Circadian clock gene variants may affect circadian fluctuations of anaerobic performance

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Abstract
Aim: Circadian rhythm (CR) may be followed by almost all biochemical and physiological processes that occur in the human body. Physical activities involving anaerobic fitness have displayed a clear CR. The aim of this study was to investigate genotype and allele frequencies of (PER3 VNTR, PER2 VNTR and TIM1) gene polymorphisms in athletes and control groups and the second aim was to examine the anaerobic performance according to the genotype distribution of male footballers.

Material and Methods: The study included 20 male footballers and 30 voluntary male controls. To examine anaerobic performance, the footballers performed three randomized 30-s Wingate anaerobic power tests (WAnT) in the morning, at noon and in the evening with minimal one-week recovery time between each testing day. Genomic DNA was isolated from the blood samples. Polymerase chain reaction-based and restriction fragment length polymorphism (PCR-RFLP) analysis was used to identify gene variants from DNA material. Also, the differences between the basic performance characteristics of the athletes and the genotype distribution of these variants were investigated according to the daytime.

Results: The allele and genotype distribution of these variants was not significantly different between male footballers and the control group. Peak power and average power in the morning increased in athletes carrying TIM1 G/G variant (p<0.001) than in athletes carrying G/C and CC variant. Also, the athletes carrying the TIM1 G/G variant had a higher minimum power in the morning and evening (p<0.001) than athletes carrying G/C and CC variant.

Discussion: This is the first study that evaluates variants of circadian rhythm genes and the anaerobic performance of elite Turkish athletes. Our results suggested that the TIM1 gene variant may affect circadian fluctuations in anaerobic performance. People who carry the G/G genotype from the TIM1 gene have a higher anaerobic power. To understand ethnic and environmental differences, further studies are needed in this field.

Keywords
Circadian rhythm; Anaerobic performance; Variant; PCR-RFLP

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Introduction
The human genome project has been recently carried to investigate the effects of several genes on athletic performance. These studies showed the impact of the genetic features on athletic performance and also the components such as strength, agility, force, nerve-muscle coordination, and flexibility were found to affect the identification of the athletic performance among the athletes and that they were regarded as a criterion for encouraging athletes to do sport, which they tend to [1]. Biologic rhythms are the cyclic changes that regularly occur gradually, and cyclic rhythms that are related to the sunlight are called the circadian rhythms (CRs) [2]. In other words, cyclic physiological changes, which occur within 24 hours are called CRs. The relationship between CR and several behavioral and physiological functions was mentioned in early publications [3]. Extensive studies have been conducted on the effect of CRs on several physiological variables associated with athletic performance. The studies have reported the circadian variations of maximum aerobic power, blood pressure, heart rate, and central temperature during physical exercise [2]. The daytime effect of responses on short-term anaerobic exercise has also been studied, but there have been conflicting results. According to Melhim [4], and Hill and Smith [5], athletes had higher values of Wingate anaerobic test performance in the afternoon than in the morning. As stated by Souissi et al., [6] athletes significantly improved peak power, mean power, and maximal power from morning to afternoon during the Wingate and Force-Velocity tests.

There are several genes that control biological clock such as cryptochrome 1-2, clock (CRY1-2), period 1-3 (PER1-3), timeless (TIM), casein kinase 1 epsilon (CK1E), reverse strand of erb alpha (REV-ERBA), retinoic acid-related orphan receptor A (ROAR)-α, and brain and muscle aryl hydrocarbon receptor nuclear translocator-(ARNT)-like 1 (BMAL1). The aim of this study was to evaluate the CR difference between male footballers and controls and to examine differences in the anaerobic performance of male footballers according to genotype distribution.

Material and Methods
The study was organized and conducted in accordance with Helsinki protocol. The ethical approval was taken from the Clinical Research Committee of Ondokuz Mayis University, number 2017/794.

Study population
The study was conducted on 20 male footballers (age 23.58±3.27 years; body height 176.30±4.73 cm; weight 74.60±6.31 kg) who are studying at the university. All subjects in the study were engaged in physical activity regularly. Only the footballers and demographics features of groups. For comparison of the

Written informed consent was obtained from each participant before the blood sampling.

Wingate Anaerobic Test (WAnT)
To examine anaerobic performance, the footballers performed three randomized 30-s WAnT. The three testing days involved three randomized testing times with minimal one-week recovery time between each testing day (morning: 09:00h, noon: 14:00h, and evening: 19:00h). A bicycle ergometer and a bicycle-mounted computer assembly were used in the anaerobic performance of the athletes (Monark 894E, Varberg, Sweden). Before each test, the seat and handlebar adjustments were made to the subjects to provide the optimal cycling position, and WAnT was applied with the standard methods prescribed [7]. WAnT was applied for 30 seconds with a weight of 7.5% per subject’s body weight. The athletes warmed up by pedaling for 5 minutes at a speed of 60-70 (RPM) and sprinted for 5 seconds on the 3rd and 5th minutes of warm-up. The athletes rested until the pulse dropped to 100 beats/min before the test to relieve the fatigue that occurs after the warm-up. After the rest period, the test started, and the athletes pedaled at the highest speed for 30 seconds against the determined resistance. The subjects were verbally encouraged during the test. As a result of the test, the maximal anaerobic power, average powers, and minimum powers of the subjects were obtained. The Borg scale was used to determine the rate of perceived exertion (RPE) [8].

