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RESEARCH ARTICLE

Association of *CLOCK* gene variants with obesity and adiposity-related anthropometric, metabolic, and behavioral parameters

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Abstract

The *CLOCK* gene is a core component of the circadian clock and regulates various aspects of metabolism. Therefore, any variation that affects the function/expression of the *CLOCK* gene may contribute to the manifestation of metabolic disorders such as obesity. This study investigated whether the *CLOCK* variants rs4864548 and rs6843722 are associated with obesity and related traits in Pakistanis. A total of 306 overweight/obese cases and 306 age- and gender-matched control subjects were recruited (males 336 and females 276, age range 12–63 years). Anthropometric and metabolic parameters were taken by standard procedures and biochemical analyses, respectively. Behavior-related information was collected with a questionnaire. The genotypes of the variants were determined by allelic discrimination Taqman assays. Both variants were found to have a significant association with overweight/obesity according to the over-dominant model. The rs4864548 and rs6843722 were observed to escalate the risk of overweight/obesity by 1.611 ($p = 0.004$) and 1.657 ($p = 0.002$) times, respectively. These variants were also seen to be significantly associated with various other adiposity-related anthropometric parameters ($p < 0.05$). However, no association of both variants with metabolic and behavioral parameters was observed ($p > 0.05$). Thus, these variants may contribute to increasing the risk of overweight/obesity and related anthropometric traits in Pakistanis.

Key words: *CLOCK* gene, rs4864548, rs6843722, obesity, anthropometry, metabolic parameters

Introduction

Circadian rhythms are daily oscillations of multiple physiological processes with nearly 24 h periodicity. These rhythms are the external expression of an internal timing system engendered by a circadian clock that is synchronized by the day–night cycle. The circadian timing system includes the central clock that exists in the suprachiasmatic nucleus of the hypothalamus and various peripheral clocks found in different tissues and organs (Richter et al. 2004). The central clock is chiefly entrained by light, whereas peripheral clocks within organs that govern glucose and energy homeostasis like skeletal muscle, adipose tissue, liver, and pancreas for instance, are entrained by external cues such as temperature, feeding–fasting cycles, hormones, and exercise (Partch et al. 2014; Asher and

Sassone-Corsi 2015; Bass and Lazar 2016; Gabriel and Zierath 2019). Thus, the primary function of the circadian timing system is to drive overt circadian rhythms in the physiology of the organisms to ensure that main physiological functions are in synchrony with the external environment, for example, circadian clocks drive the whole body metabolism under homeostatic conditions (Maury 2019). At the molecular level, circadian rhythms are generated by a network of transcriptional–translational feedback loops that drive nearly 24 h rhythmic expression patterns of core clock components. Thus, circadian molecular machinery is based on core clock genes and regulates the expression of various clock-controlled genes such as metabolic genes that are coordinated in a tissue-specific manner (Fatima and Rana 2020; Stehle et al. 2021). Therefore, any variation in these core clock genes that either affects their expression or function can result in circadian disruption of various physiological processes including metabolism (Fatima and Rana 2020).

The *CLOCK* (circadian locomotor output cycle kaput) gene is a core component of the molecular circadian clock. A whole-genome linkage scan employing 380 microsatellite markers for identification of genomic regions that may contain quantitative-trait loci for obesity has revealed that the region 4q12, the chromosomal site of the *CLOCK* gene, may be linked to obesity (Deng et al. 2002). Therefore, the association of *CLOCK* gene variants with obesity can be explored in various populations. As genetic variants are known to affect a particular trait in some populations but not in others, many genetic associations can thus be population specific. The Pakistani population provides distinct opportunities to study various genetic and environmental factors to elucidate the complex pathophysiological mechanisms underlying obesity, because the Pakistani population has diverse genetic architecture as a consequence of several migratory events, large pedigrees, consanguineous marriages, large population size, and transitional shift in nutrition (Pigeyre et al. 2018). In addition, the prevalence of obesity in Pakistan is continuously rising at an alarming rate (Ng et al. 2014). Furthermore, the association of *CLOCK* gene variants with obesity has never been explored before in the Pakistani population. The *CLOCK* gene variant rs4864548 is in the promoter region of the *CLOCK* gene; therefore, it is likely that it may influence the rate of transcription. In contrast, the variant rs6843722 is an intronic variant. Although intronic variants do not change the protein sequence they may be critical in the regulation of gene expression (Hindorff et al. 2009). Moreover, the intronic mutations may disrupt noncoding RNA genes and transcription regulatory motifs (Vaz-Drago et al. 2017). In addition, the intronic variants may influence the alternative RNA splicing by affecting the recognition of the splice site (Slaugenhaupt et al. 2001; Neklason et al. 2004). Furthermore, intronic variants may modify the binding affinities of RNA binding proteins to *cis*-regulatory sequences (Kashima et al. 2007; Lin et al. 2019). However, the role of variant rs6843722 in any of the aforementioned mechanisms is not known yet. Thus, the current study was undertaken to investigate the association of the two *CLOCK* gene variants namely rs6850524 (C > G) and rs6843722 (A > C) with overweight/obesity and adiposity-related anthropometric, metabolic, and behavioral parameters in a sample of the Pakistani population.

Material and methods

Study population

The study was executed at the International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan. The study protocol was approved by the Independent Ethics Committee of the ICCBS and Advanced Studies and Research Board of the University. A total of 306 overweight/obese cases and an equal number of their age- and gender-matched control subjects having normal body mass index (BMI) were recruited for the study. Together, cases and controls constituted a total sample population of 612 individuals with an age range of 12–63 years. The sample population included 276 females and 336 males. Subjects were recruited from the city of Karachi and

the written informed consent from each participant/guardian of the participant was secured before participation in the study.

