

## Per3 VNTR polymorphism and chronic heart failure

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**Aims.** The aim of this study was to investigate the relationship between gene Period3 (Per3) variable number tandem repeat (VNTR) polymorphism and chronic heart failure (CHF).

**Methods.** The study subjects (372 patients of Caucasian origin with CHF and 332 healthy controls) were genotyped for Per3 VNTR polymorphism using an allele-specific PCR.

**Results.** No significant differences in genotype or Per3 VNTR allele frequencies were found between CHF cases and controls ( $P_g=0.30$ ,  $P_a=0.52$ ). No significant differences were uncovered either between CHF cases according to etiology (DCMP vs. IHD;  $P_g=0.87$ ,  $P_a=0.91$ ). In the multivariate regression modeling, no predictive function of VNTR Per3 polymorphism on ejection fraction or NYHA class, hyperlipidaemia or type II diabetes risk was found.

**Conclusion.** Per3 VNTR polymorphism is not a major risk factor for chronic heart failure or a factor modulating the severity of the CHF in this population.

**Key words:** circadian clock, Per3, polymorphism, case-control study, chronic heart failure

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### INTRODUCTION

Many physiological processes in the organism exhibit daily rhythmicity, which is generated by an internal "clock" setting about 24-h periodic expression of clock genes and their products. Such reliable biological oscillators allow almost every cell to predict environmental changes and thus accurately react to them. In humans, autonomic processes such as the control of sleep, body temperature, and hormone secretion, renal and cardiovascular functions are subject to circadian variation<sup>1-5</sup>.

Cell "clocks" are governed by a primary clock system in the suprachiasmatic nuclei (SCN), which reacts to daylight changes. At a molecular level this endogenous system consists of a series of positive and negative transcriptional feedback loops interacting with many clock-dependent proteins and thus affecting different metabolic pathways<sup>6,7</sup>. According to the basic model of the function of "core clock proteins", the principal components of the positive loop BMAL1 and CLOCK periodically activate the transcription of Per and Cry genes. Therefore, the Per and Cry complexes, after reaching threshold concentration, interact with BMAL1 and CLOCK and repress their transcription therefore generating nearly antiphasic expression<sup>8,9</sup>.

Despite the lack of precise knowledge of signaling pathways of all components integrated in the clock machinery, it has been experimentally proven in mammals that mutations in the "core clock genes" may lead to changes in circadian rhythmicity, disturbance of sleep-wake cycles and may also affect heart control and blood

pressure<sup>10,11</sup>. Period 3 (Per3), one of the key components of the negative limb of the human clock system, is a member of the Period protein family, described first in *Drosophila*<sup>12</sup>, and has recently been associated with metabolic dysfunctions. According to functional studies, deletion of Per3 in mice has a mild influence on circadian activity<sup>13</sup>. However, in humans, several mutations in Per3 have been described to affect sleep homeostasis and mood disorders<sup>14-17</sup>. Among the most surveyed Per3 polymorphisms in the last five years is the biallelic length change near the putative phosphorylation site of exon 18, composed of 4 or 5 indirect repeats. Numerous studies have linked this VNTR polymorphism to overall control of autonomic balance during sleep and wake cycles and indirectly to cardiac autonomic control<sup>18-20</sup>.

However, little is known about the exact clock mechanism in cardiovascular biology, and circadian rhythms in the heart have not yet been characterized at the transcriptional level, although several studies have shown that clock genes have an important function in myocardial contractile function and metabolism. Experiments in this area conducted to date have mostly involved manipulations of the light-dark cycle of mice clock mutants and their influence on myocardial functions<sup>21,22</sup>. Possible imbalance or impairment in the clock mechanism within the cardiomyocyte may alter the cardiac metabolism and function and thus increase the susceptibility of cardiovascular diseases. Therefore, we chose the Per3 gene and decided to investigate the differences in allelic representation of VNTR polymorphism among subject with chronic heart failure (CHF).