Genotyping
Genomic DNA was obtained from peripheral blood using a commercial DNA isolation kit. The isolated DNA was kept at -20°C until analysis. PER3 VNTR and PER2 VNTR variants were genotyped through polymerase chain reaction (PCR) analysis, as described previously [9-10]. Each 25-μL PCR reaction mixture contained 150 ng genomic DNA, 10 pM of each primer, 10x PCR mix buffer, 200 μM dNTP, and one unit of Taq DNA polymerase. After an initial step of 5 min at 95 °C, 33 cycles of amplification (45 s at 94 °C, 30 s at 60 °C, 45 s at 72°C) and a final extension step of 12 min at 72 °C were performed. The amplified products were analyzed by 2 % agarose gel electrophoresis. The TIM1 G/C was genotyped through polymerase chain reaction and analysis restriction fragment length polymorphism (PCR-RFLP), as described previously [11]. The amplified products were analyzed by 2 % agarose gel electrophoresis. In order to validate the accuracy and reproducibility of this method, each PCR reaction included negative and positive controls for each genotype. Second PCR was performed to confirm samples which results are not clear.

Statistical analysis
Statistical analysis was performed using the Statistical Package Program for the Social Sciences (IBM SPSS, version 20) and the OpenEpi Info software package version 2.3.1 (www.openepi.com). Continuous data were given as mean±SD (standard deviation) and (min-max). Chi-square tests were used to evaluate the significance of differences between the two study groups in the allele frequency and genotype distribution. Odds ratio (OR) and 95% confidence intervals (CIs) were calculated. A p-value p<0.05 was considered statistically significant. The nonparametric Mann-Whitney U test was used in the comparisons between baseline clinical and demographics features of groups. For comparison of the
Results

In this study, a total of 52 subjects, including 20 elite athletes and 30 controls, were genotyped for the PER3 VNTR, PER2 VNTR, and TIM1 G/C variant. The baseline demographic characteristics of the subjects are shown in Table 1. The genotype and allele distribution of PER3 VNTR, PER2 VNTR, and TIM1 G/C variants in Turkish footballers versus controls are presented in Table 2. The allele and genotype distribution of these variants were not significantly different between the male footballers and the control group (p>0.05).

In Table 3, the comparisons are made between the clinical parameter averages of individuals with genotypes of each gene. It was found that the peak power and average power in the morning increased in athletes carrying TIM1 G/G variant.

Table 1. Baseline clinical and demographics features of the study groups (mean ± SD)

| Characteristic | Athletes n=20 | Control Group n=30 | P |
|----------------|---------------|---------------------|---|
| Gender (male)  | n=20          | n=30                |   |
| Age (years)    | 23.84±3.27    | 22.00±2.31          | >0.05 |
| Height (cm)    | 173.00±6.48   | 173.20±6.48         | >0.05 |
| Weight (kg)    | 74.60±6.31    | 78.60±8.21          | >0.05 |

Table 2. Genotype and allele frequencies of PER3 VNTR, PER2 VNTR and TIM1 rs9313422 G/C gene polymorphisms between athletes and control groups.

| Gene          | Athletes n=20 | Healthy Controls n=30 | p     | OR (CI 95%) |
|---------------|---------------|----------------------|-------|-------------|
| PER3 VNTR 4/5 |               |                      |       |             |
| Genotypes     |               |                      |       |             |
| 4/4           | 6 (30%)       | 15 (50%)             | >0.05 |             |
| 4/5           | 10 (50%)      | 11 (36.7%)           | >0.05 |             |
| 5/5           | 4 (20%)       | 4 (13.3%)            | >0.05 |             |
| Alleles       |               |                      |       |             |
| 4             | 22 (59%)      | 41 (68.3%)           | >0.05 |             |
| 5             | 18 (45%)      | 19 (31.7%)           | >0.05 |             |

| PER2 VNTR 4/5 |               |                      |       |             |
| Genotypes     |               |                      |       |             |
| 4/4           | 8 (40%)       | 14 (46.7%)           | >0.05 |             |
| 4/5           | 10 (50%)      | 10 (33.3%)           | >0.05 |             |
| 5/5           | 2 (10%)       | 6 (20%)              | >0.05 |             |
| Alleles       |               |                      |       |             |
| 4             | 26 (65%)      | 58 (63.3%)           | >0.05 |             |
| 5             | 14 (35%)      | 22 (36.7%)           | >0.05 |             |

