NPAS2 and PER2 are linked to risk factors of the metabolic syndrome

Ani Englund*†1, Leena Kovanen†1, Sirkku T Saarikoski1, Jari Haukka1,3, Antti Reunanen2, Arpo Aromaa2, Jouko Lönnqvist1 and Timo Partonen1

Address: 1Department of Mental Health and Alcohol Research, National Public Health Institute, Mannerheimintie 166, FI-00300 Helsinki, Finland, 2Department of Health and Functional Capacity National Public Health Institute, Mannerheimintie 166, FI-00300 Helsinki, Finland and 3Department of Biostatistics and Epidemiology Cluster, International Agency for Research on Cancer, Lyon, France (Haukka)

Email: Ani Englund* - ani.englund@thl.fi; Leena Kovanen - leena.kovanen@thl.fi; Sirkku T Saarikoski - sirkku.saarikoski@thl.fi; Jari Haukka - jari.haukka@thl.fi; Antti Reunanen - antti.reunanen@thl.fi; Arpo Aromaa - arpo.aromaa@thl.fi; Jouko Lönnqvist - jouko.lonnqvist@thl.fi; Timo Partonen - timo.partonen@thl.fi

* Corresponding author †Equal contributors

Abstract

Background: Mammalian circadian clocks control multiple physiological events. The principal circadian clock generates seasonal variations in behavior as well. Seasonality elevates the risk for metabolic syndrome, and evidence suggests that disruption of the clockwork can lead to alterations in metabolism. Our aim was to analyze whether circadian clock polymorphisms contribute to seasonal variations in behavior and to the metabolic syndrome.

Methods: We genotyped 39 single-nucleotide polymorphisms (SNP) from 19 genes which were either canonical circadian clock genes or genes related to the circadian clockwork from 517 individuals drawn from a nationwide population-based sample. Associations between these SNPs and seasonality, metabolic syndrome and its risk factors were analyzed using regression analysis. The p-values were corrected for multiple testing.

Results: Our findings link circadian gene variants to the risk factors of the metabolic syndrome, since Npas2 was associated with hypertension (P-value corrected for multiple testing = 0.0024) and Per2 was associated with high fasting blood glucose (P-value corrected for multiple testing = 0.049).

Conclusion: Our findings support the view that relevant relationships between circadian clocks and the metabolic syndrome in humans exist.
metabolic futile cycle can provide the animal with those circadian signals needed for reset [5]. When there exists no light-dark transitions to reset the principal clock, reindeer living above the Arctic Circle use the metabolic cycles as the reference instead [6].

The molecular circadian clock consists of multiple positive and negative feedback loops that generate the 24-hour oscillation of target genes. In the positive loop NPAS2 (MOP4) protein [7], which plays an overlapping role with the CLOCK protein [8], pairs up with ARNTL (BMAL1 or MOP3) protein. These heterodimers activate the transcription of target genes (for review, see [9]). Downstream, PER and CRY proteins pair up and execute the negative loop. Nuclear receptor co-activators and repressors and several post-transcriptional modifications are necessary for clock precision. In addition, clockwork output molecules can provide an input to the following cycles [10].

Circadian clocks and energy metabolism are linked because the disruptions of the clockwork lead to alterations in metabolism and vice versa (for review, see [11]). Mutation in the Clock gene leads to metabolic syndrome in mice [12], and in humans Clock polymorphisms have been associated with obesity and metabolic syndrome [13,14]. Cellular metabolic states can serve as a link between stimuli from the habitat and drive for the clockwork, because the reduced forms of nicotinamide adenine dinucleotide cofactors stimulate DNA binding of the NPAS2-ARNTL [15] and CLOCK-ARNTL [16] heterodimers, whereas the oxidized forms inhibit the binding [17]. Npas2-deficient mice have reduced ability to adapt to restricted feeding [18], whereas Clock-deficient mice adapt to it even better than do wild-type mice [19], suggesting a key role of NPAS2.

Herein, we hypothesized that circadian clock polymorphisms contribute to the routine seasonal variations and to the metabolic syndrome. Our earlier finding that sea-

Methods

The study was part of a nationwide health interview and examination survey, the Health 2000 Study, which was carried out in Finland, a north-eastern (60–70°N, 20–31°E) European country with about 5 million inhabitants, from September 2000 to June 2001. The two-stage stratified cluster sampling design was planned by Statistics Finland. The sampling frame comprised adults living in mainland Finland. This frame was regionally stratified according to the five university hospital regions, or catchments areas, each containing roughly one million inhabitants. From each university hospital region, 16 health care districts were sampled as clusters (80 health care districts in the whole country, including 160 municipalities, or groups of municipalities with joint primary care). The 15 biggest health care districts in the country were all selected in the sample and their sample sizes were proportional to population size. The remaining 65 health care districts were selected by systematic probability proportional to size sampling in each stratum, and their sample sizes (ranging from 30 to 100) were equal within each university hospital region, the total number of persons drawn from a university hospital region being proportional to the corresponding population size. The 80 health care districts were the primary sampling units, and the ultimate sampling units were persons who were selected by systematic sampling from the health centre districts. From these 80 health care districts, a random sample of individuals was drawn using the data provided by Population Register Centre. Its population information system contains the official information for the whole country on the Finnish citizens and aliens residing permanently in Finland. All the persons aged 30 and over (n = 8028) who were identified from the nationally representative sample by The Social Insurance Institution of Finland were contacted in person. Interviewers attended training sessions on the specific themes that were to be covered in the computer-assisted interviews. Of the final sample of 7979 persons, 6986 (88%) were interviewed at home or in the examination at home or in an institution. Overall, 84% participated either in the health status examination proper or in the examination at home. All the methods are reported in more detail on the Internet site of the Health 2000 http://www.ktl.fi/health2000.

