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**ARNTL** (*BMAL1*) and **NPAS2** Gene Variants Contribute to Fertility and Seasonality

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**Abstract**

**Background:** Circadian clocks guide the metabolic, cell-division, sleep-wake, circadian and seasonal cycles. Abnormalities in these clocks may be a health hazard. Circadian clock gene polymorphisms have been linked to sleep, mood and metabolic disorders. Our study aimed to examine polymorphisms in four key circadian clock genes in relation to seasonal variation, reproduction and well-being in a sample that was representative of the general population, aged 30 and over, living in Finland.

**Methodology/Principal Findings:** Single-nucleotide polymorphisms in the ARNTL, ARNTL2, CLOCK and NPAS2 genes were genotyped in 511 individuals. 19 variants were analyzed in relation to 31 phenotypes that were assessed in a health interview and examination study. With respect to reproduction, women with ARNTL rs2278749 TT genotype had more miscarriages and pregnancies, while NPAS2 rs11673746 T carriers had fewer miscarriages. NPAS2 rs2305160 A allele carriers had lower Global Seasonality Scores, a sum score of six items i.e. seasonal variation of sleep length, social activity, mood, weight, appetite and energy level. Furthermore, carriers of A allele at NPAS2 rs6725296 had greater loadings on the metabolic factor (weight and appetite) of the global seasonality score, whereas individuals with ARNTL rs6290035 TT genotype experienced less seasonal variation of energy level.

**Conclusions/Significance:** ARNTL and NPAS2 gene variants were associated with reproduction and with seasonal variation. Earlier findings have linked ARNTL to infertility in mice, but this is the first time when any polymorphism of these genes is linked to fertility in humans.

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**Introduction**

Circadian rhythms are the approximate 24-hour oscillations in behavioral or physiological processes that allow organisms to anticipate routine environmental changes and to prepare for the appropriate alignment in order to adapt. Circadian rhythms are generated by the intrinsic clocks whose principal pacemaker is located in the suprachiasmatic nuclei of the anterior hypothalamus. This internal clock is synchronized to the external 24-hour clock by following time-giving cues, primarily the daily light-dark transitions, in the habitat. The principal circadian clock coordinates peripheral oscillators that maintain the timing for a range of physiological functions such as hormone release, body temperature, cardiovascular function and physical activity. Recently, there has been growing interest in the impact of disruption of these rhythms on health.

At the molecular level, circadian rhythms are generated by a network of proteins. The clock protein (CLOCK) [1] pairs up with aryl hydrocarbon receptor nuclear translocator-like (ARNTL or BMAL1) protein [2]. Neuronal PAS domain protein 2 (NPAS2) [3] can substitute for CLOCK and aryl hydrocarbon receptor nuclear translocator-like 2 (ARNTL2 or BMAL2) [4] for ARNTL. Paired CLOCK/NPAS2 – ARNTL/ARNTL2 heterodimers thereafter activate the transcription of their target genes [5-7].

So far, genetic variations in circadian clock genes have been associated with sleep, mood, and metabolic disorders [8]. Concerning these disorders, CLOCK gene variants have been linked to diurnal preference [9,10], delayed sleep phase syndrome [11], metabolic syndrome and obesity [12], ARNTL gene variants to bipolar disorder [13], type 2 diabetes and hypertension [14], and NPAS2 gene variants to diurnal preference and seasonal affective disorder [15,16]. However, in some cases the aforementioned associations are conflicting [17,18], and the established links between circadian clock gene polymorphisms and disease susceptibility therefore remain incomplete.

Our aim was to examine the role of 19 single-nucleotide polymorphisms (SNPs) of four canonical circadian clock genes (ARNTL, ARNTL2, CLOCK, NPAS2) in relation to a range of health-related phenotypes that were assessed with structured methods as part of a population-based health interview and...
examination study. Here, we report associations with seasonal variation, reproduction, and social activity.

**Methods**

**Subjects**

This study was part of a nationwide health interview and examination survey whose design and methods are described in detail on the http://www.survey2000.fi/indexe.html site. The ethical approval of the survey has been accepted by the ethics committee of the National Public Health Institute and all participants provided written informed consent. Of the 7415 participants aged 30 or over, 5480 took part to a health status examination and a diagnostic mental health interview (M-CIDI [19]), a valid and reliable instrument for the assessment of alcohol use, mood and anxiety disorders, yielding the diagnosis according to DSM-IV, filled in the self-report questionnaires, and gave a venous blood sample for DNA extraction. The sample that was drawn from the study for our analysis included 511 individuals (9.3% of the eligible participants) having no diagnosis of mental disorder. Of the study subjects that were originally selected as healthy controls for those with alcohol use disorder, 412 were men and 99 women.