| TIM1 rs9313422|               |                      |       |             |
| Genotypes     |               |                      |       |             |
| G/G           | 8 (40%)       | 17 (56.7%)           | >0.05 |             |
| G/C           | 7 (35%)       | 10 (33.3%)           | >0.05 |             |
| C/C           | 5 (25%)       | 3 (10%)              | >0.05 |             |
| Alleles       |               |                      |       |             |
| G             | 23 (57.5%)    | 44 (73.3%)           | >0.05 |             |
| C             | 17 (42.5%)    | 16 (26.7%)           | >0.05 |             |

Table 3. Comparisons of the baseline clinical and demographic features of the athletes according to PER3, PER2 and TIM1 genotypes.

| Characteristic | Time of Day | PER3 VNTR | PER2 VNTR | TIM1 |
|---------------|------------|-----------|-----------|------|
|               | mean±SD    | p         | mean±SD   | p    | mean±SD | p    |
| Body Temperature (°C) |          |           |           |      |         |      |
| Morning       | 36.30±0.17 | >0.05     | 36.35±0.17| >0.05 | 36.31±0.17| >0.05|
| Noon          | 36.63±0.02 | >0.05     | 36.67±0.02| >0.05 | 36.70±0.17| >0.05|
| Evening       | 36.85±0.20 | >0.05     | 36.85±0.20| >0.05 | 36.94±0.13| >0.05|
| Peak Power (W) |          |           |           |      |         |      |
| Noon          | 832.51±12.19| >0.05    | 832.45±12.52| >0.05 | 771.83±13.60| >0.05|
| Evening       | 737.28±11.91| >0.05    | 832.26±13.38| >0.05 | 764.22±12.57| >0.05|
| Average Power (W) |        |           |           |      |         |      |
| Noon          | 538.90±66.83| >0.05    | 533.01±51.90| >0.05 | 526.62±57.73| >0.05|
| Evening       | 541.98±75.49| >0.05    | 544.07±29.58| >0.05 | 524.14±46.51| >0.05|
| Min Power (W) |          |           |           |      |         |      |
| Noon          | 325.50±55.76| >0.05    | 313.82±110.57| >0.05 | 308.94±53.48| >0.05|
| Evening       | 330.46±63.75| >0.05    | 337.81±76.24| >0.05 | 308.07±59.99| >0.05|
| Power Drop (%) |          |           |           |      |         |      |
| Noon          | 60.71±4.38 | >0.05     | 60.92±7.00| >0.05 | 61.28±6.94| >0.05|
| Evening       | 58.44±1.62 | >0.05     | 62.82±6.51| >0.05 | 62.74±9.32| >0.05|

* a b c (p<0.01) Comparison is made between genotypes within the gene itself. Statistically significant results were bolded.
Gene variants and performance

(p<0.01, p<0.01 respectively) when comparing the TIM1 G/C and TIM1 C/C genotypes. Also, athletes carrying the TIM1 G/G variant had higher minimum power in the morning and evening (p<0.01). Other variables did not show statistically significant differences (p>0.01).

Discussion

The type of selected sport is affected by either genotype or chronotype, and it is not clear how much the chronotype is affected by the sporting environment. Since no study has been carried out on the circadian rhythm genes in Turkish athletes, we aimed to evaluate PER3 VNTR, PER2 VNTR, and TIM1 variants in footballers and the control group and we aimed to examine the anaerobic performance according to genotype distribution of male footballers. The results demonstrated no differences in allele and genotype distribution of CR genes (PER3 VNTR, PER2 VNTR and TIM1) between male footballers and the control group.

In this study, we examined the genotype distribution of these variants in 20 male football players. To compare the performance of circadian phenotypes, indicators such as peak power, average power, and minimum power were measured in the daytime (morning, noon and evening). Although the allele and genotype distributions of PER3 VNTR, PER2 VNTR, and TIM1 G/G variants were not different in the footballers compared to the controls, there was a significant difference between genotype distribution and anaerobic performance status in terms of daytime. We found that the peak power and average power in the morning were higher in athletes carrying TIM1 G/G variants. Also, minimum power in the morning and evening increased in athletes carrying TIM1 G/G variant. This means that footballers who carry the G/G genotype from the TIM1 gene have better anaerobic power.

Several important factors affect CR in physical performance. The causes of these factors are both external or environmental changes and internal or physiological changes occurring during the day. It is often difficult to identify a single cause of performance fluctuations due to the effect of different physiological systems on performance fluctuations, which occur simultaneously. Reilly and Waterhouse identified three major determinants that affect a CR in sporting performance [12]. These functions include perceptual and cognitive performance, metabolic variables, several behavioral, cardiovascular, and neuromuscular variables, and sensory-motor resting levels.