Blood sample collection

The venous whole blood samples were collected after overnight fasting of 8–12 h from all the participants of the study. The collected blood samples were utilized for DNA and serum isolation.

Anthropometric parameters

Anthropometric measurements of the study participants were taken by employing standard procedures. Stadiometer (Seca 214, Germany) and a mechanical column scale (Seca 755, Germany) were utilized to find out subjects' height in centimeters (to the nearest 0.1 cm) and weight in kilograms (to the nearest 0.1 kg), respectively. Subjects were weighed barefoot wearing light clothes. BMI was calculated as weight (kg)/height (m^2). Waist circumference (WC), hip circumference (HC), and skinfold thicknesses (SFTs) from biceps, triceps, abdomen, supra iliac, sub-scapular, and thighs were measured from each participant. By dividing the WC values by the values of height, the waist-to-height ratio (WHtR) was calculated. Similarly, the measurements of waist and hip circumference were used to find out the waist-to-hip ratio (WHR). Body fat percentage (%BF) was calculated by using the sum of SFTs via gender-specific formulae (Jackson and Pollock 1985).

Metabolic parameters

Metabolic parameters included fasting blood glucose (FBG), fasting insulin levels, homeostasis model assessment of insulin resistance (HOMA-IR), systolic blood pressure (SBP), diastolic blood pressure (DBP), and total lipid profile. From the upper right arm of each participant, arterial SBP and DBP were taken. A standard Hg sphygmomanometer having 1 mm Hg accuracy (CR-2001, Certeza medical) was utilized for this purpose. A blood glucose monitoring system including blood glucose strips and Glucometer (Abbott, UK) was used to estimate FBG level (mg/dL). Fasting serum insulin levels were estimated on Thermo Scientific™ Multiskan™ FC Microplate Photometer (MA, USA) by using an ELISA (Enzyme-linked ImmunoSorbent Assay) based commercial kit (Catalog number KAP1251, The *DIA source INS*-ELISA) following protocol provided by the manufacturing company. HOMA-IR was evaluated by values of FBG and fasting serum insulin by using the appropriate formula (Matthews et al. 1985). The enzymatic colorimetric method was used to estimate the fasting lipid profile of each participant in the study. Appropriate commercial kits (Merck, Germany) and Roche's automatic chemistry analyzer (Japan) were used for the estimation of the lipid profile. Fasting lipid profile included serum triglycerides, total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and very-low-density lipoprotein cholesterol (VLDL-C). Moreover, additional metabolic parameters including TyG (product of triglyceride and glucose) index (used as a substitute to detect insulin resistance), lipid accumulation product (LAP; mmol/L), visceral adiposity index (VAI; mmol/L), LDL-C/HDL-C ratio, triglyceride-to-HDL-C ratio and coronary risk index (CRI) also were calculated by computing values of the aforementioned variables.

Behavioral parameters

Information for obesogenic behavioral factors was collected by using a self-reported questionnaire. Behavioral factors included sleep–wake timings, sleep duration, food timings, diet consciousness, the tendency towards fat-dense food (TFDF), and physical activity.

DNA isolation and genotyping

DNA was extracted from blood samples using commercially available DNA extraction kits (Bio Basic, Canada). Genotyping of the *CLOCK* gene variants rs4864548 and rs6843722 was performed by using TaqMan assays with allele-specific primers and probes (Assay ID C__11821330_10, Catalogue number: 4351379 and Assay ID C__11821276_10, Catalogue number: 4351379 ABI, Foster City, CA, USA) and TaqMan® genotyping master mix (Cat. no. 4371355, ABI, Foster City, CA, USA) as per the manufacturer's instructions on the Applied Biosystems Quant Studio™ 5 Real-Time PCR System (ABI, Foster City, CA, USA). For each batch, two negative controls (no template control) and positive control for each genotype were included.

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics for Windows, version 19 (IBM Corp., Armonk, NY, USA). Genotypic frequencies were stated as counts and percentages. Genotypic frequencies were checked for compliance to Hardy–Weinberg Equilibrium (HWE) by a web-based calculator (Rodriguez et al. 2009). The normality of quantitative variables was tested by using Shapiro–Wilk test. For non-normal quantitative variables, the rank-based inverse normal transformation was conducted. Modified values of continuous variables were employed for subsequent analyses. The association of the *CLOCK* gene variants with overweight/obesity was found by logistic regression by considering multiple genetic models (additive, pairwise comparison of genotypes, dominant, recessive, and over-dominant). The *h*-index (degree of dominance) was determined to select the best-fit model in case of getting association in more than one genetic model (Zintzaras and Santos 2011). Odds ratio (OR) and 95% confidence intervals (CI) (adjusted for age and gender) were calculated to find out the risk of overweight/obesity associated with the variants. The association of the variants with all continuous and categorical variables was found by linear and logistic regression, respectively. Linear/logistic regression was employed to find the effect size (β)/OR with 95% CI and p values. All tests for detecting the association of the variants with obesity-related anthropometric, metabolic, and behavioral parameters were adjusted for confounders such as age and gender. However, the tests seeking association of the variants with the adiposity-related metabolic and behavioral parameters were also adjusted for BMI. In addition, to reduce the risk of false-positive findings, Benjamini–Hochberg's method was employed for the correction of multiple comparisons (Benjamini and Hochberg 1995). All tests where $p < 0.05$ were considered statistically significant.

Power calculation

The power of the current study was calculated for both variants (rs4864548and rs6843722) separately by an online program "Sampsize" (available at sampsize.sourceforge.net/iface/s3.html#ccp) (Ngamjarus 2016). In the case of rs4864548, the present study was found to have a power of 84% at a significance level of 0.05 ($\alpha = 0.05$) and odds ratio of 1.611, where 43% of controls were found to be exposed to risk genotype (GA). Similarly, in the case of rs6843722, the estimated power was 88% with an odds ratio of 1.657, when 45% of the controls were observed to have risk genotype (AC).