Phenotype data

All participants had been asked to come to the health status examination fasting at least 4 hours and without drinking on the same day. In the laboratory, a nurse recorded how these instructions had been followed and then took the blood samples. The samples were centrifuged at the examination site and placed into deep freezers at -20°C before they were transferred within one week to the National Public Health Institute and stored in deep freezers at -70°C. Routine fasting laboratory tests included the concentrations of blood glucose and those of serum total cholesterol and triglycerides (Glucose Hexokinase, Cholesterol CHOD PAP and Triglycerides GPO PAP, Olympus System Reagent, Germany), those of HDL cholesterol and low-density lipoprotein (LDL) cholesterol (HDL-C Plus and LDL-C Plus, Roche Diagnostics GmbH, Germany), and those of gamma-glutamyltransferase (GGT) and uric acid (IFCC/ECCLS and URIKAASI PAP, Konelab, Thermo Electron Oy, Finland).
The diagnostic mental health interview was performed at the end of the comprehensive health examination. The computerized version of the CIDI (M-CIDI) was used. The program uses algorithms to meet the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria and allows the estimation of DSM-IV diagnoses for major disorders [21]. The translation of the M-CIDI into Finnish was made pair wise by psychiatric professionals and revised by others. The official Finnish translation of the DSM-IV classification was used as a basis for formulating the interview. The process included consensus meetings, third expert opinions, an authorized translator’s review, and testing with both informed test subjects and unselected real subjects [22]. Interviews were performed to determine the 12-month prevalence rates of major depressive episodes and disorder, dysthymia, general anxiety disorder, panic disorder with or without agoraphobia, social phobia, alcohol abuse and dependence, and other substance dependence and abuse.

As part of the assessment, the participants filled in the items of lifetime seasonal variations in mood and behavior taken and adapted from the Seasonal Pattern Assessment Questionnaire (SPAQ) [23]. The questionnaire was translated into Finnish and then back-translated to revise the linguistic accuracy. Each of the six items of sleep length, social activity, mood, weight, appetite, and energy level was scored from 0 to 3 (none, slight, moderate or marked change), not from 0 to 4 (none, slight, moderate, marked or extremely marked change), with the sum or global seasonality score (GSS) ranging from 0 to 18. A dichotomous variable depicting seasonality was derived from the distribution of global scores on the modified questionnaire and based on the provisional criteria similar to the original ones [24], the GSS ranging from 0–7 (not affected) and 8–18 points (affected).

There are several definitions for metabolic syndrome and its risk factors. In this study we used US Adult Treatment Panel III of the National Cholesterol Education Program (NCEP-ATPIII) criteria [NCEP 2002] and the International Diabetes Federations (IDF) criteria [IDF 2005] to determine metabolic syndrome.

The US Adult Treatment Panel III of the National Cholesterol Education Program (NCEP-ATPIII) criteria for metabolic syndrome is [NCEP 2002] defined as having at least three of the following components: the fasting blood glucose level 6.1 mmol/l or higher, the high blood pressure (systolic pressure 130 mmHg or more or diastolic pressure 85 mmHg or more), the serum triglycerides level 1.7 mmol/l or higher, the serum high-density lipoprotein cholesterol level lower than 1.0 mmol/l for men or lower than 1.3 mmol/l for women, or the waistline 102.1 cm or more for men or 88.1 cm or more for women.

The International Diabetes Federations (IDF) criteria for metabolic syndrome [IDF 2005] is defined as having waistline of 94 cm or more for men or 80 cm or more for women and at least two of the following components: the serum triglycerides level 1.7 mmol/l or higher, the serum high-density lipoprotein cholesterol level lower than 1.02 mmol/l for men or lower than 1.29 mmol/l for women, high blood pressure in terms of systolic pressure 130 mmHg or more or diastolic pressure 85 mmHg or more or treatment for previously diagnosed hypertension and raised fasting plasma glucose level 5.6 mmol/l or higher, or previously diagnosed type 2 diabetes.

The individual risk factor variables are listed below. These include the variables forming the criteria’s above and in addition supplemental variables, that World Health Organization (WHO) and European Group for the Study of Insulin Resistance (EGIR) consider as risk factors for metabolic syndrome and American Association of Clinical Endocrinologists (AACE) use to define Insulin Resistance Syndrome.

