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Original Article

Abnormal circadian rhythms are associated with plaque instability in acute coronary syndrome patients

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Abstract: Aim: Acute coronary syndrome (ACS), a leading cause of morbidity and mortality worldwide, is among the most serious cardiovascular diseases. Circadian rhythms are present in almost all organisms. In clinical practice, we have found that ACS is closely related to these circadian rhythms. However, the relationship between circadian rhythms and plaque instability in ACS patients is incompletely understood. The aim of this study is to provide new insights into the relationship between circadian rhythms and plaque instability in ACS patients. Methods: We enrolled patients with ACS and individuals with normal coronary artery function in this study. The Athens Insomnia Scale (AIS), Pittsburgh Sleep Quality Index (PSQI), International Physical Activity Questionnaire (IPAQ) and Healthy Diet Score (HDS) were used to evaluate circadian rhythms. Furthermore, quantitative real-time polymerase chain reaction (qRT-PCR) was used to assess the mRNA expression levels of muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (Bmal1), circadian locomotor output cycles kaput (Clock), Cryptochrome1 (Cry1), Period2 (Per2), nuclear receptor subfamily 1, group D, member 1 (Rev-erbα), and matrix metalloproteinases MMP2 and MMP9. Results: AIS scores and PSQI scores were significantly higher in patients with ST segment elevation myocardial infarction (STEMI), non-ST segment elevation myocardial infarction (NSTEMI), and unstable angina pectoris (UA) than in the normal controls (NCs) ($P < 0.05$). The IPAQ scores of the NCs and patients with UA were significantly higher than in patients with STEMI and NSTEMI ($P < 0.05$). Notably higher HDS scores were recorded for the NCs compared to those of patients with UA, NSTEMI, and STEMI ($P < 0.05$). Consistent with these findings, compared with the NCs, the lowest levels of Bmal1, Clock, Cry1, Per2 and Rev-erbα mRNAs were detected in patients with STEMI, followed by patients with NSTEMI and then patients with UA ($P < 0.05$). Furthermore, the levels of MMP2 and MMP9 mRNA were significantly higher in the patients with STEMI, NSTEMI, and UA than those in the NCs ($P < 0.05$). In addition, we found that the levels of MMP mRNA negatively correlated with the levels of clock genes mRNAs ($P < 0.05$, respectively). Conclusions: Based on our data, the circadian rhythms and clock genes are correlatively with the occurrence of ACS, and the expression levels of clock genes are negatively correlated with plaque stability in ACS patients.

Keywords: Acute coronary syndrome (ACS), circadian rhythms, plaque stability

Introduction

Acute coronary syndrome (ACS) is a serious clinical syndrome that includes unstable angina pectoris (UA), ST segment elevation myocardial infarction (STEMI), and non-ST segment elevation myocardial infarction (NSTEMI). It is caused by partial or complete occlusion of the coronary arteries due to sudden rupture of atherosclerotic plaques and subsequent coronary thrombosis [1]. Myocardial infarct and unstable angina tend to occur between the early morning and noon [2]. Epidemiological research has consistently shown the circadian rhythmicity of the time of ACS onset [2]. Therefore, we speculated that this phenomenon may be related to circadian rhythms; however, the mechanism underlying the phenomenon remains unclear.

All organisms, including bacteria, plants and mammals, have intrinsic 24-h rhythms that adapt to changes in the surrounding environment. In humans, the central pacemaker of the circadian rhythm is the suprachiasmatic nucle-

us (SCN) of the hypothalamus, which orchestrates the 24-h cycles present in most cells of the body that are of particular relevance to behavioral and physiological functions, including sleep/wake cycles, feeding behavior, and rhythmic activity [3]. The core genes of the circadian clock include Clock and Bmal1, and the regulatory genes include Crys, Pers and Reverbs, which play important roles in regulating physiologic activities. Many animal experiments and clinical studies have shown that abnormal circadian rhythms may lead to atherosclerosis [4], obesity [5], diabetes mellitus [6], hyperlipidemia [7], endothelial cell dysfunction [8] and abnormal immune response [9]. These conditions are closely related to the occurrence of ACS.

The aim of our study was to investigate interactions between abnormal circadian rhythms and plaque instability in acute coronary syndrome patients. We hypothesized that abnormal circadian rhythms and clock genes contribute to the development of ACS.

Materials and methods

Patient population

This study analyzed the relationship between abnormal circadian rhythms and ACS. In our study, 218 patients were recruited from the Central People's Hospital in Yichang, Hubei Province, China. Their ages ranged from 29 to 85 years (mean age: 60.06 ± 11.15 years). Subjects were provided with detailed oral and written information about the research project and provided written consent prior to participation. The Ethics Committee of the Central People's Hospital of Yichang approved the study. Patients were classified into four groups: group 1: normal control group (NC); group 2: UA group; group 3: acute STEMI group; and group 4: acute NSTEMI group. The diagnostic criteria were derived from the American Heart Association (AHA), the American College of Cardiology (ACC), and European Society of Cardiology (ESC) [10, 11].

Inclusion and exclusion criteria

NC group: patients were diagnosed by coronary angiography, and no vascular diseases were observed.

UA group: 1) negative serum troponin level; 2) clinical manifestation of chest pain; and 3) the electrocardiogram (ECG) showed transient ST segment depression and/or a flat or inverted T-wave flat and rare ST segment elevation.

Acute STEMI group: 1) typical ischemic clinical manifestation of chest pain lasting ≥ 20 min; 2) ST segment elevation (≥ 1 mm) in ≥ 1 electrocardiogram leads; 3) a cardiac troponin (T or I), or a creatine kinase-myoglobin (CKMB) level greater than the institutional upper limit of normal; and 4) a coronary angiography revealed stenosis in the coronary artery.

Acute NSTEMI group: 1) typical clinical manifestation of chest pain during the 24 h prior to receiving medical treatment; 2) changes observed on ECG indicative of myocardial ischemia: ST segment depression (≥ 1 mm) and/or T-wave inversion; 3) a cardiac troponin (T or I) or a CKMB level greater than the institutional upper limit of normal; and 4) coronary angiography revealed stenosis in the coronary artery.

The exclusion criteria were as follows: 1) cardiovascular events for < 1 year, such as a stroke or myocardial infarction; 2) low-density lipoprotein (LDL)-cholesterol level ≥ 4.3 mmol/L; 3) presence of malignant diseases; 4) renal failure; 5) liver diseases; 6) various chronic and acute infections; 7) connective tissue diseases; 8) received treatment with anti-inflammatory drugs and/or immunosuppressive agents; 9) pregnant or lactating patients; and 10) subjects who were participating in another clinical trial or refused to sign the informed consent form.

Circadian rhythm assessments

The circadian rhythms of humans mainly manifest as sleep/wake cycles, feeding behavior and rhythmic activity. Therefore, we evaluated circadian rhythms among the enrolled patients in terms of sleep, diet and exercise. The Athens Insomnia Scale (AIS) shown in Table S1 in the Supplementary Appendix and the Pittsburgh Sleep Quality Index (PSQI) shown in Table S2 in the Supplementary Appendix were used to assess sleep quality during the last month. The International Physical Activity Questionnaire (IPAQ) shown in Table S3 in the Supplementary Appendix was used to assess the amount of physical activity in the last 7 days, and the sum

of the MET (metabolic equivalent of task) min/week was calculated for each participant. In the IPAQ, 3.3 METs correspond to 1 minute of walking, 4.0 METs correspond to 1 minute of moderate activity and 8.0 METs correspond to 1 minute of vigorous activity. The Healthy Diet Score (HDS) shown in Table S4 in the Supplementary Appendix was used to assess daily energy intake during the last month.