Results

Genotypic frequencies of the *CLOCK* variants and HWE

The genotypic frequencies of the *CLOCK* variants rs4864548 and rs6843722 are shown in **Table 1**. The genotypic frequencies of the rs4864548 and rs6843722 were observed to conform with HWE in controls ($p = 0.141$ and $p= 0.199$, respectively), whereas the corresponding genotypic frequencies did not conform to HWE in cases ($p = 0.006$ and $p= 0.003$, respectively). Significant differences were

Table 1. Genotypic frequencies and association of the *CLOCK* variants with overweight/obesity.

Genetic Model	Genotype/alleles	Controls	Cases	Unadjusted			Adjusted for age and gender			*h*- index
				OR	95% CI	*p*	OR	95% CI	*p*	
			rs4864548							
Additive	G vs A	G = 347 (43%)	G = 337 (55%)	1.071	0.851–1.348	0.558	1.090	0.864–1.375	0.466	—
		A = 265 (57%)	A = 275 (45%)							
Pairwise Comparison										
Wild type homozygous vs. mutant homozygous	GG vs. AA	—	—	1.029	0.642–1.648	0.906	1.063	0.660–1.712	0.803	3.3
Wild type homozygous vs. heterozygous	GG vs. GA	—	—	1.656	1.148–2.388	**0.007**	1.648	1.142–2.379	**0.008**	3.3
Dominant	AA + GA	200 (47.1%)	225 (52.9%)	1.458	1.031–2.063	**0.033**	1.471	1.038–2.084	**0.030**	3.3
	GG	105 (56.5%)	81 (43.5%)							
Over- dominant	GA	131 (42.8%)	175 (57.2%)	1.638	1.190–2.255	**0.002**	1.611	1.169–2.221	**0.004**	3.3
	GG + AA	168(55.1%)	137 (44.9%)							
Recessive	AA	63 (55.8%)	50 (44.2%)	0.750	0.498–1.131	0.170	0.750	0.512–1.176	0.232	3.3
	GG + GA	242 (48.6%)	256 (51.4%)							
Hardy–Weinberg Equilibrium										
Cases									*p* = 0.006	
Controls									*P* = 0.141	
			rs6843722							
Additive	A vs C	A = 373 (61%)	A = 344 (56 %)	1.230	0.972–1.556	0.084	1.249	0.985–1.585	0.067	
		C = 239 (39 %)	C = 268 (44 %)							
Pairwise Comparison										
Wild type homozygous vs. mutant homozygous	AA vs. CC	—	—	1.267	0.779–2.062	0.341	1.311	0.801–2.147	0.281	1.864
Wild type homozygous vs. heterozygous	AA vs. AC			1.805	1.262–2.581	**0.001**	1.812	1.264–2.597	**0.001**	1.864
Dominant	CC + AC	188 (61.4%)	222 (72.5%)	1.659	1.180–2.330	**0.004**	1.681	1.192–2.371	**0.003**	1.864
	AA	118 (36.8%)	84 (27.5%)							
Over- dominant	CC + AA	169 (55.2%)	130 (42.5%)	1.670	1.213–2.299	**0.002**	1.657	1.203–2.284	**0.002**	1.864
	AC	137 (44.8%)	176 (57.5%)							
Recessive	AA + AC	255 (83.3%)	260 (85%)	0.885	0.573–1.366	0.580	0.908	0.586–1.408	0.667	1.864
	CC	51 (16.7%)	46 (15.0%)							

(*continued*)

Table 1. (*concluded*)

Genetic Model	Genotype/alleles	Controls	Cases	Unadjusted OR	Unadjusted 95% CI	Unadjusted *p*	Adjusted for age and gender OR	Adjusted for age and gender 95% CI	Adjusted for age and gender *p*	*h*- index
Hardy–Weinberg Equilibrium										
Cases										*p*-value = 0.003
Controls										*p*-value = 0.199

Note: The genotypic/allelic frequencies for *CLOCK* gene variants, rs6843722 and rs4864548, are shown in counts (percentage in parentheses). The unadjusted and age- and gender-adjusted association of the *CLOCK*gene variants with overweight/obesity was also determined by logistic regression assuming additive, pair-wise comparison (wild type homozygous vs. mutant homozygous and wild type homozygous vs. heterozygous), dominant, recessive, and over-dominant genetic models. The *h*-index was computed for determining the appropriate mode of inheritance. The odds ratios along with 95% confidence intervals and *p* values were calculated to seek the impact and strength of the association, respectively. $p < 0.05$ was considered statistically significant. Statistically significant *p* values are shown in **bold**. OR, odds ratio; CI, confidence interval; *h*-index, degree of dominance index. G vs A, wild type allele vs mutant allele; GG vs. AA, Wild type homozygous vs. mutant homozygous; GG vs. GA, Wild type homozygous vs. heterozygous; AA + GA, mutant A allele carrier; GG, homozygous wild type genotype carrier; GA, heterozygous genotype carrier; GG + AA, homozygous wild type/mutant genotype carrier; AA, homozygous mutant genotype carrier; GG + GA, wild type G allele carrier; A vs C, wild type allele vs mutant allele; AA vs. CC, wild type homozygous vs. mutant homozygous; AA vs. AC, wild type homozygous vs. heterozygous; CC + AC, mutant C allele carrier; AA, homozygous wild type genotype carrier; AC, heterozygous genotype carrier; CC + AA, homozygous wild type/mutant genotype carrier; CC, homozygous mutant genotype carrier; AA + AC, wild type A allele carrier.

observed in genotypic frequencies of rs4864548 ($p = 0.004$) and rs6843722 ($p = 0.002$) between overweight/obese cases and control subjects who had normal BMI.