**Analyzed phenotypes**

The study sample that was derived from an epidemiological nation-wide cohort, representative of the population, is well characterized. This allowed an extensive phenotype analysis concerning data on reproduction, seasonal variation and mental well-being. Altogether 31 phenotypes were analyzed (Table 1).

Regarding reproduction, the menstrual cycle (irregular or no periods versus regular periods in women aged 54 or under), the number of pregnancies (ended in a delivery, a miscarriage, or an abortion), elevated blood pressure during a pregnancy, the number of miscarriages, and infertility (failure to get pregnant within 12 months) were analyzed. Sample size for these phenotypes was small due to the small number of women in our study.

With regard to seasonal variation in mood and behavior, the participants filled in a modified Seasonal Pattern Assessment Questionnaire (SPAQ) [20]. Seasonal variation in sleep length, social activity, mood, weight, appetite, and energy level were scored from 0 to 3 (none, slight, moderate or marked change) instead of 0 to 4 (none, slight, moderate, marked or extremely marked change). The sum score of the six items is the global seasonality score (GSS). The psychometric properties of this modified questionnaire have been tested [21]. The factor analysis produced two factors: factor one (GSS1) including the items of weight, and appetite; and factor two (GSS2) including the items of sleep length, social activity, mood, and energy level. The participants who reported of having any variation were divided into those who did not experience the variations as a problem and those who experienced them as mild (n = 37), moderate (n = 2), marked (n = 0) and severe (n = 1) problems.

Social activity was assessed with the sum of two questions asking for the frequencies of social contacts at home and those at visit. Concerning mental well-being, the phenotypes analyzed are listed in Table 1 and described in more detail in the supporting information (see Text S1).

**SNP selection and genotyping**

Five SNPs for ARNTL, ARNTL2 and NPAS2 and four SNPs for CLOCK were selected (Table 2). SNPs included Hap-Map tag-SNPs and candidate SNPs selected on the basis of previous association studies with health-related condition or behavior.

Genomic DNA was isolated from whole blood according to standard procedures. SNPs were genotyped in 10 μl reactions with a fluorogenic 5′ nuclease assay method (TaqMan™) with pre-designed primer-probe kits using Applied Biosystems 7300 Real Time PCR System according to manufacturers’ instructions. All the samples were successfully genotyped, and those 5% of the samples that were re-genotyped did confirm the genotyping results to have no error. Genotyping of the first 276 samples indicated that ARNTL2 rs35678285 was not polymorphic in our sample, and it was therefore excluded from further analysis, leaving 10 SNPs for the statistical analysis.

**Statistical analysis**

Statistical analyses of the data were performed by using the PLINK software, version 1.05 (http://pngu.mgh.harvard.edu/~purcell/plink/) [22]. The genotype and allele frequencies and the