Blood sample collection

We collected 8-10 mL of peripheral blood from all participants after an overnight fast. Blood samples were treated with ethylene diamine tetraacetic acid (EDTA) and examined within 4 h. The anti-coagulated blood was used for quantitative real-time polymerase chain reaction (qRT-PCR).

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from 5 mL of ethylene diamine tetraacetic acid-treated venous blood samples by Ficoll-Hypaque gradient centrifugation (1,600 rpm at room temperature for 19 min). After the centrifugation, the cells from the interface were collected and washed twice in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Auckland, NZ, USA).

qRT-PCR

Levels of the Bmal1, Clock, Per2, Cry1, Rev-erbα, Mmp2 and Mmp9 mRNAs were determined by qRT-PCR. Total RNA was extracted from peripheral blood mononuclear cells (PBMCs) using the RNAsimple Total RNA Kit (Lot DP419, TIANGEN Biotech, China) and reverse transcribed into cDNAs using the Revert Aid First Strand cDNA Synthesis Kit (Lot 00422877, Thermo Scientific, USA), according to the manufacturer's instructions. Levels of the Bmal1, Clock, Per2, Cry1, Rev-erbα, Mmp2 and Mmp9 mRNAs were quantified using the SYBRP® Premix Ex Taq™ II (Lot RR820A, Takara, Japan) on an Agilent Strata Gene Mx3005P system (Agilent, American); Gapdh expression served as a control. Amplification was performed in a 20-μL reaction for 40 cycles of 15 s at 95°C and 30 s at 60°C after an initial denaturation step (72°C, 30 s). The following primer sequences were used: GAPDH forward: AGGTCCACCACTGACACGTT; GAPDH reverse: GCCTCAAGATCATCAGCAAT; Bmal1 forward: GAGCAGCTCTCCTCCTCTGA; Bmal1 reverse: CGTCGTGCTCCAGAACATAA; Clock forward: AGAGCACCTTCCCTCAGTCA; Clock reverse: GCACGTGTGCTACTGTGGTT; Per2 forward: ACTCAGCGAAGTGTCGGACAC; Per2 reverse: TTCGATCCTGTGATTCAAGGG; Cry1 forward: AGTTGCTCTCAAGGGAGTGG; Cry1 reverse: GACTAGGACGTTTCCCACCA; Rev-erbα forward: CTGGGAGGATTTCTCCATGA; Rev-erbα reverse: GCCTTAAGCAGGGTGACTTG; MMP2 forward: ACCTGAAGCTGGAGAACCAA; MMP2 reverse: TATCGAAGGCAGTGGAGAGG; MMP9 forward: CGACGTCTTCCAGTACCGAG; and MMP9 reverse: TTGTATCCGGCAAACTGGCT. Samples were analyzed in triplicate. The relative expression levels of mRNAs were determined using the $2^{-\Delta\Delta t}$ method.

Blood biochemistry measurements

Levels of total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), fasting plasma glucose (FPG), serum creatinine, C-reactive protein (CRP), alanine aminotransferase (ALT), aspartate transaminase (AST), creatine kinase (CK), CKMB, lactate dehydrogenase (LDH), and α-hydroxybutyrate dehydrogenase (α-HBDH) were measured using enzymatic methods in the clinical chemistry laboratory of the Central People's Hospital in Yichang.

Statistical analysis

Data analyses were performed using the SPSS statistical software (version 18.0). Data for continuous variables are presented as means ± standard deviations (SD). Group comparisons were performed using a one-way ANOVA followed by Tukey's test. Associations were evaluated using Pearson's correlation coefficient. For discrete variables, differences were expressed as counts and percentages and were analyzed with a χ^2 test (or Fisher's exact test). A two-tailed $P < 0.05$ was considered significant.

Results

Participants' characteristics

The clinical characteristics of the participants included in this study are presented in **Table 1**. Two hundred eighteen participants completed the study: 34 participants in the normal control

Table 1. Clinical characteristics of the four groups

Characteristics	NC (n = 34)	UA (n = 67)	STEMI (n = 89)	NSTEMI (n = 28)
Age, mean ± SD years	54.12 ± 14.32	61.12 ± 9.44	60.42 ± 10.31	63.57 ± 11.08
BMI (Kg/m^2)	24.25 ± 7.78	24.17 ± 3.02	24.10 ± 2.68	24.38
FPG (mmol/L)	5.21 ± 1.24	5.25 ± 1.50	6.62 ± 2.12[a,b]	6.77 ± 2.20[a,b]
Current Smoker, n (%)	11 (32.4)	26 (38.3)	41 (46.1)	10 (35.7)
Current drinker, n (%)	8 (23.5)	19 (28.4)	30 (33.7)	7 (25.0)
Diabetes Mellitus, n (%)	4 (11.8)	11 (16.4)	21 (23.6)	6 (21.4)
Hyperlipidemia, n (%)	5 (14.7)	7 (10.4)	10 (11.2)	3 (10.7)
Hypertension, n (%)	6 (17.6)	10 (14.9)	18 (20.2)	5 (17.9)
CRP (mg/L)	2.91 ± 1.48	5.73 ± 5.05	17.34 ± 13.52[a,b]	16.62 ± 14.93[a,b]
ALT (U/L)	25.61 ± 12.27	25.95 ± 19.33	51.20 ± 38.04[a,b]	30.25 ± 14.65[c]
AST (U/L)	23.94 ± 6.79	25.68 ± 11.33	210.88 ± 162.87[a,b]	92.71 ± 86.83[a,b,c]
SCR (umol/L)	65.22 ± 14.70	71.27 ± 21.74	72.46 ± 25.12	74.63 ± 14.99
TC (mmol/L)	4.47 ± 0.97	4.25 ± 0.79	4.38 ± 1.07	4.29 ± 1.35
TG (mmol/L)	1.68 ± 0.74	1.52 ± 1.11	1.34 ± 0.62	1.47 ± 0.72
HDL (mmol/L)	1.40 ± 0.30	1.36 ± 0.37	1.25 ± 0.28	1.15 ± 0.29[a,b]
LDL (mmol/L)	2.65 ± 0.79	2.43 ± 0.68	2.65 ± 0.91	2.60 ± 0.81
CK (IU/L)	101.07 ± 55.43	116.00 ± 102.45	1914.50 ± 2212.71[a,b]	836.00 ± 875.39[c]
CKMB (IU/L)	14.77 ± 13.02	15.13 ± 9.02	166.51 ± 183.81[a,b]	80.89 ± 82.47[c]
LDH (IU/L)	217.97 ± 52.03	231.58 ± 65.57	676.45 ± 469.05[a,b]	382.04 ± 171.80[c]
α-HBDH (IU/L)	157.17 ± 30.17	166.93 ± 49.54	585.39 ± 445.09[a,b]	306.56 ± 157.73[c]

Data are presented as mean ± SD or number (percentage). NC: normal control group; UA: unstable angina pectoris group; STEMI: acute ST segment elevation myocardial infarction group; NSTEMI: acute non-ST segment elevation myocardial infarction group. SCR: serum creatinine; TC: total cholesterol; TG: triglyceride; HDL-C: high-density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; CRP: C-reactive protein; ALT: Alanine aminotransferase; AST: Aspartate transaminase; CK: Creatinekinase; CKMB: Creatine kinase-MB; LDH: lactate dehydrogenase; α-HBDH: α-hydroxybutyrate dehydrogenase. a, P < 0.05 vs. NC. b, P < 0.05 vs. UA. c, P < 0.05 vs. STEMI.

group, 67 participants in the UA group, 89 participants in the acute STEMI group and 28 participants in the acute NSTEMI group. None of the following parameters differed among the four groups: age, BMI, smoking, drinking, diabetes mellitus, hyperlipidemia, hypertension, serum creatinine, and lipid profiles, including LDL-c, TG, and TC levels. Compared to NCs and patients with UA, patients with STEMI and NSTEMI were more likely to display significantly higher FPG, CRP, ALT and AST levels ($P < 0.05$), but the levels of these measures were not significantly different between the NCs and the patients with UA ($P > 0.05$) or between the patients with STEMI and NSTEMI ($P > 0.05$). Markedly higher levels of myocardial enzymes (CK, CKMB, LDH and α-HBDH) were observed in the STEMI group than those in the NC, NSTEMI and UA groups ($P < 0.05$). Compared to the NC and UA groups, higher levels of myocardial enzymes were detected in the NSTEMI group, but the difference was not significant ($P > 0.05$).