Association of the *CLOCK* variants rs4864548 and rs6843722 with overweight/obese phenotype

The significant association of both variants rs4864548 and rs6843722 with overweight/obesity was observed in pairwise genotypic comparison (wild type homozygous vs. heterozygous), dominant, and over-dominant genetic models ($p < 0.05$) (Table 1). Since the association was seen in more than one genetic model, the *h*-index was computed to determine the appropriate mode of inheritance. The *h*-index value indicated the over-dominant model of inheritance as the best-fit model for both *CLOCK* gene variants, rs4864548 (*h*-index = 3.3) and rs6843722 (*h*-index = 1.864). According to the over-dominant model, the GA genotype of the rs4864548 increased the risk of being overweight/obese 1.611 times ($p = 0.004$). Likewise, the AC genotype of the rs6843722 increased the risk of having an overweight/obese phenotype 1.657 times ($p = 0.002$) (Table 1).

Association of the *CLOCK* variants rs4864548 and rs6843722 with adiposity-related anthropometric traits

The *CLOCK* variant rs4864548 (Table 2) was found to be significantly associated with all the adiposity-related anthropometric traits including body weight, WC, HC, WHR, WHtR, SFTs, and % BF ($p < 0.05$). Similarly, the rs6843722 (Table 3) was also found to be significantly associated with nearly all the anthropometric traits ($p < 0.05$) except biceps SFT ($p = 0.165$).

Lack of association of the *CLOCK* variants with adiposity-related metabolic and behavioral parameters

No association was seen between the *CLOCK* gene variants (rs6843722 and rs4864548) and any of the adiposity-related metabolic (SBP, DBP, LAP, VAI, TG, FBG, fasting insulin, HOMA-IR, TG, TC, LDL-C, VLDL-C HDL-C, CRI, LDL-C/HDL-C, TG/HDL-C) (Tables 4 and 5) and behavioral parameters (random eating behavior, diet unconsciousness, high TFDF, low physical activity, insufficient sleep duration, and irregular sleep–wake cycle) (Tables 6 and 7) ($p > 0.05$).

Table 2. Association of the rs4864548 with anthropometric parameters.

Parameters (unit of measurement)	Over-dominant model	Mean	SD	Unadjusted			Adjusted for age and gender			
				β	95% CI	*p*	β	95% CI	*p*	*p* value corrected[a]
Weight (kg)	GA	76.83	20.76	5.828	2.524–9.131	**0.001**	5.696	2.529–8.863	**<0.001**	**<0.001**
	GG + AA	70.80	20.87							
WC (cm)	GA	99.28	18.36	5.173	2.286–8.059	**<0.001**	4.847	1.995–7.699	**0.001**	**0.002**
	GG + AA	94.03	17.98							
HC (cm)	GA	106.70	13.57	4.232	2.012–6.400	**<0.001**	4.046	1.836–6.256	**<0.001**	**<0.001**
	GG + AA	102.30	14.40							
WHR	GA	0.93	0.08	0.020	0.007–0.033	**0.002**	0.018	0.006–0.031	**0.004**	**0.005**
	GG + AA	0.91	0.08							
WHtR	GA	0.80	0.33	0.117	0.065–0.170	**<0.001**	0.110	0.058–0.161	**<0.001**	**<0.001**
	GG + AA	0.67	0.33							
Biceps SFT (mm)	GA	14.97	8.93	1.606	0.231–2.981	**0.022**	1.487	0.204–2.770	**0.023**	**0.023**
	GG + AA	13.32	8.37							
Triceps SFT (mm)	GA	24.73	11.93	3.249	1.408–5.090	**0.001**	3.145	1.328–4.963	**0.001**	**0.002**
	GG + AA	21.42	11.23							
Abdominal SFT (mm)	GA	38.21	17.09	4.527	1.844–7.211	**0.001**	4.160	1.530–6.789	**0.002**	**0.003**
	GG + AA	33.61	16.69							
Supra-iliac SFT (mm)	GA	30.99	15.29	4.265	1.861–6.670	**0.001**	4.096	1.795–6.397	**0.001**	**0.002**
	GG + AA	26.67	14.96							
Thigh SFT (mm)	GA	36.09	18.58	4.403	1.432–7.373	**0.004**	4.273	1.338–7.208	**0.004**	**0.005**
	GG + AA	31.62	18.80							
Sub-scapular SFT (mm)	GA	26.70	11.86	2.831	0.954–4.708	**0.003**	2.637	0.780–4.494	**0.005**	**0.005**
	GG + AA	23.79	11.75							
%BF	GA	28.92	9.92	2.527	0.990–4.060	**0.001**	2.321	0.814–3.820	**0.003**	**0.004**
	GG + AA	26.35	9.43							

Note: Association of the *CLOCK* gene variant rs4864548 with anthropometric parameters was determined using over-dominant genetic model by linear regression. The β, CI, and *p* values are provided before and after age and gender adjustment. Benjamini-Hochberg method was used for the correction of multiple comparisons. The effect size (β) along with 95% confidence intervals and *p*-values were calculated to seek the impact and strength of the association, respectively. $p < 0.05$ was considered statistically significant. The statistically significant *p* values are shown in **bold**. Abbreviations: WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; WHtR, waist-to-height ratio; SFT, skin fold thickness; %BF, body fat percentage; SD, standard deviation; β, effect size; CI, confidence interval; GA, heterozygous genotype carrier; GG + AA, homozygous wild type/mutant genotype carrier.

[a]*p* values after correction for multiple comparisons.

Discussion

The present study reports a significant association of two *CLOCK* (an important gene within the circadian machinery) gene variants, rs6843722 and rs4864548, with the increased risk of overweight/obesity in the Pakistani population. Similarly, our study also reveals the significant association of the aforementioned variants with adiposity-related anthropometric traits including body

Table 3. Association of the rs6843722 with anthropometric parameters.