The blood pressure was defined high when mean value of systolic blood pressure was 140 mmHg or more or diastolic blood pressure was 90 mmHg or more. A variable taking into account high blood pressure and in addition a treatment for previously diagnosed hypertension was created. We also used a variable which defined blood pressure high when mean value of systolic blood pressure was 130 mmHg or more or diastolic blood pressure was 85 mmHg or more. A variable with preceding and hypertension medication was also included in the study.

The serum high-density lipoprotein (HDL) cholesterol level was considered low when it was lower than 1.02 mmol/l for men or lower than 1.29 mmol/l for women. We also used another variable with thresholds of 1.0 mmol/l and 1.3 mmol/l, respectively. The triglyceride levels were considered raised if they were higher than 1.7 mmol/l in both genders. A variable taking into account raised triglyceride levels and also the low HDL cholesterol in terms of 0.9 mmol/l or less in men and 1.0 mmol/l in women was used. A variable with triglycerides termed high when higher than 2 mmol/l or HDL was less than 1.0 mmol/l or person was using lipid medication was also used in this study.

Plasma glucose levels were measured after fasting at least for 4 hours. The first variable considered fasting plasma glucose levels raised if they were 6.1 mmol/l or higher. The second variable was positive if the fasting glucose levels were between 6.1–6.9 mmol/l. The third variable was positive if fasting plasma glucose levels were 5.6 mmol/l or higher, or the individual had previously diagnosed type 2 diabetes.
Waist circumference was measured in centimeters. We also used two additional variables to define the waistline status: In the first variable circumference was considered high when it was 102 cm or more for men or 88 cm or more for women, in the second variable the values were 94 cm or more for men or 80 cm or more for women. The waist/hips circumference ratio was determined high when it was 0.9 or more for men or 0.85 or more for women.

**Study sample**

Overall, the 5480 individuals participated in the health status examination and the diagnostic mental health interview, filled in the self-report of seasonal changes in mood and behavior and gave venous blood samples for DNA extraction and were screened with the M-CIDI interview to have no mental illness according to the DSM-IV criteria. Among these individuals, 517 were randomly selected to form the final study sample.

**Gene and SNP selection**

A total of 39 single-nucleotide polymorphisms (SNPs) of 19 genes were genotyped (Table 1). Herein, we wanted to focus on the circadian clock and selected genes which were either canonical circadian clock genes (Arntl, Arntl2, Clock, Cry2, Npas2, Per2 and Timeless) or genes having their influence on pathways related to the circadian clockwork (Adcyap1, Drd2, Opn4, Npy, Vip, Vipr2, Fdft1). Since the circadian clockwork and sleep are interactive, specific sleep-related genes were included (Acads, Ada and Glo1). Arntl2 was included in the study because it has significant homology with Arntl1 and Ncoa because it has significant sequence homology with Clock and therefore a possible role in the circadian clock [25,26]. Both candidate SNPs and tag-SNPs were included in this study. Candidate SNPs were selected based on their possible functional potential including variation resulting in amino acid change (i.e. missense, Table 1) and SNPs previously reported to have significant results are presented in Table 2.

**Genomic DNA analysis**

Genomic DNA was isolated from the whole blood according to standard procedures. SNPs were genotyped with a fluorogenic 5' nuclease assay method (TaqMan™) with pre-designed primer-probe kits (TaqMan® Pre-Designed SNP Genotyping Assays) using the Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Foster City, California, USA) according to the instructions provided by the manufacturer.

Custom TaqMan® SNP Genotyping Assays were used for three SNPs. The primers sequences were CGCACCCAT and TGGGCCCCCTAAGC and the reporter sequences ACITTTGGCTGTGC and ACITTTGGGCITTTGGA for ADA 22G>A (Asp8Asn), AAGCCGACCTTGCCCTG and ACAAGGAGCCGGGTTCGT and the reporter sequences CTTGGGCAATTTCAT and TTTGCCGGTGTTTCAT and PER2 10870, and GCTCAGCAGCAGCTT GAA and CGAATCTGCCATGTTTCTATT and the reporter sequences CTTGCTACAAGTGCTCCTC and TTGCTACAGGTAATCTC for FDF1 rs11549147.

All samples were successfully genotyped, yielding the success rate of 100% for all SNPs, and about 5% of samples were re-genotyped to confirm the genotyping results. The following three SNPs were not in the Hardy-Weinberg equilibrium: ARNTL rs1982350 (P = 0.01), ARNTL rs6486120 (P = 0.009) and PER2 rs934945 (P = 0.05).

**Statistical analysis**

Genotype frequencies, allele frequencies and Hardy-Weinberg p-values were calculated with the Pearson exact test. Only those haplotypes occurring with a frequency >0.05 were considered. The linkage disequilibrium (LD) between the SNPs analyzed was estimated. The remaining 35 SNPs were tested using additive model. Coefficients, odds ratios (OR) and their 95% confidence intervals (CI) were calculated. The sex and age were controlled for these analyses. The p-values were corrected to reduce the false positives resulting from multiple testing by using an approximation of Bonferroni-p-values: we selected associations with significant p-values and low false discovery rates (FDR below 0.05) and then corrected the p-values with the number of the genes analyzed (17). Statistical analysis was performed using the R software, version 2.5.0 [27], and the PLINK software, version v1.04 [28].