Findings from angiography and percutaneous coronary interventions

All participants underwent coronary angiography. The findings from angiography and percutaneous coronary interventions are summarized in **Table 2**. Compared with the NCs, the most severe coronary lesions were observed in the patients with STEMI ($P < 0.05$), followed by the patients with NSTEMI ($P < 0.05$); the lowest lesion burden was observed in the patients with UA ($P < 0.05$). **Figure 1A** is an example of normal coronary angiography in NC group patients. **Figure 1B** is an example of abnormal coronary angiography in UA group patients. **Figure 1C** is an example of abnormal coronary angiography in acute STEMI group patients. **Figure 1D** is an example of abnormal coronary angiography in Acute NSTEMI group patients.

Circadian rhythm assessments

All the participants completed the AIS, PSQI, IPAQ, and HDC to assess circadian rhythms. As

Table 2. Results of coronary angiography in four groups

	NC (n = 34)	UA (n = 67)	STEMI (n = 89)	NSTEMI (n = 28)	χ^2	*P*
No vessel disease, n (%)	34 (100.0)	13 (19.4)	2 (2.7)	1 (4.5)	162.58	< 0.001
1 vessel disease, n (%)		30 (43.1)	19 (21.6)	11 (40.9)		
2 vessel disease, n (%)		8 (12.5)	29 (32.4)	8 (27.3)		
3 vessel disease, n (%)		16 (25.0)	39 (43.3)	8 (27.3)		

Data given as n (%), mean ± SD. NC: normal control group; UA: unstable angina pectoris group; STEMI: acute ST segment elevation myocardial infarction group; NSTEMI: acute non-ST segment elevation myocardial infarction group.

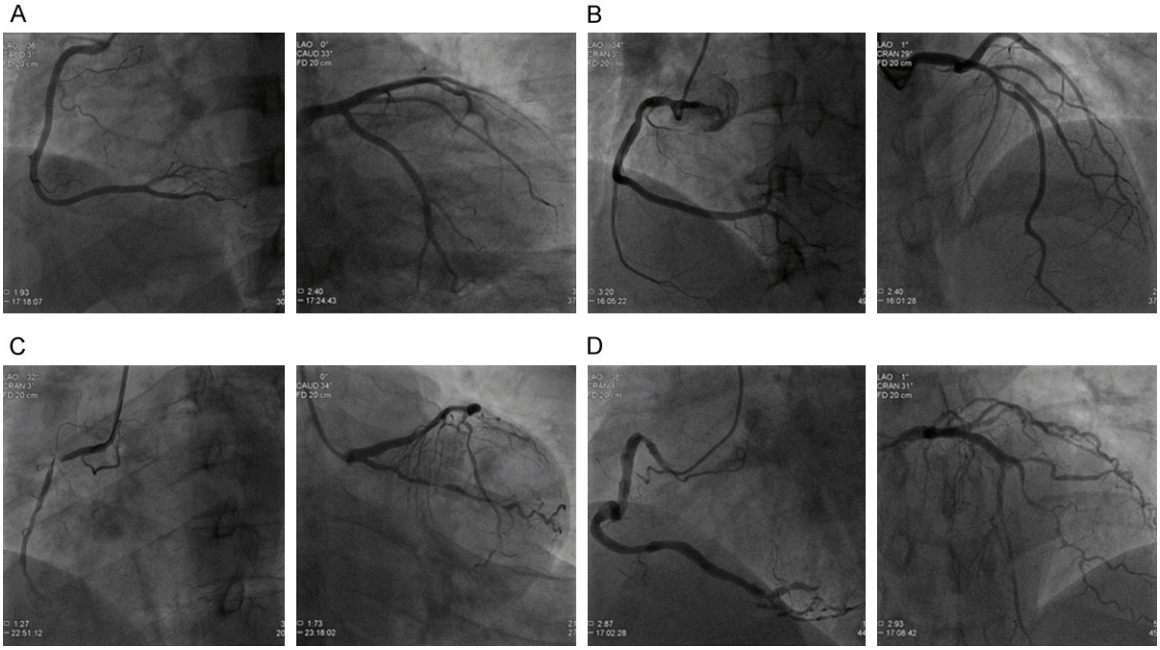

Figure 1. Examples of results of coronary angiography in four groups of patients. A. No obvious stenosis by the coronary arteriography in an NC group patient. B. 80% stenosis in the proximal segment of LAD and 30% stenosis in the proximal segment of RCA in a UA group patient. C. 90% stenosis in the mid LAD, 95% stenosis in LCA, and 99% stenosis in RCA in an acute STEMI group patient. D. 85% stenosis in the mid of LAD, 90% stenosis in the mid of LCA and 80% stenosis in the proximal segment of RCA in an acute NSTEMI group patient.

shown in the results of the sleep quality evaluation presented in **Table 3**, significantly higher AIS scores were recorded for the patients with STEMI (9.02 ± 4.27), NSTEMI (8.61 ± 5.03), and UA (5.60 ± 4.30) compared to those of the NCs (2.24 ± 3.64) (*P < 0.05*). Similarly, the highest PSQI scores were observed for the STEMI group (9.85 ± 4.53) followed by the NSTEMI (8.82 ± 4.97), UA (7.60 ± 4.55), and NC (4.41 ± 3.55) groups, respectively (*P < 0.05*). Regarding the results of activity evaluation, the IPAQ scores of the NC and UA groups were significantly higher than those in the STEMI and NSTEMI groups (*P < 0.05*). However, the differences between the STEMI and NSTEMI groups were not statistically significant (*P > 0.05*). Based on the results of the daily energy intake evaluation, notably higher HDS scores were recorded for the NCs than for the patients with UA, NSTEMI, and STEMI (NC, 39.41 ± 4.68; UA, 36.51 ± 3.84; NSTEMI, 34.63 ± 4.97; STEMI, 31.58 ± 3.88; *P < 0.05*).

Levels of the clock gene mRNAs in PBMCs

The core of the molecular clock is the heterodimeric transcription factor heterodimer Clock/Bmal1. The genes regulating the molecular clock include Crys, Pers and Rev-erbs. In this study, we measured the levels of the Bmal1, Clock, Cry1, Per2, and Rev-erbα mRNAs in PBMCs from all participants. As shown in **Figure 2**, compared with NCs, the lowest levels of all clock gene mRNAs were observed in the

Table 3. Assessments of circadian rhythm in four groups

	NC (n = 34)	UA (n = 67)	STEMI (n = 89)	NSTEMI (n = 28)
Score of AIS	2.24 ± 3.64	5.60 ± 4.30[a]	9.02 ± 4.27[a,b]	8.61 ± 5.03[a,b]
Score of PSQI	4.41 ± 3.55	7.60 ± 4.55[a]	9.85 ± 4.53[a,b]	8.82 ± 4.97[a,b]
Score of IPAQ	4018.19 ± 2046.80	3335.50 ± 2005.76	1912.55 ± 1769.62[a,b]	2096.67 ± 1751.25[a,b]
Healthy diet score	39.41 ± 4.68	36.51 ± 3.84[a]	31.58 ± 3.88[a,b]	34.63 ± 4.97[a,b,c]

Data are presented as mean ± SD. AIS: Athens Insomnia Scale; PSQI: Pittsburgh sleep quality index; IPAQ: International physical activity questionnaire. NC: normal control group; UA: unstable angina pectoris group; STEMI: acute ST segment elevation myocardial infarction group; NSTEMI: acute non-ST segment elevation myocardial infarction group. a, P < 0.05 vs. NC. b, P < 0.05 vs. UA. c, P < 0.05 vs. STEMI.