Table 1. Phenotypes analyzed in the study.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reproduction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men: Infertility</td>
<td>35</td>
<td>376</td>
</tr>
<tr>
<td>Women: Infertility</td>
<td>19</td>
<td>80</td>
</tr>
<tr>
<td>Irregular or no periods in women less than 54 years old</td>
<td>20</td>
<td>61</td>
</tr>
<tr>
<td>Number of pregnancies</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>Number of miscarriages</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>High blood pressure during pregnancy</td>
<td>23</td>
<td>59</td>
</tr>
<tr>
<td>Seasonal variation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Global seasonality score (GSS)</td>
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<td></td>
</tr>
<tr>
<td>Global seasonality score factor 1 (GSS1)</td>
<td>511</td>
<td></td>
</tr>
<tr>
<td>Global seasonality score factor 2 (GSS2)</td>
<td>511</td>
<td></td>
</tr>
<tr>
<td>Seasonal variation in sleep length</td>
<td>364</td>
<td>147</td>
</tr>
<tr>
<td>Seasonal variation in social activity</td>
<td>348</td>
<td>163</td>
</tr>
<tr>
<td>Seasonal variation in mood</td>
<td>364</td>
<td>147</td>
</tr>
<tr>
<td>Seasonal variation in weight</td>
<td>225</td>
<td>286</td>
</tr>
<tr>
<td>Seasonal variation in appetite</td>
<td>187</td>
<td>324</td>
</tr>
<tr>
<td>Seasonal variation in energy</td>
<td>362</td>
<td>149</td>
</tr>
<tr>
<td>If seasonal variation experienced in above, a problem?</td>
<td>40</td>
<td>391</td>
</tr>
<tr>
<td>Vitamin D, S-D-25, nmol/l</td>
<td>504</td>
<td></td>
</tr>
<tr>
<td>Well-being</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beck Depression Inventory (BDI)</td>
<td>510</td>
<td></td>
</tr>
<tr>
<td>Beck Depression Inventory factor 1 (BDI1)</td>
<td>499</td>
<td></td>
</tr>
<tr>
<td>Beck Depression Inventory factor 2 (BDI2)</td>
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<td></td>
</tr>
<tr>
<td>General Health Questionnaire (GHQ)</td>
<td>510</td>
<td></td>
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<tr>
<td>General Health Questionnaire factor 1 (GHQ1)</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>General Health Questionnaire factor 2 (GHQ2)</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>Health related quality of life factor 1 (15-D 1)</td>
<td>510</td>
<td></td>
</tr>
<tr>
<td>Health related quality of life factor 1 (15-D 2)</td>
<td>510</td>
<td></td>
</tr>
<tr>
<td>Health related quality of life factor 1 (15-D 3)</td>
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</tr>
<tr>
<td>Epworth Sleepiness Scale factor 1 (ESS1)</td>
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<td></td>
</tr>
<tr>
<td>Epworth Sleepiness Scale factor 2 (ESS2)</td>
<td>490</td>
<td></td>
</tr>
<tr>
<td>Hours of sleep in 24 hours</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>Social activity</td>
<td>194</td>
<td>119</td>
</tr>
<tr>
<td>Maslach Burnout Inventory (MBI)</td>
<td>396</td>
<td></td>
</tr>
</tbody>
</table>

*Positive (A) and negative (B) status with respect to the phenotype analyzed. In case of continuous variables, the number of subjects is given in the column A. doi:10.1371/journal.pone.0010007.t001
Hardy-Weinberg equilibrium (HWE) estimates were calculated. SNPs were compared between participants with a condition and those without the condition, or in relation to a quantitative measure. Additive, dominant and recessive models were calculated using linear and logistic regression models for the quantitative and categorized traits respectively. Age and sex were controlled for in the analyses. To correct SNP analyses for multiple testing across all the tests performed, the R software (http://www.r-project.org/) was used to calculate false discovery rate (FDR) q-values [23]. The tests performed, the R software (http://www.r-project.org/) was used to calculate the linkage disequilibrium (LD) estimates. The formed haplotype blocks were analyzed by using the PLINK software, with the sliding window approach, regression models, and controlling for age and sex.

**Results**

The calculated genotype and allele frequencies and HWE estimates are given in Table 2. All the SNPs were in HWE (P>0.01) in the 511 subjects. The most significant associations (q<0.4) are listed in Table 3, and they are discussed below (see Table S1, for all the associations having P<0.01). One of these associations had a false discovery rate q-value under 0.05. It is of note that there was no significant association with ARNTL2. In the haplotype analysis (see Table S2), P-values for haplotypes in the formed blocks (see Figure S1) were higher than for the individual SNPs within the haplotype and thus are not discussed.

The TT allelic status (AA and AG genotypes) of NPAS2 rs2305160 was over-represented among individuals not experiencing seasonal variation (P<0.001) as measured with the GSS. The A allele (P<0.001) and the TT (AA or AG genotypes) allelic status (P<0.01) of NPAS2 rs6725290 were associated with the GSS1 and with the single item of seasonal variation in weight. In addition, people with the TT genotype of ARNTL rs2290035 were more likely not to experience seasonal variation in energy level (P<0.001).

The TT genotype of ARNTL rs2278749 was associated with both a higher number of pregnancies (P<0.01) and a higher number of miscarriages (P<0.000001), whereas the TT (TT or CT genotypes) allelic status of NPAS2 rs11673746 was linked to a lower number of miscarriages (P<0.01). Individuals with the TT genotype of CLOCK rs2412646 were more likely to have a lower level of social activity (P<0.01).