Parameters (unit of measurement)	Over-dominant model	Mean	SD	Unadjusted			Adjusted for age and gender			
				β	95% CI	*p*	β	95% CI	*p*	*p* value corrected[a]
Weight (kg)	AC	76.33	20.5	4.946	1.629–8.262	**0.004**	4.553	1.372–7.734	**0.005**	**0.018**
	AA + CC	71.39	21.24							
WC (cm)	AC	98.42	18.01	3.514	0.611–6.417	**0.018**	3.363	0.501–6.225	**0.021**	**0.029**
	AA + CC	94.91	18.55							
HC (cm)	AC	106.02	13.7	2.930	0.694–5.165	**0.010**	2.931	0.711–5.151	**0.010**	**0.024**
	AA + CC	103.09	14.40							
WHR	AC	0.93	0.08	0.014	0.001–0.027	**0.030**	0.014	0.001–0.026	**0.036**	**0.039**
	AA + CC	0.91	0.08							
WHtR	AC	0.70	0.34	0.085	0.032–0.138	**0.002**	0.085	0.034–0.137	**0.001**	**0.012**
	AA + CC	0.70	0.34							
Biceps SFT (mm)	AC	14.53	8.61	0.746	−0.634–2.126	0.289	0.908	−0.376–2.193	0.165	0.165
	AA + CC	13.79	8.77							
Triceps SFT (mm)	AC	24.19	11.70	2.202	0.349–4.054	**0.020**	2.297	0.472–4.121	**0.014**	**0.027**
	AA + CC	21.99	11.61							
Abdominal SFT (mm)	AC	37.86	17.09	3.909	1.219–6.600	**0.004**	3.729	1.090–6.350	**0.006**	**0.018**
	AA + CC	33.95	16.78							
Supra-iliac SFT (mm)	AC	30.66	15.21	3.655	1.244–6.065	**0.003**	3.359	1.053–5.665	**0.004**	**0.018**
	AA + CC	27.01	15.14							
Thigh SFT (mm)	AC	35.59	18.34	3.453	0.475–6.430	**0.023**	3.61	0.681–6.551	**0.016**	**0.027**
	AA + CC	32.14	19.16							
Sub-scapular SFT (mm)	AC	26.27	11.56	2.021	0.134–3.907	2.048	0.036	0.187–3.909	**0.031**	**0.037**
	AA + CC	24.25	12.15							
%BF	AC	28.52	9.90	1.743	0.198–3.280	1.769	0.027	0.250–3.270	**0.022**	**0.029**
	AA + CC	26.77	9.54							

Note: Association of the *CLOCK* gene variant rs6843722 with anthropometric parameters was found using over-dominant genetic model by linear regression. The β, CI, and *p* values are provided before and after age and gender adjustment. Benjamini–Hochberg method was used for the correction of multiple comparisons. The effect size (β) along with 95% confidence intervals and *p* values were calculated to seek the impact and strength of the association, respectively. $p < 0.05$ was considered statistically significant. The statistically significant *p* values are shown in **bold**. WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; WHtR, waist-to-height ratio; SFT, skin fold thickness; %BF, body fat percentage; SD, standard deviation; β, effect size; CI, confidence interval; AC, heterozygous genotype carrier; AA + CC, homozygous wild type/mutant genotype carrier.
[a]The *p* values after correction for multiple comparisons.

weight, WC, HC, WHR, WHtR, SFTs, and %BF. In agreement with our study, Garaulet et al. (2010a, 2010b) reported the association between the rs4864548 and increased risk of obesity and related variables including weight, BMI, and waist circumference in the Spanish population . Similarly, the association of rs6843722 and rs4864548 with overweight/obesity and increased BMI was also observed in Argentineans of European ancestry (Sookoian et al. 2008). Conversely, Sookoian et al. (2008) found no association of both variants with waist circumference in the Argentinean population. Likewise, a

Table 4. Association of the *CLOCK* variant rs4864548 with adiposity-related metabolic traits.

Parameters (unit of measurement)	Over-dominant model	Mean	SD	Unadjusted			Adjusted for age, gender, and BMI		
				β	95% CI	*p*	β	95% CI	*p*
SBP (mmHg)	GA	117.29	14.31	0.927	−1.305–3.160	0.415	−0.363	−2.394–1.669	0.726
	GG + AA	116.35	13.74						
DBP (mmHg)	GA	77.54	10.30	0.384	−1.209–1.978	0.636	−0.596	−2.093–0.901	0.435
	GG + AA	77.14	9.71						
VAI (mmol L^{-1})	GA	3.08	2.00	0.083	−0.244–0.409	0.619	−0.132	−0.441–0.177	0.403
	GG + AA	2.99	2.10						
LAP (mmol L^{-1})	GA	55.11	40.07	10.04	3.506–16.59	0.003	1.102	−3.463–5.668	0.636
	GG + AA	44.79	42.46						
TyG index	GA	8.58	0.66	0.010	−0.086–0.106	0.841	−0.040	−0.133–0.052	0.392
	GG + AA	8.57	0.54						
FBG (mg dL^{-1})	GA	102.75	19.10	1.637	−1.544–4.818	0.313	0.390	−2.726–3.505	0.806
	GG + AA	101.06	20.91						
Fasting insulin (µl U mL^{-1})	GA	23.59	14.02	1.576	−0.630–3.783	0.161	−0.200	−2.177–1.776	0.842
	GG + AA	21.94	13.77						
HOMA-IR	GA	6.087	4.14	0.486	−0.161–1.133	0.140	−0.061	−0.639–0.517	0.836
	GG + AA	5.579	4.00						
TC (mg dL^{-1})	GA	150.27	38.94	−2.349	−8.634–3.936	0.463	−4.815	−11.032–1.402	0.129
	GG + AA	152.48	40.16						
TG (mg dL^{-1})	GA	121.33	66.52	3.165	−7.703–14.03	0.568	−2.590	−12.865–7.685	0.621
	GG + AA	117.94	70.26						
HDL-C (mg dL^{-1})	GA	30.55	8.76	0.409	−1.062–1.879	0.585	0.503	−0.970–1.976	0.503
	GG + AA	30.10	9.74						
LDL-C (mg dL^{-1})	GA	92.07	35.76	1.151	−4.602–6.903	0.695	−2.287	−7.769–3.195	0.413
	GG + AA	90.79	36.67						
VLDL-C (mg dL^{-1})	GA	24.25	13.16	0.407	−1.742–2.556	0.710	−0.665	−2.701–1.371	0.521
	GG + AA	23.80	13.88						
CRI	GA	5.22	1.65	−0.138	−0.411–0.136	0.324	−0.255	−0.519–0.009	0.058
	GG + AA	5.365	1.79						
LDL-C/HDL-C	GA	3.13	1.24	−0.020	−0.216–0.176	0.839	-.0151	−0.329–0.028	0.099
	GG + AA	3.15	1.22						
TG/HDL-C	GA	4.29	2.99	0.019	−0.468–0.505	0.940	−0.226	−0.678–0.225	0.325
	GG + AA	4.28	3.13						