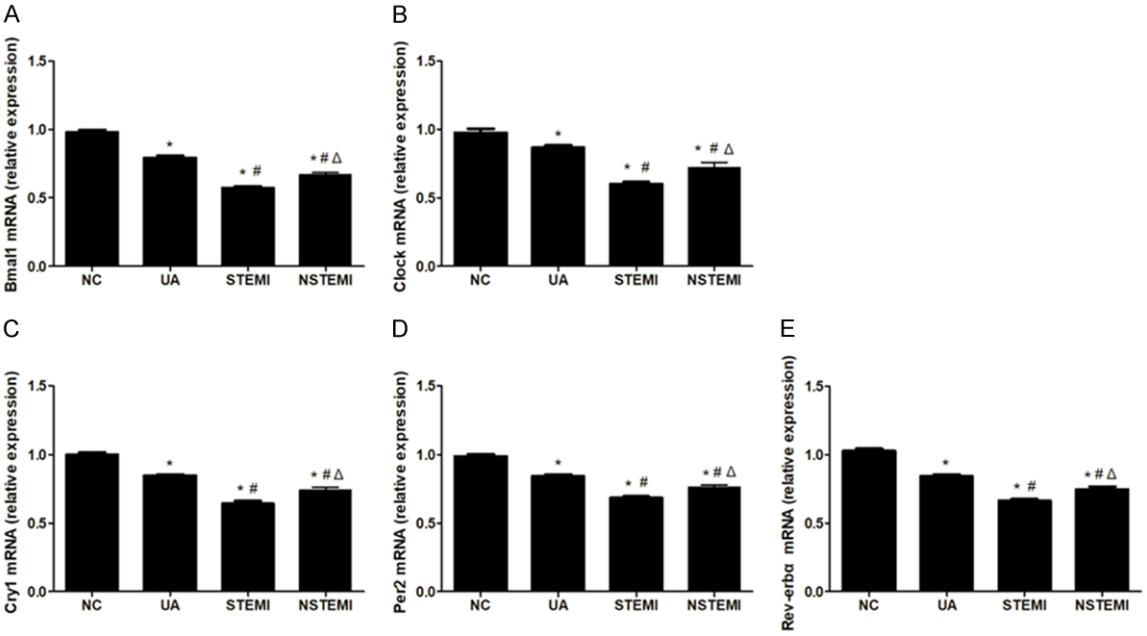

Figure 2. The expressions of Bmal1, Clock, Cry1, Per2 and Rev-erbα mRNA in PBMC were identified by real-time PCR. NC: normal control group; UA: unstable angina pectoris group; STEMI: acute ST segment elevation myocardial infarction group; NSTEMI: acute non-ST segment elevation myocardial infarction group. A. The relative expression of Bmal1 mRNA in four groups; B. The relative expression of Clock mRNA in four groups; C. The relative expression of Cry1 mRNA in four groups; D. The relative expression of Per2 mRNA in four groups; E. The relative expression of Rev-erbα mRNA in four groups. Data are shown as mean ± SEM. *P < 0.05 vs. NC. #P < 0.05 vs. UA. ΔP < 0.05 vs. STEMI.

patients with STEMI (*P < 0.05*), followed by the patients with NSTEMI (*P < 0.05*) and finally the patients with UA (*P < 0.05*).

Expression of Mmp mRNAs in PBMCs

In our study, we measured the levels of MMP2 and MMP9 mRNAs in PBMCs from all participants. As shown in **Figure 3**, statistically significant differences in MMP2 and MMP9 mRNA expression levels were observed among the four groups. Significantly increased mRNA levels of MMP2 and MMP9 were detected in the STEMI, NSTEMI and UA groups compared with those in the NC group (*P < 0.05*). Patients with STEMI and NSTEMI displayed significantly higher mRNA levels of MMP2 and MMP9 compared to the patients with UA (*P < 0.05*), but compared with the patients with NSTEMI, the expression levels of MMP2 and MMP9 mRNAs were higher in the patients with STEMI (*P < 0.05*).

Correlation between clock genes and MMPs

As shown in **Figure 4**, the level of MMP2 mRNA showed negative correlation with the levels of the Bmal1, Clock, Cry1, Per2 and Rev-erbα mRNAs among STEMI, NSTEMI, and UA groups (r = -0.7282, *P* < 0.0001; r = -0.6430, *P* <

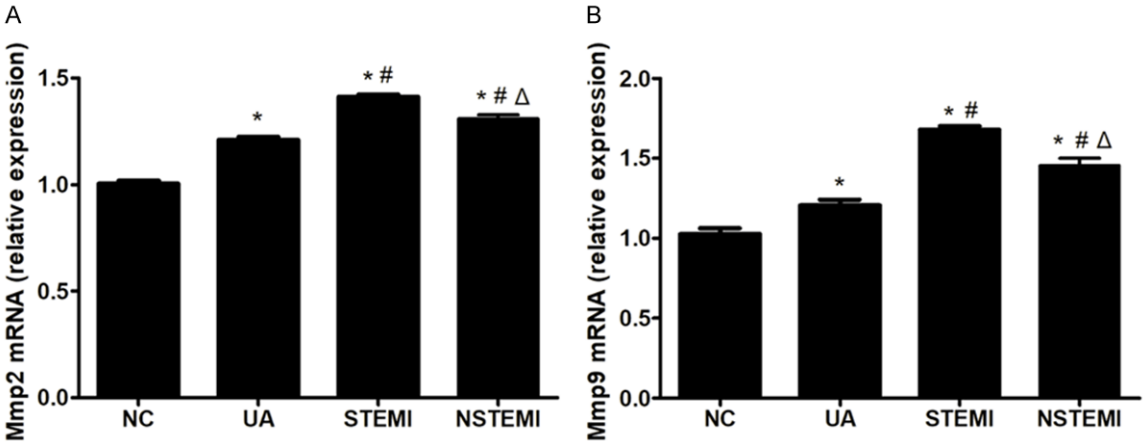

Figure 3. The expressions of MMP2 and MMP9 mRNA in PBMC were identified by real-time PCR. NC: normal control group; UA: unstable angina pectoris group; STEMI: acute ST segment elevation myocardial infarction group; NSTEMI: acute non-ST segment elevation myocardial infarction group. The expressions of MMP2 and MMP9 mRNA in PBMC were identified by real-time PCR. A. The relative expression of MMP2 mRNA in four groups; B. The relative expression of MMP9 mRNA in four groups. Data are shown as mean ± SEM. *P < 0.05 vs. NC. #P < 0.05 vs. UA. ΔP < 0.05 vs. STEMI.