**Discussion**

Our aim was to investigate genetic variants of the four key circadian clock genes ARNTL, ARNTL2, CLOCK and NPAS2 in relation to a range of health related phenotypes assessed in a representative nation-wide sample of the population aged 30 or over. These genes together with other circadian clock genes generate the circadian rhythms and the seasonal cycles, such as reproduction related activities, as well as have control for the cell-division cycle and the metabolic cycle [25,26].

Circadian rhythms and clock genes have previously been connected to reproductive functions in animal models [27], but no studies on the role of these genes in human fertility have been published. In studies with ARNTL knockout mice [28], both male and female homozygous knockout mice were infertile or had reduced fertility [29–32]. Their results indicated an inability to carry on viable pregnancies and some degree of embryo lethality in these mice. The reason for this infertility remains unclear, but Alvarez et al. (2008) speculated that it was most likely to result from altered levels of reproductive hormones. We found that the TT genotype of ARNTL rs2278749 was linked to a higher number of pregnancies and a higher number of miscarriages, whereas and the
Here, we thus hypothesize that the effect of \textit{NPAS2} rs2305160 on seasonality may, at least in part, be mediated via an influence on testosterone levels.

\textit{NPAS2} rs2905160 is in LD with \textit{NPAS2} rs1541353 (S471L) that has been demonstrated to associate with hypertension as part of the metabolic syndrome [42]. Here, our results demonstrate that the \textit{A}^+\text{ allelic status of \textit{NPAS2} rs6725296 was associated with loadings on the metabolic factor of the global seasonality score, which is composed of seasonal variation of weight and appetite. The role of \textit{NPAS2} in eating behavior is supported by an observation that \textit{NPAS2} deficient mice have impaired ability to adapt to restricted feeding schedule [43]. As the circadian, metabolic and cell-division cycles appear to be coordinated in a similar way, deficient \textit{NPAS2} may have an adverse effect on these functions [25,44,45]. On the other hand, experienced poor lighting increases the risk of the metabolic syndrome through its contribution to the metabolic factor of the global seasonality score [46]. Our findings now suggest that seasonal weight gain bridges \textit{NPAS2} and hypertension to the pathogenesis of the metabolic syndrome. Exposures to light or lighting conditions may modify the pathogenesis through their actions on testosterone production [47] and its subsequent effects. Here, no significant association was observed for \textit{NPAS2} rs11541353 even though this SNP has been linked to seasonal affective disorder [15], the association however being only with the clinical diagnosis but not with the GSS. In the current study, we analyzed individuals whose seasonal variations were assessed but who had no diagnosis of mental disorder, and therefore the findings do not disagree. With respect to \textit{ARNTL}, the \textit{TT} genotype of rs2290035 was associated with less seasonal variation in energy level. In our earlier study, it was the heterozygote genotype that was overrepresented among patients with winter depression [16] of which 96% report routine seasonal variations in mood, weight, appetite and energy level.

As a new finding, we found an association of \textit{CLOCK} rs2412646 with social activity. Support for the role of \textit{CLOCK} in the behavioral activity is provided by a study in which another \textit{CLOCK} variant, rs1801260 (T311C), was linked to the diurnal activity levels in depressed patients [49,50], and on the other hand in mice disruption of \textit{CLOCK} contributes to overactive or manic-like behavior [51]. Social withdrawal is a common feature in depression and \textit{CLOCK} gene polymorphisms have been associated with mood disorders [52,53] even though contradictory studies exist [54–56]. In addition, \textit{NPAS2} is a close analog of \textit{CLOCK} and its polymorphisms have earlier been associated with autism [57], a disorder characterized among others with impaired social interaction and communication.