## MATERIAL AND METHODS

### Subjects

The study cohort consisted of 372 consecutive patients (median of age 56 y, age range 21 - 91 y, 107 females/ 265 males) inclusive of chronic heart failure diagnosis (functional class NYHA II-IV, ejection fraction median 25%, cardiothoracic index more than 50%) both of ischemic heart disease (IHD) or dilated cardiomyopathy (DCMP) origin. To estimate the population frequencies of the examined genotypes and alleles, the control cohort was recruited consisting of 332 healthy individuals of similar age and gender distribution, without clinical signs of cardiovascular diseases and without a family history of early cardiovascular disease (median of age 51, age range 15.8 - 86, 218 females/ 189 males). The prevalence of diabetes mellitus and hyperlipidaemia in the controls was approximately 6% and 8%, respectively, which corresponds well with the general prevalence in the Czech population. In all subjects, a complete medical history with respect to conventional cardiovascular risk factors was obtained. All patients had chronic heart failure of at least of 3 months in duration and were stable on unchanged medication for at least 1 month. All patients originated from the Czech Caucasian population and were recruited at the 1<sup>st</sup> Cardioangiological and 2<sup>nd</sup> Internal Departments, St. Ann's Hospital, Brno. The study was approved by the Committee for Ethics of Medical Experiments on Human Subjects, Faculty of Medicine, Masaryk University, Brno (no. 64/93, 1993) and was performed in adherence to the Declaration of Helsinki Guidelines. Participants gave their written informed consent before they entered the study.

### Clinical examination

Clinical examination, basic and special laboratory, including renal functions, was done early in the morning followed by echocardiographic examination (SONOS 5500, Hewlett-Packard). The echocardiographic measurements were performed using a 2.5 MHz phased array transducer, volumes were measured from apical four-chamber view and ejection fraction was calculated using the single plain Chapman method. Electrocardiography and standard X-ray (with evaluation of cardio-thoracic index and pulmonary congestion by the method of Meszaros) were obtained. Hyperlipidaemia was defined as plasma total cholesterol level of >200 mg/dL, plasma triglyceride level of >150 mg/dL, or current use of antilipidemics with the previously established diagnosis of hyperlipidaemia. The patients were treated with heart failure therapies including cardiac transplantation. After venous blood sample (5-10 mL) collection from each subject, white cell fraction was used to extract DNA according to standard procedure using proteinase K.

### VNTR polymorphism genotyping

Genotyping was performed by polymerase chain reaction (PCR) using the primers previously described<sup>15</sup>. The PCR reaction was carried out in total volume of 25 µL containing 150 ng genomic DNA and optimized for the

following amplification conditions: 94 °C for 6 min, then 34 cycles of 94 °C for 40 s, 55 °C for 30 s, 70 °C for 40 s, and 70 °C for 8 min. Respective alleles (401 bp product - 5 repeats allele and 347 bp product - 4 repeats allele) were examined by agarose gel electrophoresis.

### Statistics

The observed number of each genotype was compared with that expected for a population in Hardy-Weinberg equilibrium using the  $\chi^2$  test. Fisher's exact test with Tukey-Kramer's method of adjustment for multiple comparisons was used for evaluation of categorical variables. Differences between continuous variables were evaluated using ANOVA with the corresponding post hoc test for more than two groups; multiple regression analysis was applied in all cases of associations between the genotypes and clinical parameters, significant in the univariate analysis. The data analysis was performed using Statistica v. 7.0 (Statsoft Inc., Tulsa, USA) program package. Power calculations were performed using Quanto software<sup>23</sup>.

## RESULTS

A total of 704 unrelated Caucasian subjects (372 cases and 332 controls) were enrolled in the study. The basic demographic, clinical and laboratory parameters of the cases are listed in Table 1.

**Table 1.** Baseline characteristics of the CHF cohorts according to etiology.

Variable	IHD patients	DCMP patients
LVEF (%)	25 (10-40)	24 (10-40)
NYHA (No. in class II/ III/ IV)	71/130/24	57/68/9
DM I (%)	10.6	9.6
DM II (%)	29.6	15.3
HLPP (%)	60.4	34.0
Anticoagulant agents	19.5	25.6
Digoxin	54.6	83.3
CAA	5.5	2.56
Diuretics	85.18	92.3
BB	69.4	70.5
ACEI	94.4	89.7
AT1	3.7	10.25

Values are given as median + min-max range

CHF - chronic heart failure, IHD - ischemic heart disease, DCMP - dilated cardiomyopathy, LVEF - left ventricular ejection fraction, NYHA - New York Heart Association Classification, HLPP - hyperlipoproteinemia, CAA - Calcium antagonists, BB - Beta blockers, ACEI - ACE inhibitors, AT1 - Angiotensin type I receptor blockers