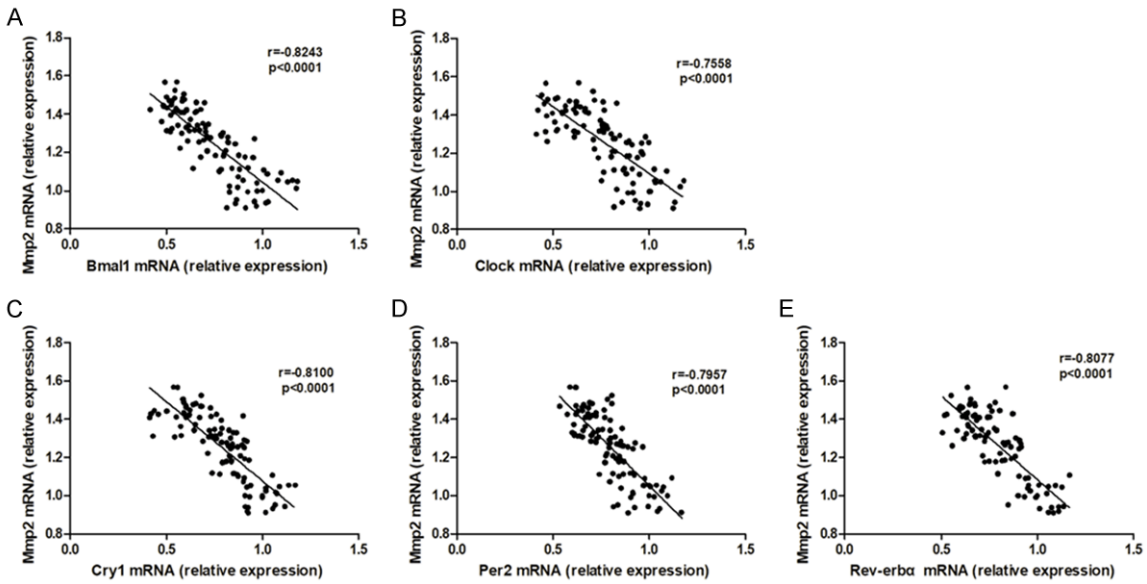

Figure 4. The correlations between the expressions of clock genes and the expression of MMP2. A. The correlation between the expression of Bmal1 and the expression of MMP2. B. The correlation between the expression of Clock and the expression of MMP2. C. The correlation between the expression of Cry1 and the expression of MMP2. D. The correlation between the expression of Per2 and the expression of MMP2. E. The correlation between the expression of Rev-erbα and the expression of MMP2. Pearson's correlation coefficient (normal distributed data) was used to assess interrelationships; P < 0.01 is considered statistically significant. (r, correlation coefficient; *P* values are shown).

0.0001; r = -0.6607, *P* < 0.0001; r = -0.6379, *P* < 0.0001; r = -0.5090, *P* < 0.0001; respectively). Consistently, as shown in **Figure 5**, the level of MMP9 mRNA showed negative correlation with the levels of the Bmal1, Clock, Cry1, Per2, and Rev-erbα mRNAs among STEMI, NSTEMI and UA groups (r = -0.7507, *P* < 0.0001; r = -0.6732, *P* < 0.0001; r = -0.6640, *P* < 0.0001; r = -0.6972, *P* < 0.0001; r = -0.7166, *P* < 0.0001; respectively).

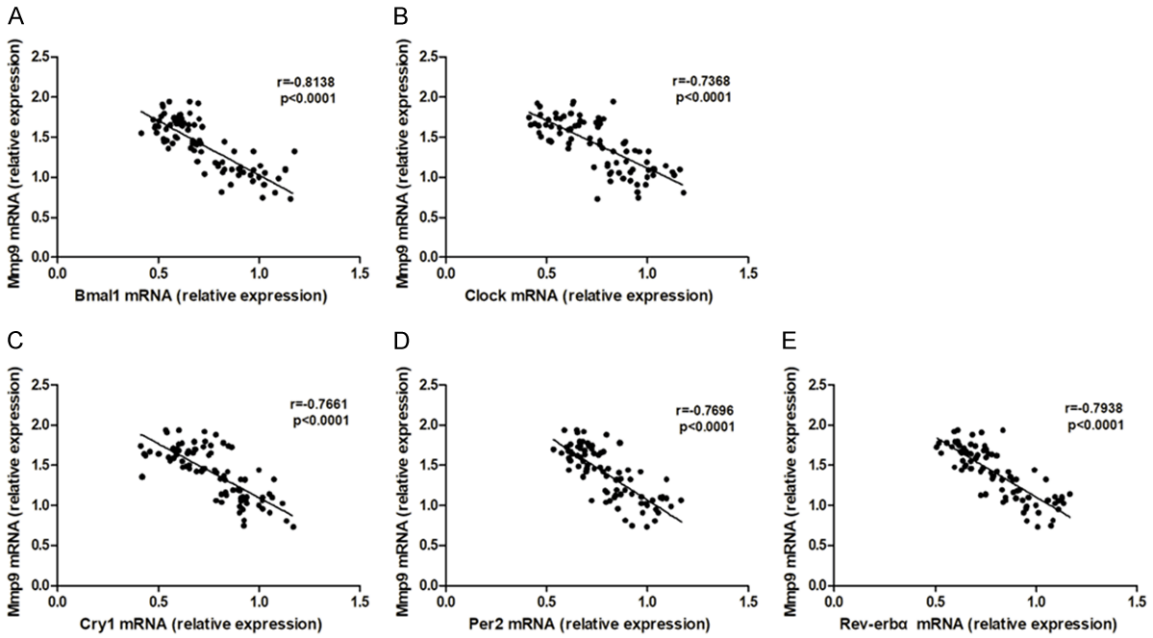

Figure 5. The correlations between the expressions of clock genes and the expression of MMP9. A. The correlation between the expression of Bmal1 and the expression of MMP9. B. The correlation between the expression of Clock and the expression of MMP9. C. The correlation between the expression of Cry1 and the expression of MMP9. D. The correlation between the expression of Per2 and the expression of MMP9. E. The correlation between the expression of Rev-erbα and the expression of MMP9. Pearson's correlation coefficient (normal distributed data) was used to assess interrelationships; $P < 0.01$ is considered statistically significant. (r, correlation coefficient; *P* values are shown).

Discussion

To the best of our knowledge, this study is the first to investigate the relationship between abnormal circadian rhythms and ACS, including UA, STEMI and NSTEMI. Our data indicated that abnormal circadian rhythms could promote the occurrence of ACS. In addition, patients with ACS exhibited significantly decreased levels of Bmal1, Clock, Cry1, Per2 and Rev-erbα mRNAs in PBMCs. Furthermore, patients with ACS also showed notably increased levels of MMP2 and MMP9 mRNAs compared to NCs. Therefore, the levels of clock genes were negatively correlated with the severity of coronary artery lesions. In contrast, the levels of MMP mRNAs were positively correlated with the severity of plaque instability. Based on these results, the expression levels of Bmal1, Clock, Cry1, Per2 and Rev-erbα may diminish the process of atherosclerosis and correlate with the severity of atherosclerosis. More importantly, the levels of MMP mRNAs negatively correlated with the levels of clock genes mRNAs. These results suggest that the clock genes may diminish the plaque instability of plaque by disturbing the levels of MMPs. These findings may contribute to improving our understanding of and ability to monitor ACS, simultaneously suggesting possible therapeutic methods to prevent the occurrence of ACS.