Note: Association between rs4864548 and adiposity-related metabolic traits was determined by linear regression using over-dominant genetic model. The *p* values before and after adjustment for age, gender and BMI are provided. SBP, systolic blood pressure; DBP, diastolic blood pressure; VAI, visceral adiposity index; LAP, lipid accumulation product; TyG, product of triglycerides and glucose; FBG, fasting blood glucose; HOMA–IR, homeostatic model assessment–insulin resistance; TC, total cholesterol; TG, triglyceride; HDL-C, high-density-lipoprotein cholesterol; LDL-C, low-density-lipoprotein cholesterol; VLDL-C, very-low density-lipoprotein cholesterol; CRI, Coronary Risk Index; LDL-C/HDL-C, LDL-C/HDL-C ratio; TG/HDL-C, triglyceride-to-HDL-C ratio; BMI, body mass index; SD, standard deviation; CI, confidence interval; GA, heterozygous genotype carrier; GG + AA, homozygous wild type/mutant genotype carrier.

Table 5. Association of the *CLOCK* variant rs6843722 with adiposity-related metabolic traits.

Parameters (unit of measurement)	Over-dominant model	Mean	SD	Unadjusted			Adjusted for age, gender, and BMI		
				β	95% CI	*p*	β	95% CI	*p*
SBP (mmHg)	AC	117.42	14.03	1.210	−1.018–3.439	0.286	−0.128	−2.150–1.894	0.901
	AA + CC	116.21	14.03						
DBP (mmHg)	AC	77.42	10.91	0.019	−1.219–1.965	0.646	−0.582	−2.072–0.909	0.444
	AA + CC	77.19	10.68						
VAI (mmol L^{-1})	AC	3.04	1.96	−0.001	−0.327–0.326	0.997	0.536	−0.460-.155	0.331
	AA + CC	3.04	2.14						
LAP (mmol L^{-1})	AC	53.47	38.81	7.010	.430–13.591	0.037	−0.547	−5.097–4.002	0.813
	AA + CC	46.46	44.01						
TyG index	AC	8.58	0.66	0.021	−0.075–0.117	0.671	−0.022	−0.115–0.069	0.624
	AA + CC	8.56	0.54						
FBG (mg dL^{-1})	AC	102.70	19.00	1.595	−1.583–4.773	0.325	0.892	−2.209–3.993	0573
	AA + CC	101.11	21.01						
Fasting insulin (μl U mL^{-1})	AC	23.38	13.58	1.218	−0.991–3.428	0.279	−0.397	−2.367–1.572	0.692
	AA + CC	22.16	14.24						
HOMA-IR	AC	6.022	4.03	0.377	−0.271–1.025	0.254	−0.099	−0.675–0.477	0.736
	AA + CC	5.64	4.13						
TC (mg dL^{-1})	AC	151.45	40.01	0.216	−6.067–6.499	0.946	−1.593	−7.795–4.608	0.614
	AA + CC	151.24	39.09						
TG (mg dL^{-1})	AC	121.99	66.04	4.747	−6.110–15.60	0.391	−0.809	−11.043–9.42	0.877
	AA + CC	117.24	70.71						
HDL-C (mg dL^{-1})	AC	30.68	8.84	0.724	-.746–2.194	0.334	0.895	−0.572–2.363	0.231
	AA + CC	29.96	9.66						
LDL-C (mg dL^{-1})	AC	92.47	36.53	2.110	−3.637–7.856	0.471	−0.922	−6.384–4.539	0.740
	AA + CC	90.36	35.81						
VLDL-C (mg dL^{-1})	AC	24.26	13.22	0.485	−1.662–2.632	0.657	−0.574	−2.602–1.454	0.579
	AA + CC	23.78	13.82						
CRI	AC	5.23	1.68	−0.112	−0.386–0.161	0.421	−0.221	0.484–0.042	0.100
	AA + CC	5.35	1.76						
LDL-C/HDL-C	AC	3.13	1.23	−0.015	−0.211–0.181	0.879	−0.139	−0.317–0.039	0.127
	AA + CC	3.15	1.23						
TG/HDL-C	AC	4.30	2.94	0.016	−0.469–0.502	0.947	−0.238	−0.687–0.211	0.299
	AA + CC	4.28	3.17						

Note: Association between rs6843722 and adiposity-related metabolic traits was determined by linear regression using over-dominant genetic model. The *p*-values before and after adjustment for age, gender and BMI are provided. SBP, systolic blood pressure; DBP, diastolic blood pressure; VAI, visceral adiposity index; LAP, lipid accumulation product; TyG, product of triglycerides and glucose; FBG, fasting blood glucose; HOMA–IR, homeostatic model assessment–insulin resistance; TC, total cholesterol; TG, triglyceride; HDL-C, high-density-lipoprotein cholesterol; LDL-C, low-density-lipoprotein cholesterol; VLDL-C, very-low density-lipoprotein cholesterol; CRI, Coronary Risk Index; LDL-C/HDL-C, LDL-C/HDL-C ratio; TG/HDL-C, triglyceride-to-HDL-C ratio; BMI, body mass index; SD, standard deviation; CI, confidence interval; AC, heterozygous genotype carrier; AA + CC, homozygous wild type/mutant genotype carrier.