**Ethics**

The study project was coordinated by the National Public Health Institute and implemented in collaboration with social insurance organizations and the Ministry of Social Affairs and Health. It provided a written informed consent to each participant, giving a full description of the protocol before signing it. The procedures were according to the ethical standards of the responsible committee on human experimentation and with the Declaration of Helsinki, its amendments and revision.

**Results**

The allele frequencies and genotype distributions of the SNPs are shown in Table 1. The first 100 samples genotyped indicated that in our Finnish study population four SNPs were not polymorphic, including Arntl2 rs35878285, Cry2 rs2863712, Ncoa3 rs2230783 and Per2 S662G, so these were excluded from further analysis. Each polymorphic SNP was then analyzed in relation to seasonality and to metabolic syndrome risk factors. The significant results are presented in Table 2.
Table 1: Genotypes and allele frequencies.

| Gene  | SNP   | Mutation Type | Allele 1 | Allele 2 | n1 (freq) | n2 (freq) | n11 (freq) | n12 (freq) | n22 (freq) |
|-------|-------|---------------|----------|----------|-----------|-----------|------------|------------|------------|
| Acads | rs1799958 | missense | G | A | 768 (0.74) | 266 (0.26) | 283 (0.55) | 202 (0.39) | 32 (0.06) |
| Ada   | 22G>A | missense | G | A | 978 (0.95) | 56 (0.05) | 461 (0.89) | 56 (0.11) | 0          |
| Adcyap1 | rs2856966 | missense | A | G | 850 (0.82) | 184 (0.18) | 344 (0.67) | 162 (0.31) | 11 (0.02) |
| Arntl | rs6468120 | intronic | G | T | 744 (0.72) | 290 (0.28) | 280 (0.54) | 184 (0.36) | 53 (0.10) |
|       | rs1982350 | intronic | G | A | 587 (0.57) | 447 (0.43) | 181 (0.35) | 225 (0.44) | 111 (0.21) |
|       | rs3186360 | intronic | C | T | 552 (0.53) | 482 (0.47) | 152 (0.29) | 248 (0.48) | 117 (0.23) |
|       | rs2278749 | intronic | C | T | 823 (0.80) | 211 (0.20) | 328 (0.63) | 167 (0.32) | 22 (0.04) |
|       | rs2290035 | intronic | A | T | 595 (0.58) | 439 (0.42) | 175 (0.34) | 245 (0.47) | 97 (0.19) |
| Arnt2 | rs7958822 | intronic | G | A | 560 (0.54) | 474 (0.46) | 147 (0.28) | 266 (0.51) | 104 (0.20) |
|       | rs4964057 | intronic | T | G | 601 (0.58) | 433 (0.42) | 178 (0.34) | 245 (0.47) | 94 (0.18) |
|       | rs1037921 | missense | A | G | 947 (0.92) | 87 (0.08) | 433 (0.84) | 81 (0.16) | 3 (0.01) |
|       | rs2306074 | intronic | T | C | 668 (0.65) | 366 (0.35) | 213 (0.41) | 242 (0.47) | 62 (0.12) |
|       | rs35878285 | mis-sense | A | 1034 (1.00) | 517 (1.00) |        |            |            |            |
| Clock | rs2412646 | intronic | C | T | 760 (0.74) | 274 (0.26) | 280 (0.54) | 200 (0.39) | 37 (0.07) |
|       | rs11240 | intronic | C | G | 696 (0.67) | 338 (0.33) | 227 (0.44) | 242 (0.47) | 48 (0.09) |
|       | rs2412648 | intronic | T | G | 654 (0.63) | 380 (0.37) | 210 (0.41) | 234 (0.45) | 73 (0.14) |
|       | rs3805151 | intronic | T | C | 613 (0.59) | 421 (0.41) | 183 (0.35) | 247 (0.48) | 87 (0.17) |
| Cry2  | rs2863712 | missense | T | 1034 (1.00) | 517 (1.00) |        |            |            |            |
| Drd2  | rs1800497 | missense | G | A | 838 (0.81) | 196 (0.19) | 336 (0.65) | 166 (0.32) | 15 (0.03) |
|       | rs6277 | silent | G | A | 542 (0.52) | 492 (0.48) | 141 (0.27) | 260 (0.50) | 116 (0.22) |
| Fdft1 | rs11549147 | missense | A | G | 944 (0.91) | 90 (0.09) | 431 (0.83) | 82 (0.16) | 4 (0.01) |
| Glo1  | rs2736654 | missense | T | G | 662 (0.64) | 372 (0.36) | 207 (0.40) | 248 (0.48) | 62 (0.12) |
| Opn4  | rs1079610 | missense | T | C | 714 (0.69) | 320 (0.31) | 246 (0.48) | 222 (0.43) | 49 (0.09) |
| Ncoa3 | rs6094752 | missense | C | T | 1003 (0.97) | 31 (0.03) | 486 (0.94) | 31 (0.06) | 0          |
|       | rs2230782 | missense | G | C | 932 (0.9) | 102 (0.1) | 422 (0.82) | 88 (0.17) | 7 (0.01) |
|       | rs2230783 | missense | T | 1034 (1.00) | 517 (1.00) |        |            |            |            |
| Npas2 | rs11541353 | missense | C | T | 859 (0.83) | 175 (0.17) | 358 (0.69) | 143 (0.28) | 16 (0.03) |
|       | rs2305160 | missense | G | A | 727 (0.7) | 307 (0.3) | 252 (0.49) | 223 (0.43) | 42 (0.08) |
We found associations with circadian clock genes and the risk factors for metabolic syndrome. \textit{Npas} rs11541353 was associated with hypertension, the minor allele being protective against hypertension (T vs. C, OR = 0.54, Corrected P-value = 0.02). The results almost the same when people getting treatment for their hypertension were included in group (T vs. C, OR = 0.53, Corrected P-value = 0.015).