Circadian rhythms are present in most cells of the body and play important roles in biological processes. Disrupted circadian rhythms can lead to the development of cardiovascular diseases [4, 12], tumors [13], immune system diseases [14] and hematological diseases [15], among other conditions. ACS is mainly caused by abluminal remodeling, plaque erosion or thrombus formation upon rupture. Dyssomnia, changes in physical activity and disordered dietary habits can disrupt normal circadian rhythms and the expression levels of clock genes. Knutsson et al. found that insomniacs and shift workers had a higher risk of acute myocardial infarction [16]. In addition, disrupted circadian rhythms were a novel risk factor for insulin resistance and Type 2 diabetes [17, 18] and increased the levels of circulating inflammatory cytokine [19, 20]. Based on our clinical data, we found that the incidence of

ACS was increased among individuals exhibiting dyssomnia, changes in physical activity and disordered dietary habits. In addition, we found that the levels of FPG and CRP were higher in ACS patients. Therefore, we suspected that the circadian rhythm could affect the occurrence of ACS by regulating glucose metabolism and inflammatory responses. The severity of these conditions was positively correlated with the severity of coronary artery lesions.

The core genes Bmal1 and Clock play major roles in maintaining the homeostasis of circadian rhythms. In our study, markedly decreased levels of Bmal1 and Clock mRNAs were observed in the patients with UA, NSTEMI and STEMI. Pan et al. [4] found that in animal experiments, compared with $Apoe^{-/-}$ mice, $Bmal1^{-/-}$ $Apoe^{-/-}$ mice show increased hyperlipidemia and atherosclerosis, but Bmal1 overexpression in $Bmal1^{-/-}Apoe^{-/-}$ mice reduces hyperlipidemia and atherosclerosis. According to a study by Pan et al. [21], $Ldlr^{-/-}Apoe^{-/-}$ mice with a global Clock mutation develop more lesions in the aortic arches and aortic roots. Moreover, downregulated expression of Bmal1 or Clock not only induces hyperglycemia, hypertriglyceridemia and hypercholesterolemia [22-24] but also increases the expression of inflammatory cytokines [25]. Bmal1 knockout endothelial cells exhibit reduced nitric oxide production and increased superoxide levels [8, 26]. Furthermore, based on the results of the present study, high expression levels of Cry1, Per2 and Rev-Erbα protected individuals from developing ACS. Previous peer studies have reported that Cry1 overexpression in a mouse model of atherosclerosis significantly decreases the expression of proinflammatory factors and the concentrations of LDL-c [27]. Furthermore, Cry1 overexpression ameliorates the development of atherosclerosis by regulating the TLR/NF-κB pathway [27]. Similarly, $Per2^{-/-}$ mice display aortic endothelial dysfunction involving reduced nitric oxide production and increased levels of cyclooxygenase-1-derived vasoconstrictors [28]. However, in those mice, the difference in Per2 expression did not affect metabolic risk factors [28]. Rev-erbα silencing has been shown to elevate plasma lipid levels [29]. Furthermore, Rev-erbα overexpression inhibits the expression of inflammatory factors [30]. In summary, abnormal circadian rhythms can affect the occurrence of ACS by changing the expression levels of clock genes. Although the underlying mechanism remains unclear, we presume that the clock genes may reduce the incidence of ACS by maintaining normal vascular endothelial function, limiting the inflammatory response, and decreasing plasma lipid levels. These conclusions warrant further investigation in the future.

MMPs, which are highly concentrated in foam cell-rich regions, participate in the creation of plaques and remodeling of the vascular wall. MMPs play important roles in the development of atherosclerosis. Previous studies have shown that elevated MMP concentrations may contribute to plaque rupture [31]. Furthermore, enhanced MMP activity leads to activation of the pro-inflammatory factor IL-1β [32]. One important finding in the present study was that the increased levels of MMP2 and MMP9 mRNAs observed in patients with ACS correlated with the severity of plaque instability. Interestingly, increased expression levels of clock genes in macrophages downregulate MMP levels [33]. In our study, we found that the levels of MMP2 and MMP9 mRNAs negatively correlated with the levels of Bmal1, Clock, Cry1, Per2, and Rev-Erbα mRNAs. Moreover, compared to other clock genes, the levels of Bmal1 had the highest correlation. Therefore, we suspected that clock genes could increase the stability of atherosclerotic plaques by reducing the expression levels of MMPs. However, the mechanism by which clock genes affect MMPs expressions in patients with ACS remains unclear and warrants further investigation.

In conclusion, abnormal circadian rhythms and clock genes contributed to the development of ACS, and the expression levels of clock genes were negatively correlated with the severity of coronary artery lesions. Meanwhile, the expression levels of MMPs were higher in the patients with ACS. Moreover, the levels of MMP mRNAs negatively correlated with the levels of clock genes mRNAs. Therefore, we hypothesized that abnormal circadian rhythms and clock genes can increase the instability of atherosclerotic plaques by disturbing the levels of MMPs. Our findings can be regarded as preliminary due to a rather small sample and need replication in a large cohort of ACS patients. It must be noted, though, that because the patients with ACS had all been treated with various psychophar-

macological drugs during a number of years, we cannot exclude that the here observed alterations are due to long term epigenetic drug effects on the levels of glucose and lipid in the ACS patients. Because of the limitation of conditions, we could not collect blood samples at different time intervals to observe changes of the expressions of circadian rhythm genes. There are some limitations such as the mechanism by which clock genes moderated glucose metabolism and inflammatory responses and MMPs. Unfortunately, the results do not explain the mechanism underlying the relationships between circadian rhythms and clock genes and the stability of atherosclerotic plaques in patients with ACS. We will continue to investigate this mechanism in subsequent experiments. Furthermore, the regulation of circadian rhythms and clock genes may be targets for therapeutic intervention in patients with ACS.

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Disclosure of conflict of interest

None.

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References

[1] Libby P. Current concepts of the pathogenesis of the acute coronary syndromes. Circulation 2001; 104: 365-372.

[2] Alessi MC, Poggi M and Juhan-Vague I. Plasminogen activator inhibitor-1, adipose tissue and insulin resistance. Curr Opin Lipidol 2007; 18: 240-245.

[3] Shi SQ, Ansari TS, McGuinness OP, Wasserman DH and Johnson CH. Circadian disruption leads to insulin resistance and obesity. Curr Biol 2013; 23: 372-381.

[4] Pan X, Bradfield CA and Hussain MM. Global and hepatocyte-specific ablation of Bmal1 induces hyperlipidaemia and enhances atherosclerosis. Nat Commun 2016; 7: 13011.

[5] Antunes LC, Levandovski R, Dantas G, Caumo W, and Hidalgo MP. Obesity and shift work: chronobiological aspects. Nutr Res Rev 2010; 23: 155-168.

[6] Prasai MJ, George JT, and Scott EM. Molecular clocks, type 2 diabetes and cardiovascular disease. Diab Vasc Dis Res 2008; 5: 89-95.

[7] Rivera-Zavala JB, Molina-Aguilar C, Pérez-Mendoza M, Olguín-Martínez M, Hernández-Muñoz R, Báez-Ruiz GA and Díaz-Muñoz M. Daytime restricted feeding modifies the daily regulation of fatty acid β-oxidation and the lipoprotein profile in rats. Br J Nutr 2017; 117: 930-941.

[8] Anea CB, Zhang M, Stepp DW, Simkins GB, Reed G, Fulton DJ and Rudic RD. Vascular disease in mice with a dysfunctional circadian clock. Circulation 2009; 119: 1510-1517.

[9] Majumdar T, Dhar J, Patel S, Kondratov R and Barik S. Circadian transcription factor BMAL1 regulates innate immunity against select RNA viruses. Innate Immun 2017; 23: 147-154.

[10] Alpert JS, Thygesen K, Antman E and Bassand JP. Myocardial infarction redefined--a consensus document of the joint european society of cardiology/American college of cardiology committee for the redefinition of myocardial infarction. J Am Coll Cardiol 2000; 36: 959-969.