Table 6. Association of the rs4864548 with behavioral characteristics.

Parameters	Category	Over-dominant model	Counts (%)	Unadjusted			Adjusted for age, gender, and BMI			
				OR	95% CI	*p*	OR	95% CI	*p*	*p* value corrected[a]
Eating behavior	Random	GG + AA	179 (49.3%)	0.977	0.708–1.350	0.889	0.937	0.672–1.306	0.700	0.865
		GA	184 (50.6%)							
	Specific	GG + AA	121 (48.5%)							
		GA	128 (51.4%)							
Diet consciousness	No	GG + AA	219 (50.2%)	0.849	0.597–1.206	0.360	0.825	0.577–1.179	0.291	0.865
		GA	217 (49.8%)							
	Yes	GG + AA	81 (46.0%)							
		GA	95 (54.0%)							
TFDF	High	GG + AA	82 (47.7%)	1.013	0.681–1.508	0.949	0.952	0.631–1.437	0.816	0.865
		GA	90 (52.3%)							
	Low	GG + AA	108 (48.0%)							
		GA	117 (52.0%)	0.889	0.611–1.293	0.539	0.907	0.615–1.337	0.621	0.865
	Moderate	GG + AA	110 (36.7%)							
		GA	105 (48.8 %)							
Physical activity	Low	GG + AA	88 (39.3%)	0.495	0.325–0.753	0.001	1.788	1.157–2.763	0.009	0.865
		GA	136 (60.7 %)							
	Moderate	GG + AA	127 (53.4%)	0.570	0.394–0.825	0.003	1.116	0.731–1.703	0.612	0.865
		GA	111 (46.6%)							
	High	GG + AA	85 (56.7%)							
		GA	65 (43.3%)							
Sleep-wake cycle	Irregular	GG + AA	150 (47.5%)	1.129	0.822–1.552	0.453	1.053	0.760–1.459	0.756	0.865
		GA	166 (52.5%)							
	Regular	GG + AA	150 (50.7%)							
		GA	146 (49.3%)							
Sleep duration	Insufficient[b]	GG + AA	167 (48.0%)	1.092	0.793–1.505	0.590	1.029	0.741–1.428	0.865	0.865
		GA	181 (52.0%)							
	Sufficient[c]	GG + AA	133 (50.4%)							
		GA	131 (49.6%)							

Note: Association between the rs4864548 and behavioral parameters was sought by logistic regression using over-dominant genetic model. The OR, CI, and *p* values before and after adjusting for age and gender are provided. The odds ratio along with 95% confidence intervals and *p* values were calculated to seek the impact and strength of the association, respectively. Benjamini-Hochberg method was used for the correction of multiple comparisons. $p < 0.05$ was considered statistically significant. Regular sleep timings were taken between 8:00 pm –12:00 and wake timings between 5:00–7.30 am TFDF, Tendency towards fat-dense food; OR, odds ratio; CI, confidence interval; GA, heterozygous genotype carrier; GG + AA, homozygous wild type/mutant genotype carrier.

[a]The *p*-values after correction for multiple comparisons.

[b]Less than 7 h sleep.

[c]7–9 h sleep.

Table 7. Association of the rs6843722 with behavioral characteristics.

				Unadjusted			Adjusted for age, gender, and BMI			
Parameters	Category	Over-dominant model	Counts (%)	OR	95% CI	*p* value	OR	95% CI	*p* value	*p* value corrected[a]
Eating behavior	Random	AA + CC	174 (49.8%)	1.095	0.793–1.512	0.582	1.062	0.74–1.477	0.493	0.907
		AC	189 (52.1%)							
	Specific	AA + CC	125 (50.2%)							
		AC	124 (49.8%)							
Diet consciousness	No	AC	220 (50.5%)	0.909	0.640–1.291	0.594	0.882	0.619–1.257	0.487	0.627
		AA + CC	216 (49.5%)							
	Yes	AA + CC	83 (47.2%)							
		AC	93 (52.8%)							
TFDF	High	AA + CC	82 (47.7%)	1.031	0.693–1.534	0.879	0.976	0.649–1.469	0.907	0.907
		AC	90 (52.3%)							
	Low	AA + CC	109 (48.4%)							
		AC	116 (51.6%)	0.931	0.641–1.353	0.708	0.969	0.659–1.424	0.872	0.907
	Moderate	AA + CC	108 (50.2%)							
		AC	107 (49.8)							
Physical activity	Low	AA + CC	93 (41.5%)	1.745	1.149–2.649	0.009	1.569	1.019–2.417	0.041	0.369
		AC	131 (58.5%)							
	Moderate	AA + CC	123 (51.7%)	1.158	0.769–1.746	0.483	1.148	0.754–1.747	0.520	0.907
		AC	115 (48.3%)							
	High	AA + CC	83 (55.3%)							
		AC	67 (44.7%)							
Sleep-wake cycle	Irregular	AA + CC	151 (47.8%)	1.093	0.796–1.501	0.584	1.021	0.739–1.412	0.898	0.907
		AC	165 (52.2%)							
	Regular	AA + CC	148 (50.0%)							
		AC	148 (50.0%)							
Sleep duration	Insufficient[b]	AA + CC	162 (46.6%)	1.140	0.828–1.570	0.421	1.086	0.785–1.504	0.618	0.907
		AC	186 (53.4%)							
	Sufficient[c]	AA + CC	137 (51.9%)							
		AC	127 (48.1%)							