\textit{Per2} 10870 was associated with glucose metabolism. 10870 minor allele reduced the risk of raised plasma glucose (G vs. A, Beta coefficient = -0.010, Corrected P-value = 0.049).

**Discussion**

Our main results herein are that \textit{Npas2} is linked to hypertension and that \textit{Per2} is associated with blood glucose levels.

---

### Table 1: Genotypes and allele frequencies. (Continued)

| Gene | SNP      | Type     | T  | C  | 956 (0.92) | 78 (0.08) | 444 (0.86) | 68 (0.13) | 5 (0.01) |
|------|----------|----------|----|----|------------|-----------|------------|-----------|----------|
| \textit{Npy} | rs16139  | missense |    |    |            |           |            |           |          |
| \textit{Per2} | rs934945 | missense | C  | T  | 917 (0.89) | 117 (0.11) | 402 (0.78) | 113 (0.22) | 2 (0.004) |
|                | rs2304672 | UTR 5'   | G  | C  | 865 (0.84) | 169 (0.16) | 361 (0.70) | 143 (0.28) | 13 (0.03) |
|                | S662G    | missense | T  |    | 1034 (1.00) |            | 517 (1.00) |           |          |
| \textit{Plcb4} | rs6077510 | missense | A  | G  | 552 (0.53) | 482 (0.47) | 142 (0.27) | 268 (0.52) | 107 (0.21) |
| \textit{Timeless} | rs2291739 | missense | A  | G  | 624 (0.6)  | 410 (0.4)  | 193 (0.37) | 238 (0.46) | 86 (0.17) |
|                | rs2291738 | intronic | C  | T  | 546 (0.53) | 488 (0.47) | 147 (0.28) | 252 (0.49) | 118 (0.23) |
| \textit{Vip}  | rs3823082 | intronic | C  | T  | 854 (0.83) | 180 (0.17) | 351 (0.68) | 152 (0.29) | 14 (0.03) |
|                | rs688136  | UTR 3'   | T  | C  | 676 (0.65) | 358 (0.35) | 221 (0.43) | 234 (0.45) | 62 (0.12) |
| \textit{Vipr2} | rs885863 | UTR 3'   | T  | C  | 518 (0.50) | 516 (0.50) | 126 (0.24) | 266 (0.51) | 125 (0.24) |

a) dbSNP symbols [http://www.ncbi.nlm.nih.gov/SNP](http://www.ncbi.nlm.nih.gov/SNP)

b) Alleles extracted from HapMap [http://www.HapMap.org](http://www.HapMap.org)
c) Total number of alleles in study sample, frequencies in parenthesis.

---

### Table 2: Results from one-SNP analysis.

| Variable                      | Gene | SNP      | P-value | P-value corrected for multiple testing | Beta-coefficient | 95% CI       |
|-------------------------------|------|----------|---------|----------------------------------------|-----------------|-------------|
| Fasting blood glucose level (Logarithmic) a | \textit{Per2} | #10870  | 0.002   | 0.049                                  | -0.010          | -0.016—-0.035 |

| Variable                      | Gene | SNP      | P-value | P-value corrected for multiple testing | Odds ratio    | 95% CI       |
|-------------------------------|------|----------|---------|----------------------------------------|---------------|-------------|
| High blood pressure b         | \textit{Npas2} | rs11541353 | 0.001   | 0.02                                   | 0.54          | 0.37—0.79    |
| High blood pressure or hypertension medication c | \textit{Npas2} | rs11541353 | <0.001  | 0.015                                  | 0.53          | 0.36—0.77    |

**Table 2: Results from one-SNP analysis.**

| Variable                      | Gene | SNP      | P-value | P-value corrected for multiple testing | Odds ratio    | 95% CI       |
|-------------------------------|------|----------|---------|----------------------------------------|---------------|-------------|
| Fasting blood glucose level (Logarithmic) a | \textit{Per2} | #10870  | 0.002   | 0.049                                  | -0.010          | -0.016—-0.035 |

| Variable                      | Gene | SNP      | P-value | P-value corrected for multiple testing | Odds ratio    | 95% CI       |
|-------------------------------|------|----------|---------|----------------------------------------|---------------|-------------|
| High blood pressure b         | \textit{Npas2} | rs11541353 | 0.001   | 0.02                                   | 0.54          | 0.37—0.79    |
| High blood pressure or hypertension medication c | \textit{Npas2} | rs11541353 | <0.001  | 0.015                                  | 0.53          | 0.36—0.77    |

- Single SNPs were analyzed using linear regression for continuous variables and logistic regression for dichotomous variables. Beta-coefficients were calculated for continuous variables, odds ratios for dichotomous variables. The sex and age were controlled for these analyses. P-values corrected for multiple testing were calculated.

