered SNPs in LD ($r^2 > 0.8$), with are known to exert a high correlation in the same gene, thereby posing a challenge for the calculation of the GRS. However, by removing SNPs in LD while keeping the most significant variants, we obtained the same results. Indeed, weighted GRS seem to counteract LD effects in this kind of models [61].

In conclusion, within this EPIC-Spain study, we identified some nominally significant associations between circadian-related SNPs and sleeping duration, anthropometric measures and chrononutrition variables. Associations with our SNP-based genetic score support that circadian disruptions leading to diverse outcomes are intrinsically related by genetic markers, and that clock gene variants might predispose individuals to a given chronotype and obesity. Further investigations in studies of larger sample size are needed to develop a deeper understanding of genetic variation of the circadian system and its impact on cronobiological outcomes.

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Author contributions

All authors meet the criteria for authorship. They have read and approved the last version of the manuscript. Contributors: Writing original draft: EMM prepared the initial draft of the manuscript and protocol, and conducted the analyses. MRB, JRQ and MJS provided substantial intellectual input. Writing, review & editing: All authors contributed to critical revisions of the manuscript and approved the final version for submission. EMM and MRB acts as guarantor of the manuscript. Conceptualization and funding acquisition: JRQ. The corresponding author attests that all listed authors meet authorship criteria and that no others meeting the criteria were omitted.

Data availability statement

The data of this study is preserved by the EPIC-Spain research group. Data are subject to data sharing agreements and are not publicly available.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2022.07.027.

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