Note: Association between the rs6843722 and behavioral parameters was assessed by logistic regression using over-dominant genetic model. The OR, CI, and *p* values before and after adjusting for age and gender are provided. The odds ratio along with 95% confidence intervals and *p* values were calculated to seek the impact and strength of the association, respectively. Benjamini-Hochberg method was used for the correction of multiple comparisons. $p < 0.05$ was considered statistically significant.Regular sleep timings were taken between 8:00 pm –12:00 and wake timings between 5:00–7.30 am. TFDF, Tendency towards fat-dense food; OR, odds ratio; CI, confidence interval; AC, heterozygous genotype carrier; AA + CC, homozygous wild type/mutant genotype carrier.

[a]The *p* values after correction for multiple comparisons.

[b]Less than 7 h sleep.

[c]7–9 h sleep.

lack of association between rs4864548 and overweight/obesity was reported in the Japanese population (Uemura et al. 2016). Moreover, Krishnan et al. (2017b) observed a lack of association of the rs4864548 with BMI and %BF in New Zealand children (Krishnan et al. 2017b). The effects of genetic variants that enhance the predisposition of an individual to a phenotype may differ from population to population; therefore, the association of the genetic variants with the study outcomes among different populations may or may not appear. The differences in the results of different genetic association studies are also attributable to differences in study design, the sample size of the study, age and gender of the study participants, and adjustment of confounders in the association tests. It is important to note that we sought the association of the variants assuming multiple genetic models and computed the *h*-index to find the best-fit model of inheritance for each variant. In contrast, none of the other studies mentioned calculated the *h*-index to indicate the appropriate mode of inheritance. To the best of our knowledge, the associations of rs6843722 and rs4864548 with HC, WHR, WHtR, and SFTs were sought for the first time in this study. The WC and WHtR are considered as the useful predictor of abdominal obesity and cardiovascular health (Zhu et al. 2002; Hsieh et al. 2003; Shen et al. 2017). Thus, the current study highlights the possible role of *CLOCK* gene variants for the increased risk of abdominal obesity and cardiovascular disease along with general obesity via increasing WC and WHtR. Additionally, the association of the rs6843722 and rs4864548 with different skinfold thicknesses and %BF signified that both the variants might be involved in fat deposition throughout the body.

According to the results reported in our study, lack of association of the rs4864548 with HDL-C, total cholesterol, HOMA-IR, and SBP was observed in the White European population (Scott et al. 2008). Furthermore, Sookoian et al. (2008) observed no significant association of rs6843722 and rs4864548 with any metabolic parameters including total cholesterol, LDL-C, HDL-C, triglycerides, fasting blood glucose, fasting insulin, and HOMA IR in Europeans. This observation shows that rs4864548 and rs6843722 contribute to the developing susceptibility of our population to overweight/obesity but do not directly influence the metabolic parameters. We could not find any related study in the literature that explored the association of the aforementioned variants with other metabolic parameters such as TyG, DBP, VLDL-C, TG/HDL-C, LDL-C/HDL-C, CRI, VAI, and LAP.

A significant relationship has been demonstrated between the obesity risk and various behavioral factors related to eating and sleeping habits as well as physical inactivity (Taheri et al. 2004; Pietiläinen et al. 2008; Sayon-Orea et al. 2013; Wang et al. 2014a; Wang et al. 2014b; Murakami and Livingstone 2015; Aparicio et al. 2017). We aimed to evaluate whether the genetic variations in the *CLOCK* gene influence the obesogenic behavioral traits including the random eating pattern, diet unconsciousness, the tendency towards energy-rich foods, lack of physical activity, inadequate sleep duration, and irregular sleep–wake cycle. In our study, no association of the variants (rs4864548 and rs6843722) was established with any of the aforementioned behavioral parameters. In line with the results reported in our study, lack of association of the rs4864548 with low physical activity and inadequate sleep duration was observed in New Zealand children (Krishnan et al. 2017a; Krishnan et al. 2018). Moreover, a lack of association between the rs6843722 and sleep duration was observed in the people of European ancestry (Lane et al. 2013). However, Garaulet et al. (2010b) reported the significant association of the rs4864548 variant with the total energy intake in European individuals. The lack of association of the *CLOCK* variants (rs4864548 and rs6843722) with the obesogenic behaviors might be due to self-reporting (under-reporting) bias of the overweight/obese participants since the behavior-related information was collected by a questionnaire. Moreover, behavioral traits such as eating habits may differ among individuals across different populations; Garaulet et al. (2010b) reported a significant association between the rs4864548 and energy intake is from Europeans. We could not find any related study like ours that investigated the associations of

rs4864548 and rs6843722 with other obesogenic behavioral traits, such as the random eating pattern, diet unconsciousness, moderate-to-high TFDF, and irregular sleep–wake cycle.

Conclusions

In conclusion, we report the association of two *CLOCK* variants (rs6843722 and rs4864548) with obesity and adiposity-related anthropometric phenotypes including body weight, WC, HC, WHR, WHtR, SFTs, and %BF in the Pakistani population. There was a lack of association of the *CLOCK* variants with obesity-related metabolic and behavioral traits.

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Author contributions

SR conceived and designed the study. NF and AAB performed the experiments/collected the data. NF and AAB analyzed and interpreted the data. SR contributed resources. SR, NF, and AAB drafted or revised the manuscript.

Competing interest statement

Authors declare that they have no conflict of interest.

Data availability statement

All data generated or analyzed during this study are included in this published article.

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