- a) The concentrations of blood glucose (mmol/L) after fasting at least 4 hours and without drinking on the same day. The variable was log-transformed to obtain the normal distribution.

- b) The blood pressure was defined high when systolic pressure was 140 mmHg or higher or diastolic pressure was 90 mmHg or higher.

- c) High blood pressure (b) or treatment for previously diagnosed hypertension.
Seasonality and disruption of circadian molecular clockwork are risk factors for metabolic syndrome ([12, 20]). We now found that the common risk factors for metabolic syndrome are associated with polymorphisms in circadian clock genes. Npas2 rs11541353 was associated with hypertension in the Finnish population. Earlier, Arntl was linked to hypertension and type 2 diabetes mellitus [29]. Now, we demonstrate herein the associations of Npas2 with hypertension and of Per2 with blood glucose levels.

Together these earlier findings and those of ours emphasize the importance of the circadian system and its core genes in regulation of blood pressure, and point to a role in pathological situations. Moreover, they parallel to SAD in which there is a strong metabolic component and with which this unit of ARNTL, NPAS2 and PER2 is associated [30]. There are often not only disturbances in the metabolic networks [31] but also disruptions of the circadian rhythms [32] together with pronounced seasonal changes in mood and behavior [33] in individuals having affective disorders. Now, this may concern the general population as well.

Npas2 rs11541353 is a missense mutation, leading to substitution of serine with leusine in the amino acid position 471. Npas2 rs11541353 minor allele was protective against hypertension and heterozygosity of Npas2 rs11541353 is protective against Seasonal affective disorder (SAD) [30]. These findings reveal that protection from seasonal variations and protection from high blood pressure go hand in hand in some cases. However, Partonen et al. also found that homozygosity for both Npas2 rs11541353 minor and major alleles was a major risk factor for SAD. Combining these results, persons with two major alleles of Npas2 rs11541353 have substantially increased risk not only for SAD but also for hypertension. However, when a person has two Npas2 rs11541353 minor alleles, the results are difficult to interpret, as the homozygosity increases the odds for SAD, but protects against hypertension. Next, the phenotypes in terms of SAD and hypertension in Npas2 rs11541353 homozygous and heterozygous persons need to be analyzed.

Our results indicate, that Per2 10870 contributed to changes in glucose metabolism. Per2 10870 is an intronic mutation originally found by Spanagel et al. (2005), when searching for the Per2 SNPs modulating alcohol intake in mice. Its minor allele G was protective against high alcohol intake in humans [34] but increased the odds for SAD [30]. In our current study, the minor allele G reduced the risk for raised plasma glucose levels. Lamia et al. previously demonstrated that Per1−/−;Per2−/− mice have altered blood glucose homeostasis [35]. Another recent study demonstrated that administration of metformin, one of the most commonly used drugs for type 2 diabetes, leads to the degradation of PER2 and to a phase advance in the

### Table 3: Single SNP analysis with corrected p-values = 0.10.

| Variable                               | Gene | SNP   | P-value | P-value corrected for multiple testing | Beta-coefficient | 95% CI  |
|----------------------------------------|------|-------|---------|----------------------------------------|-----------------|---------|
| Fasting glucose level (Logarithmic) a  | D2   | rs6277| 0.003   | 0.051                                  | -0.008          | -0.012–0.003 |
| Waist circumference b                  | PLC4 | rs6077510 | 0.004  | 0.085                                  | 2.0             | 0.63–3.4    |
| High waist circumference c             | Per2 | rs934945 | 0.003  | 0.062                                  | 1.9             | 1.2–3.0     |
| Low HDL cholesterol d                  | Vip2 | rs885863 | 0.004  | 0.069                                  | 1.5             | 1.1–2.0     |
| Metabolic syndrome [IDF] f             | Per2 | rs934945 | 0.004  | 0.070                                  | 1.9             | 1.2–2.9     |

Single SNPs were analyzed using linear regression for continuous variables and logistic regression for dichotomous variables. Betacoefficients were calculated for continuous variables, odds ratios for dichotomous variables. The sex and age were controlled for these analyses. P-values corrected for multiple testing were calculated.