[11] Luepker RV, Apple FS, Christenson RH, Crow RS, Fortmann SP, Goff D, Goldberg RJ, Hand MM, Jaffe AS, Julian DG, Levy D, Manolio T, Mendis S, Mensah G, Pajak A, Prineas RJ, Reddy KS, Roger VL, Rosamond WD, Shahar E, Sharrett AR, Sorlie P, Tunstall-Pedoe H; AHA Council on Epidemiology and Prevention; AHA Statistics Committee; World Heart Federation Council on Epidemiology and Prevention; European Society of Cardiology Working Group on Epidemiology and Prevention; Centers for Disease Control and Prevention; National Heart, Lung, and Blood Institute. Case definitions for acute coronary heart disease in epidemiology and clinical research studies: a statement from the AHA council on epidemiology and prevention; AHA statistics committee; world heart federation council on epidemiology and prevention; the european society of cardiology working group on epidemiology and prevention; centers for disease control and prevention; and the national heart, lung, and blood institute. Circulation 2003; 108: 2543-2549.

[12] Huo M, Huang Y, Qu D, Zhang H, Wong WT, Chawla A, Huang Y and Tian XY. Myeloid Bmal1 deletion increases monocyte recruitment and worsens atherosclerosis. FASEB J 2017; 31: 1097-1106.

[13] Kiessling S, Beaulieu-Laroche L, Blum ID, Landgraf D, Welsh DK, Storch KF, Labrecque N and Cermakian N. Enhancing circadian clock

function in cancer cells inhibits tumor growth. BMC Biol 2017; 15: 13.
[14] O'Keeffe SM, Beynon AL, Davies JS, Moynagh PN and Coogan AN. NF-κB signalling is involved in immune-modulation, but not basal functioning, of the mouse suprachiasmatic circadian clock. Eur J Neurosci 2017; 45: 1111-1123.
[15] Johansson AS, Owe-Larsson B, Hetta J and Lundkvist GB. Altered circadian clock gene expression in patients with schizophrenia. Schizophr Res 2016; 174: 17-23.
[16] Knutsson A. Health disorders of shift workers. Occup Med (Lond) 2003; 53: 103-108.
[17] Spiegel K, Knutson K, Leproult R, Tasali E and Van Cauter E. Sleep loss: a novel risk factor for insulin resistance and type 2 diabetes. J Appl Physiol (1985) 2005; 99: 2008-2019.
[18] Taheri S, Lin L, Austin D, Young T and Mignot E. Short sleep duration is associated with reduced leptin, elevated ghrelin, and increased body mass index. PLoS Med 2004; 1: e62.
[19] Ramesh V, Nair D, Zhang SX, Hakim F, Kaushal N, Kayali F, Wang Y, Li RC, Carreras A and Gozal D. Disrupted sleep without sleep curtailment induces sleepiness and cognitive dysfunction via the tumor necrosis factor-α pathway. J Neuroinflammation 2012; 9: 91.
[20] Lange T, Dimitrov S, Fehm HL, Westermann J and Born J. Shift of monocyte function toward cellular immunity during sleep. Arch Intern Med 2006; 166: 1695-1700.
[21] Pan X, Jiang XC and Hussain MM. Impaired cholesterol metabolism and enhanced atherosclerosis in clock mutant mice. Circulation 2013; 128: 1758-1769.
[22] Staels B. When the clock stops ticking, metabolic syndrome explodes. Nat Med 2006; 12: 54-55; discussion 55.
[23] Kennaway DJ, Owens JA, Voultsios A, Boden MJ and Varcoe TJ. Metabolic homeostasis in mice with disrupted Clock gene expression in peripheral tissues. Am J Physiol Regul Integr Comp Physiol 2007; 293: R1528-1537.
[24] Rudic RD, McNamara P, Curtis AM, Boston RC, Panda S, Hogenesch JB and Fitzgerald GA. BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. PLoS Biol 2004; 2: e377.
[25] Gibbs J, Ince L, Matthews L, Mei J, Bell T, Yang N, Saer B, Begley N, Poolman T, Pariollaud M, Farrow S, DeMayo F, Hussell T, Worthen GS, Ray D and Loudon A. An epithelial circadian clock controls pulmonary inflammation and glucocorticoid action. Nat Med 2014; 20: 919-926.
[26] Anea CB, Cheng B, Sharma S, Kumar S, Caldwell RW, Yao L, Ali MI, Merloiu AM, Stepp DW, Black SM, Fulton DJ and Rudic RD. Increased superoxide and endothelial NO synthase uncoupling in blood vessels of Bmal1-knockout mice. Circ Res 2012; 111: 1157-1165.
[27] Yang L, Chu Y, Wang L, Wang Y, Zhao X, He W, Zhang P, Yang X, Liu X, Tian L, Li B, Dong S and Gao C. Overexpression of CRY1 protects against the development of atherosclerosis via the TLR/NF-κB pathway. Int Immunopharmacol 2015; 28: 525-530.
[28] Viswambharan H, Carvas JM, Antic V, Marecic A, Jud C, Zaugg CE, Ming XF, Montani JP, Albrecht U and Yang Z. Mutation of the circadian clock gene Per2 alters vascular endothelial function. Circulation 2007; 115: 2188-2195.
[29] Le MG, Claudel T, Gatfield D, Schaad O, Kornmann B, Lo SG, Moschetta A, Schibler U. REV-ERBalpha participates in circadian SREBP signaling and bile acid homeostasis. PLoS Biol 2009; 7: e1000181.
[30] Sato S, Sakurai T, Ogasawara J, Shirato K, Ishibashi Y, Oh-ishi S, Imaizumi K, Haga S, Hitomi Y, Izawa T, Ohira Y, Ohno H and Kizaki T. Direct and indirect suppression of interleukin-6 gene expression in murine macrophages by nuclear orphan receptor REV-ERBα. ScientificWorldJournal 2014; 2014: 685854.
[31] Kuzuya M, Nakamura K, Sasaki T, Cheng XW, Itohara S, Iguchi A. Effect of MMP-2 deficiency on atherosclerotic lesion formation in apoE-deficient mice. Arterioscler Thromb Vasc Biol 2006; 26: 1120-1125.
[32] Schönbeck U, Mach F and Libby P. Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing. J Immunol 1998; 161: 3340-3346.
[33] Lam MT, Cho H, Lesch HP, Gosselin D, Heinz S, Tanaka-Oishi Y, Benner C, Kaikkonen MU, Kim AS, Kosaka M, Lee CY, Watt A, Grossman TR, Rosenfeld MG, Evans RM and Glass CK. Rev-Erbs repress macrophage gene expression by inhibiting enhancer-directed transcription. Nature 2013; 498: 511-515.

Table S1

ATHENS INSOMNIA SCALE

This scale is intended to record your own assessment of any sleep difficulty you might have experienced. Please, check (by circling the appropriate number) the items below to indicate your estimate of any difficulty, provided that it occurred at least three times per week during the last month.