## Associations between examined VNTR in Per3 gene and chronic heart failure

The investigated length polymorphism did not express any deviation from Hardy-Weinberg equilibrium (tested by conventional  $\chi^2$  test) in any of the investigated cohorts (the total cases, the cases subdivided according to etiology, the controls). No significant case-control differences were found ( $P_g=0.302$ ;  $P_a=0.521$ ). As some sex-linked differences in allele or genotypes of examined polymorphisms were expected, the analysis by gender was performed but no significant sex-dependent associations of genotype distributions or allele frequencies were found. When comparing the genotype and allele frequencies by the most common causes (ischemic heart disease (IHS) vs. dilation cardiomyopathy), no significant differences were found either ( $P_g=0.585$ ;  $P_a=0.720$ ).

## Multiple regression modeling across the case cohort

In the multiple regression modeling, the VNTR Per3 polymorphism showed no independent predictive role for chronic heart failure risk. In none of the constructed models, was the VNTR Per3 polymorphism associated with increased risk of chronic heart failure or with its etiology (IHD vs. dilated cardiomyopathy). Moreover, the VNTR Per3 polymorphism was not associated with any of the investigated laboratory markers (glycaemia, bilirubin, hematocrit, big endothelin, brain natriuretic factor). In the next step, we tested whether the VNTR Per3 polymorphism had any effect on the ejection fraction or CHF risk. No association of the investigated polymorphism with ejection fraction or NYHA class was found.

## DISCUSSION

Circadian clock regulation has been identified in almost every human cell to date, suggesting a wide-ranging role of a light-dark dependent modulation on physiological and pathophysiological events. According to recent findings of clock genes fluctuating expression within the cardiomyocytes and vascular smooth muscles, there is a strong hypothesis that a clock system may regulate myocardial metabolism<sup>24</sup>. This hypothesis can be further supported by the facts that there is obvious diurnal variation in blood pressure, heart rate and cardiac output. Moreover, it has been known for long time that fatal cardiovascular events, such as acute myocardial infarction, occur preferentially at certain times of the day<sup>25</sup>.

Apropos cardiovascular disorders, we present the first study investigating relationship between polymorphism in Per3 gene and chronic heart failure in a Caucasian population. We found no association of VNTR Per3 polymorphism with chronic heart failure. Further, there was no deviation from the Hardy-Weinberg equilibrium or sex-dependent association of VNTR Per3 genotypes. Our findings are in accordance with recent papers, where certain VNTR alleles were identified with sleep homeostasis rather than with cardiovascular diseases<sup>26</sup>. Recently correlation between VNTR Per3 polymorphism and the extreme diurnal preference (the 5/5 allele associated

with morningness and the 4/4 allele with eveningness) has been found<sup>18</sup>. On the other hand, a certain mutation in Per2 gene, which has a similar function in the clock machinery to Per3, has been associated with impaired vascular endothelial function in mice and with familial sleeping disorders in humans<sup>27</sup>. The connection between both could be viewed from the perspective of the possible effect of sleep deprivation on cardiac autonomic control, associating homozygosity in Per3 VNTR with heart rate (HR) and heart rate variability (HRV) (ref.<sup>20</sup>). Although no interaction of HR and HRV with VNTR Per3 genotype was found, this polymorphism has been considered a potential marker for individual differences in the autonomic nervous system. It is possible that the Per3 gene more likely modulates the reaction of the heart cell to environmental stimuli and neurohumoral factors and in this way participates in the homeostasis of cardiomyocytes.

Even though the sample consisted of chronic heart patients, we can consider the role of Per3 in circadian neurohumoral regulation and possible disease progression. At a lower level of the circadian cascade, the importance of these regulators in the modulation of cardiovascular functions has been proven in a study on mice with deletions of three clock-controlled PAR bZIP transcription factors<sup>28</sup>. These knockout mice showed increased morbidity, low blood pressure, cardiac hypertrophy and left ventricular dysfunction. Our results suggest that the VNTR Per3 does not influence the severity of the disease but may affect the onset of cardiovascular disorder.

## CONCLUSION

We conclude that Per3 VNTR polymorphism is not associated with chronic heart failure and we do not consider this polymorphism a major risk factor for chronic heart failure or a factor modulating the severity of the CHF in this Caucasian population. Further studies of the relationship of acute and chronic cardiovascular events to the Per3 locus are required.

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## CONFLICT OF INTEREST STATEMENT

The authors stated that there are no conflicts of interest regarding the publication of this article.

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