a) The concentrations of blood glucose (mmol/L) after fasting at least 4 hours and without drinking on the same day. The variable was log-transformed to obtain the normal distribution.
b) Waist circumference in centimeters
c) Waist circumference 94 cm or more for men or 80 cm or more for women

d) Serum high-density lipoprotein (HDL) cholesterol level was considered low when it was lower than 1.02 mmol/l for men or lower than 1.29 mmol/l for women.
f) Metabolic syndrome was assessed using the International Diabetes Federations (IDF) criteria [IDF 2005] and defined as having waistline of 94 cm or more for men or 80 cm or more for women and at least two of the following components: the serum triglycerides level 1.7 mmol/l or higher, the serum high-density lipoprotein cholesterol level lower than 1.02 mmol/l for men or lower than 1.29 mmol/l for women, high blood pressure in terms of systolic pressure 130 mmHg or more or diastolic pressure 85 mmHg or more or treatment for previously diagnosed hypertension and raised fasting plasma glucose level 5.6 mmol/l or higher, or previously diagnosed type 2 diabetes.
circadian gene expression [36]. It remains to be elucidated whether PER proteins are independently important for glucose homeostasis or does their role in the circadian clock lead to the effects seen.

Woon et al. found association between Arntl and hypertension and type 2 diabetes mellitus [29]. Our SNP selection did not include the SNPs used in their study, which can explain why we failed to see any associations. Recent studies have also found association between Clock-gene polymorphism and the metabolic syndrome in man [13,14]. It is of note that we did not find support to these links in our study. We did, however, find several interesting associations, which failed to show statistically significant p-values after correction (Table 3). These include associations between DRD2 rs6277 and blood glucose levels, PLCB4 rs6077510 and Per2 SNP rs934945 and waist circumference, and Vipr2 rs885863 and low HDL cholesterol level. In addition, Per2 SNP rs934945 was associated with the metabolic syndrome.

There are some limitations in our study. We relied on a self-report questionnaire when assessing the seasonal variations in mood and behavior. However, this questionnaire has been reported to have high sensitivity and specificity [37] and can be regarded as valid for the lifet ime-retrospective assessment of routine seasonal variations in mood and behavior.

Our study bears several strengths. This was a population-based and nation-wide study. Its sample size was relatively big and representative of the general population aged over 30 living in a northern European country, Finland. Hence, these data can be generalized directly to concern the whole adult population of Finland, or any population having similar living conditions. We had rich phenotype data with reliable laboratory tests and valid assessments of syndromes on our focus. The single-nucleotide polymorphisms used were selected for their potential role in the function of the gene, which augments the possibility that the genotype seen here contributes to the phenotype although experimental analysis is needed for verification.

Conclusion
Our findings herein link the circadian gene variants and risk factors of the metabolic syndrome. Npas2 was associated with hypertension and Per2 with blood glucose levels. Our findings give support to the view that there are relevant relationships between circadian clocks and metabolic syndrome.

Competing interests
JH has served as consultant to Janssen-Cilag, other authors have no conflicts of interests.

Authors' contributions
AE drafted the manuscript. LK, JH and TP participated in the design of the study and performed the statistical analysis. TP, STS, JL, AR and AA conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements
We thank Dr. Markus Perola for his assistance with the statistical analysis. This study was supported in part by the grant #210262 from the Academy of Finland (to Timo Partonen) and Grant from Finnish Cultural Foundation (to Ani Englund).

References
1. Stratmann M, Schibler U: Properties, entrainment, and physiological functions of mammalian peripheral oscillators. J Biol Rhythms 2006, 21:494-506.
2. Schibler U, Ripperger J, Brown SA: Peripheral circadian oscillators in mammals: time and food. J Biol Rhythms 2003, 18:250-60.
3. Ukai H, Kobayashi TJ, Nagano M, Masumoto KH, Sujino M, Kondo T, Yagit A, Shiroyoshi Y, Ueda HR: Melanopsin-dependent photoperturbation reveals desynchronization underlying the singularity of mammalian circadian clocks. Nat Cell Biol 2007, 9:1327-34.
4. Tu BP, McKnight SL: Metabolic cycles as an underlying basis of biological oscillations. Nat Rev Mol Cell Biol 2006, 7:967-701.
5. Zhang J, Kaasik K, Blackburn MR, Lee CC: Constant darkness is a circadian metabolic signal in mammals. Nature 2006, 439:340-3.
6. van Oort BE, Tyler NJ, Gerkmek MA, Folkow L, Stokkan KA: Where clocks are redundant: weak circadian mechanisms in reindeer living under polar photic conditions. Naturwissenschaften 2007, 94:183-94.
7. Zhou YD, Barnard M, Tian H, Li X, Ring HZ, Francke U, Shenton J, Richardson J, Russell DW, McKnight SL: Molecular characterization of two mammalian bHLH-PAS domain proteins selectively expressed in the central nervous system. Proc Natl Acad Sci USA 1997, 94:713-8.
8. DeBruyn EP, Weaver DR, Reppert SM: CLOCK and NPAS2 have overlapping roles in the suprachiasmatic circadian clock. Nat Neurosci 2007, 10:543-5.
9. Ko CH, Takahashi JS: Molecular components of the mammalian circadian clock. Hum Mol Genet 2006, 15:R271-7.
10. O'Neill JS, Maywood ES, Chesham JE, Takahashi JS, Hastings MH: cAMP-dependent signaling as a core component of the mammalian circadian pacemaker. Science 2008, 320:949-53.
11. Green CB, Takahashi JS, Bass J: The meter of metabolism. Cell 2008, 134:728-42.
12. Turek FW, Aries E, Kohatsu A, Lin E, Ivanova G, McDearmon E, Laposky A, Losee-Olson S, Easton A, Jens DR, Ecker RH, Takahashi JS, Bass J: Obesity and metabolic syndrome in circadian Clock mutant mice. Science 2005, 308:1043-5.
13. Scott EM, Carter AM, Grant PJ: Association between polymorphisms in the Clock gene, obesity and the metabolic syndrome in man. Int J Obes (Lond). 2008, 32(4):658-62.
14. Sookoian S, Gemma C, Gianotti TF, Burguete A, Castaño G, Pirola CJ: Genetic variants of Clock transcription factor are associated with individual susceptibility to obesity. Am J Clin Nutr 2008, 87:1606-15.
15. Reich M, Garcia JA, Dudley C, McKnight SL: NPAS2: an analog of clock operable in the mammalian forebrain. Science 2001, 293:1-9.
16. Oishi K, Miyazaki K, Kadota K, Kikuno R, Nagase T, Atsumi G, Okhara N, Azuma T, Mesaki M, Yumimasa S, Kobayashi H, Itaka C, Umehara T, Horikoshi M, Kudo T, Shimizu Y, Yano M, Monden M, Machida K, Matsuda J, Horie S, Todo T, Ishida N: Genome-wide expression analysis of mouse liver reveals CLOCK-regulated circadian output genes. J Biol Chem 2003, 278:41519-27.
17. Rutter J, Reick M, Wu LC, McKnight SL: Regulation of clock and Npas2 DNA binding by the redox state of NAD cofactors. Science 2001, 293:510-4.