1. SLEEP INDUCTION *(time it takes you to fall asleep after turning-off the lights)*

0	1	2	3
No problem	Slightly delayed	Markedly delayed	Very delayed or did not sleep at all

2. AWAKENINGS DURING THE NIGHT

0	1	2	3
No problem	Minor problem	Considerable problem	Serious problem or did not sleep at all

3. FINAL AWAKENING EARLIER THAN DESIRED

0	1	2	3
Not earlier	A little earlier	Markedly earlier	Much earlier or did not sleep at all

4. TOTAL SLEEP DURATION

0	1	2	3
Sufficient	Slightly insufficient	Markedly insufficient	Very insufficient or did not sleep at all

5. OVERALL QUALITY OF SLEEP *(no matter how long you slept)*

0	1	2	3
Satisfactory	Slightly unsatisfactory	Markedly unsatisfactory	Very unsatisfactory or did not sleep at all

6. SENSE OF WELL-BEING DURING THE DAY

0	1	2	3
Normal	Slightly decreased	Markedly decreased	Very decreased

7. FUNCTIONING (PHYSICAL AND MENTAL) DURING THE DAY

0	1	2	3
Normal	Slightly decreased	Markedly decreased	Very decreased

8. SLEEPINESS DURING THE DAY

0	1	2	3
None	Mild	Considerable	Intense

Table S2

Name:_____ **Date:** _____

Pittsburgh Sleep Quality Index (PSQI)

Instructions: The following questions relate to your usual sleep habits during the past month only. Your answers should indicate the most accurate reply for the majority of days and nights in the past month. **Please answer all questions.**

1. During the past month, what time have you usually gone to bed at night? _____

2. During the past month, how long (in minutes) has it usually taken you to fall asleep each night? _____

3. During the past month, what time have you usually gotten up in the morning? _____

4. During the past month, how many hours of actual sleep did you get at night? (This may be different than the number of hours you spent in bed.) _____

	Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
5. During the past month, how often have you had trouble sleeping because you...				
a. Cannot get to sleep within 30 minutes				
b. Wake up in the middle of the night or early morning				
c. Have to get up to use the bathroom				
d. Cannot breathe comfortably				
e. Cough or snore loudly				
f. Feel too cold				
g. Feel too hot				
h. Have bad dreams				
i. Have pain				
j. Other reason(s), please describe:				
6. During the past month, how often have you taken medicine to help you sleep (prescribed or "over the counter")?				
7. During the past month, how often have you had trouble staying awake while driving, eating meals, or engaging in social activity?				
	No problem at all	**Only a very slight problem**	**Somewhat of a problem**	**A very big problem**
8. During the past month, how much of a problem has it been for you to keep up enough enthusiasm to get things done?				

	Very good	Fairly good	Fairly bad	Very bad
9. During the past month, how would you rate your sleep quality overall?				
	No bed partner or room mate	Partner/room mate in other room	Partner in same room but not same bed	Partner in same bed
10. Do you have a bed partner or room mate?				
	Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
If you have a room mate or bed partner, ask him/her how often in the past month you have had: a. Loud snoring b. Long pauses between breaths while asleep c. Legs twitching or jerking while you sleep d. Episodes of disorientation or confusion during sleep e. Other restlessness while you sleep, please describe:				

Scoring the PSQI

The order of the PSQI items has been modified from the original order in order to fit the first 9 items (which are the only items that contribute to the total score) on a single page. Item 10, which is the second page of the scale, does not contribute to the PSQI score.

In scoring the PSQI, seven component scores are derived, each scored 0 (no difficulty) to 3 (severe difficulty). The component scores are summed to produce a global score (range 0 to 21). Higher scores indicate worse sleep quality.

Component 1: Subjective sleep quality-question 9

Response to Q9	Component 1 score
Very good	0
Fairly good	1
Fairly bad	2
Very bad	3

Component 1 score:_____

Component 2: Sleep latency-questions 2 and 5a

Response to Q2	Component 2/Q2 subscore
< 15 minutes	0
16-30 minutes	1
31-60 minutes	2
> 60 minutes	3

Response to Q5a	Component 2/Q5a subscore
Not during past month	0
Less than once a week	1
Once or twice a week	2
Three or more times a week	3

Sum of Q2 and Q5a subscores	Component 2 score
0	0
1-2	1
3-4	2
5-6	3

Component 2 score:_____

Component 3: Sleep duration-question 4

Response to Q4	Component 3 score
> 7 hours	0
6-7 hours	1
5-6 hours	2
< 5 hours	3

Component 3 score:_____

Component 4: Sleep efficiency-questions 1, 3, and 4

Sleep efficiency = (# hours slept/# hours in bed) × 100%

hours slept-question 4

hours in bed-calculated from responses to questions 1 and 3

Sleep efficiency	Component 4 score
> 85%	0
75-84%	1
65-74%	2
< 65%	3

Component 4 score:_____

Component 5: Sleep disturbance-questions 5b-5j

Questions 5b to 5j should be scored as follows:	
Not during past month	0
Less than once a week	1
Once or twice a week	2
Three or more times a week	3

Sum of 5b to 5j scores	Component 5 score
0	0
1-9	1
10-18	2
19-27	3

Component 5 score:_____

Component 6: Use of sleep medication-question 6

Response to Q6	Component 6 score

Not during past month	0
Less than once a week	1
Once or twice a week	2
Three or more times a week	3

Component 6 score:_____

Component 7: Daytime dysfunction-questions 7 and 8

Response to Q7	Component 7/Q7 subscore
Not during past month	0
Less than once a week	1
Once or twice a week	2
Three or more times a week	3

Response to Q8	Component 7/Q8 subscore
No problem at all	0
Only a very slight problem	1
Somewhat of a problem	2
A very big problem	3

Sum of Q7 and Q8 subscores	Component 7 score
0	0
1-2	1
3-4	2
5-6	3

Component 7 score:_____

Global PSQI Score: Sum of seven component scores:_____

Citation: Buysse, DJ, Reynolds CF, Monk TH, Berman SR, Kupfer DJ: The Pittsburgh Sleep Quality Index (PSQI): A new instrument for psychiatric research and practice. Psychiatry Research 28:193-213, 1989.

Table S3

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE (August 2002)

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling?

_____ days per week

☐ No vigorous physical activities → ***Skip to question 3***

2. How much time did you usually spend doing vigorous physical activities on one of those days?

_____ hours per day

_____ minutes per day

☐ Don't know/Not sure

Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

3. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

_____ days per week

☐ No vigorous physical activities → ***Skip to question 5***

4. How much time did you usually spend doing **moderate** physical activities on one of those days?

_____ hours per day

_____ minutes per day

☐ Don't know/Not sure

Think about the time you spent **walking** in the **last 7 days.** This includes at work and at home, walking to travel from place to place, and any other walking that you might do solely for recreation, sport, exercise, or leisure.

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?

_____ days per week

☐ No walking → ***Skip to question 7***

6. How much time did you usually spend **walking** on one of those days?

_____ hours per day

_____ minutes per day

☐ Don't know/Not sure

The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the **last 7 days**, how much time did you spend **sitting** on a **week day**?

_____ hours per day

_____ minutes per day

☐ Don't know/Not sure

This is the end of the questionnaire, thank you for participating.

In the IPAQ, 3.3 METs correspond to 1 minute of walking, 4.0 METs correspond to 1 minute of moderate activities and 8.0 METs correspond to 1 minute of vigorous activities.

Table S4. Healthy Diet Score (HDS)

How often do you consume	Frequency of consumption (servings/month)					
	Never	1-4	5-8	9-12	13-18	> 18
Non-refined cereals (whole grain bread, pasta, rice, etc.)	0	1	2	3	4	5
Potatoes	0	1	2	3	4	5
Fruits	0	1	2	3	4	5
Vegetables	0	1	2	3	4	5
Legumes	0	1	2	3	4	5
Fish	0	1	2	3	4	5
Red meat and products	5	4	3	2	1	0
Poultry	5	4	3	2	1	0
Full fat dairy products (cheese, yoghurt, and milk)	5	4	3	2	1	0
Use of vegetable oil in cooking (times/week)	Never	Rare	< 1	1-3	3-5	Daily
	0	1	2	3	4	5
Alcoholic beverages (ml/day, 100 ml = 12 g ethanol)	< 300	300	400	500	600	> 700 or 0
	5	4	3	2	1	0