Several points of genetic variance among individuals have a combined effect leading to polygenic sports performance. Circadian phenotypes, such as a person's diurnal preference for morning or evening activity patterns and chronotype, which is a person's sleep-wake phenotype, influence peak performance at the individual level [13]. Physiological responses during exercise, such as oxygen uptake, blood lactate, heart rate, etc., are based on the intensity of exercise. Generally, there is a peak of athletic performance in the afternoon during which there is a peak of the fueling metabolic activity of physiological processes [14]. Athletes with a high evening or morning phenotype want to do better endurance activities [15], and strength training [16], near their circadian peak. According to Souissi et al. [17], and Bernard et al. [18] there was a significant circadian variation in maximal anaerobic power during Force Velocity and Wingate tests and Cycle and Multi-Jump tests. Contrary to these findings, Reilly and Down [19] reported that there was no circadian fluctuation of the anaerobic performance in the Wingate test. Variations of CR genes may cause differences among studies.

A family of genes, i.e., the PER genes, influences cellular growth and differentiation, as well as, the cell cycle [20]. The PER family includes PER1, PER2, and PER3. A promising gene candidate, which affects daytime mood is the PER3 clock gene. A heterodimer with the CRY protein being translocated into the nucleus is formed by the PER3 protein product, inhibiting the CLOCK-BMAL-mediated transcription. It takes almost 24 hours to complete this feedback loop. The timing of the circadian feedback loop could be changed ostensibly due to differences in the size of the PER3 protein, considering that the PER3 gene contains an 18 amino acid-long 4 and 5 repeat homolog [21]. The VNTR polymorphism in the PER3 gene is located on chromosome 1p36.23, which has two alleles of 4 or 5 tandem 54 bp repeats (coding for a region of 18 amino acids in exon 18). It has been regarded as a possible genetic factor for circadian phenotypes and chronotypes [22]. Behavioral rhythmicity is significantly affected by the PER2, which belongs to the PER family of genes [23]. The main expression of PER2 is found in the peripheral nervous systems and the central nervous system. There is an association between human SNPs in the PER2 gene and chronotypes, depression, and abnormal circadian parameters, also higher alcohol intake. There is a VNTR polymorphism in intron 3 of the PER2 gene. There are three members, including TIM-3, TIM-1, and TIM-4 in the TIM gene family, which is located on the human chromosome 1q23-35, including TIM-3, TIM-1, and TIM-4, related to the immune dysfunction which finally induces various diseases, such as allergic diseases, chronic viral infections, autoimmune diseases, immune reactions, and even tumors [11]. According to the studies, which have been concluded in the past years, regulation of the immune responses and maintenance of the immune homeostasis can be mainly affected by TIM-1. According to the accumulating evidence, there is an association between immune dysfunction due to certain TIM1 polymorphisms and allergic reactions, chronic viral infections, autoimmune diseases, and immunologic rejections [24].

The PER2, NPAS2, CLOCK, and TIM genes polymorphisms are related to sleep timing [25], sleep disorders [26], and diurnal preference [27]. It has been shown that the individual sport athletes comprise unusually high morning-types proportions along with a higher prevalence of the PER3 VNTR allele related to morningness [28]. According to Konorozva et al. [28] there was a strong relationship between PER3 VNTR genotype and chronotype. The daytime during which athletes preferred to train was associated with their chronotype. According to their data, there are morning types of white males of European descent who took part in the endurance sports in South Africa. It was reported that there were more morning-types in the Super Rugby players than in the control group, more evening-types in the control group than in the Super Rugby player group. Still, there were no differences in allele frequencies or PER3 VNTR genotype [29]. However, there are a few limitations to the current case-control...
analysis. First, we focused on only three variants involved in the circadian rhythm pathway; other regulatory genes in this signaling pathway may also contribute to the mechanism. Second, due to the relatively small sample size, the frequencies of some homoygous variants may have reduced statistical power. Third, the samples studied were from the same geographic region and race.

Conclusion

The difference in sports performance between elite athletes is due to the genetic basis of individuals. CR affects many physiological events as well as sports performance. To the best of our knowledge, this is the first study evaluating variants of CR genes and anaerobic performance in Turkey. Those carrying the G/G genotype from this TIM1 gene have higher anaerobic power (peak power, average power and min power), especially in the morning. Again, carriers of the G/G genotype had a higher evening min power value than those carrying the G/C and C/C genotype. This means that carrying the G/G genotype from the TIM1 gene has better anaerobic power. Our results suggested that the TIM1 gene may affect circadian fluctuations in anaerobic performance. The genetic characterization of circadian fluctuations is still not entirely clear, and further studies are needed to clarify the effects of these variants in this pathway and their relationship to individual traits.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest

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