Our study sample derives from the Finnish population, which has reduced genetic and environmental heterogeneity [58]. Moreover, the sample was identified from an epidemiological cohort that is well-characterized for somatic and psychiatric wellbeing allowing detailed phenotype analysis. However, the small sample size for reproductive phenotypes in women, number of SNPs per gene and multiple testing need to be seen as limitations. The most significant association between \textit{ARNTL} rs2278749 and increased risk of miscarriages remains significant after false discovery rate analysis. To assess the significance of the findings replication in other cohorts is needed.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Gene</th>
<th>SNP</th>
<th>Test</th>
<th>Re</th>
<th>Beta</th>
<th>S.E.</th>
<th>L95% CI</th>
<th>U95% CI</th>
<th>P-value</th>
<th>q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of pregnancies</td>
<td>\textit{ARNTL}</td>
<td>rs2278749</td>
<td>REC</td>
<td>TT</td>
<td>7.2</td>
<td>2.12</td>
<td>3.04</td>
<td>11.35</td>
<td>0.001</td>
<td>0.28</td>
</tr>
<tr>
<td>Number of miscarriages</td>
<td>\textit{ARNTL}</td>
<td>rs2278749</td>
<td>REC</td>
<td>TT</td>
<td>4.65</td>
<td>0.83</td>
<td>3.03</td>
<td>6.27</td>
<td>3 \times 10^{-7}</td>
<td>0.0004</td>
</tr>
<tr>
<td>Number of miscarriages</td>
<td>\textit{NPAS2}</td>
<td>rs11673746</td>
<td>DOM</td>
<td>T+</td>
<td>−0.64</td>
<td>0.2</td>
<td>−1.03</td>
<td>−0.25</td>
<td>0.002</td>
<td>0.33</td>
</tr>
<tr>
<td>GSS</td>
<td>\textit{NPAS2}</td>
<td>rs2305160</td>
<td>DOM</td>
<td>A+</td>
<td>−0.81</td>
<td>0.24</td>
<td>−1.29</td>
<td>−0.33</td>
<td>0.0009</td>
<td>0.28</td>
</tr>
<tr>
<td>GSS factor 1</td>
<td>\textit{NPAS2}</td>
<td>rs6725296</td>
<td>ADD</td>
<td>A</td>
<td>0.27</td>
<td>0.08</td>
<td>0.11</td>
<td>0.42</td>
<td>0.0008</td>
<td>0.28</td>
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<tr>
<td>GSS factor 1</td>
<td>\textit{NPAS2}</td>
<td>rs6725296</td>
<td>DOM</td>
<td>A+</td>
<td>0.27</td>
<td>0.08</td>
<td>0.1</td>
<td>0.43</td>
<td>0.001</td>
<td>0.33</td>
</tr>
<tr>
<td>Seasonal variation in weight</td>
<td>\textit{NPAS2}</td>
<td>rs6725296</td>
<td>ADD</td>
<td>A</td>
<td>1.95</td>
<td>0.2</td>
<td>1.32</td>
<td>2.89</td>
<td>0.0009</td>
<td>0.28</td>
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<tr>
<td>Seasonal variation in weight</td>
<td>\textit{NPAS2}</td>
<td>rs6725296</td>
<td>DOM</td>
<td>A+</td>
<td>1.95</td>
<td>0.21</td>
<td>1.29</td>
<td>2.96</td>
<td>0.002</td>
<td>0.33</td>
</tr>
<tr>
<td>Seasonal variation in energy level</td>
<td>\textit{ARNTL}</td>
<td>rs2290035</td>
<td>REC</td>
<td>TT</td>
<td>0.45</td>
<td>0.24</td>
<td>0.28</td>
<td>0.72</td>
<td>0.0008</td>
<td>0.28</td>
</tr>
<tr>
<td>Social activity</td>
<td>\textit{CLOCK}</td>
<td>rs2412646</td>
<td>REC</td>
<td>TT</td>
<td>0.2</td>
<td>0.53</td>
<td>0.07</td>
<td>0.56</td>
<td>0.002</td>
<td>0.38</td>
</tr>
</tbody>
</table>

*Results presented for the rare allele/genotype.

Abbreviations: GSS, Global Seasonality Score; SNP, single-nucleotide polymorphism; REC, recessive model; DOM, dominant model; ADD, additive model; Re, reference allele/genotype(s); OR, odds ratio; S.E., standard error; L95% CI, lower bound of the 95% confidence interval; U95% CI, upper bound of the 95% confidence interval.

doi:10.1371/journal.pone.0010007.t003
To conclude, we found that genetic variations in Arntl and Npas2 genes associated with fertility and seasonality. Our results thus support the previous observation of the role of these genes in seasonal physiology, whereas this is the first time circadian clock related genetic variants are reported to associate with the human fertility.

Supporting Information

Text S1 Analyzed phenotypes related to mental well-being. Found at: doi:10.1371/journal.pone.0010007.s001 (0.02 MB PDF)

Table S1 SNPs with p-value of less than or equal to 0.01. Found at: doi:10.1371/journal.pone.0010007.s002 (0.02 MB PDF)

References