18. Dudley CA, Erbel-Sieler C, Estill SJ, Reick M, Franken P, Pitts S, McKnight SL: Altered patterns of sleep and behavioral adaptability in Npas2-deficient mice. Science 2003, 301:379-83.

19. Pitts S, Perone E, Silver R: Food-entrained circadian rhythms are sustained in arrhythmic CkI/CkII mutant mice. Am J Physiol Regul Integr Comp Physiol 2003, 285:R57-67.

20. Rintamäki R, Grimaldi S, Englund A, Haukkia J, Partonen T, Reunanen A, Aromaa A, Lonnqvist J: Seasonal changes in mood and behavior are linked to metabolic syndrome. PLoS ONE 2008, 3:e1482.

21. Witschnig H-U, Luchner G, Wunderlich U, Pfister H: Test-retest reliability of the computerized DSM-IV version of the Munich-Composite International Diagnostic Interview (M-CIDI). Soc Psychiatry Psychiatr Epidemiol 1998, 33:568-78.

22. Pirkola SP, Isometsä E, Suvisaari J, Aro H, Joukamaa M, Poikolainen K, Koskinen S, Aromaa A, Lonnqvist J: DSM-IV mood-, anxiety- and alcohol use disorders and their comorbidity in the Finnish general population: results from the Health 2000 Study. Soc Psychiatry Psychiatr Epidemiol 2005, 40:1-10.

23. Rosenthal NE, Bradt GH, Wehr TA: Seasonal Pattern Assessment Questionnaire. National Institute of Mental Health Bethesda: 1984.

24. Kasper S, Wehr TA, Bartko JJ, Gaist PA, Rosenthal NE: Epidemiological findings of seasonal changes in mood and behavior. A telephone survey of Montgomery County, Maryland. Arch Gen Psychiatry 1989, 46:823-33.

25. Hogeschn JB, Gu Y-Z, Moran SM, Shimomura K, Radcliffe LA, Takahashi JS, Bradfield CA: The basic helix-loop-helix-PAS protein MOP9 is a brain-specific heterodimeric partner of circadian and hypoxia factors. J Neurosci 2000, 20:RC83.

26. Asher G, Schlaber U: A CLOCK-less clock. Trends Cell Biol 2006, 16:547-9.

27. R Development Core Team: R: a language and environment for statistical computing. R Foundation for Statistical Computing Vienna: 2007.

28. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ, Sham PC: PLINK: a tool set for whole-genome association and population-based linkage analysis. American Journal of Human Genetics 2007, 81:559-75 [http://pngu.mgh.harvard.edu/purcell/plink/].

29. Woon PY, Kaisaki PJ, Bragança J, Bihoareau MT, Levy JC, Farrall M, Gauguier D: Aryl hydrocarbon receptor nuclear translocator-like (BMAL1) is associated with susceptibility to hypertension and type 2 diabetes. Proc Natl Acad Sci USA 2007, 104:14412-7.

30. Partonen T, Treutlein J, Alpman A, Frank J, Johannson C, Depner M, Aron L, Rietschel M, Wellek S, Soronen P, Paunio T, Koivisto V, Schalling M, Peltonen L, Schumann G: Three circadian clock genes Per2, Arntl, and Npas2 contribute to winter depression. Ann Med 2007, 39:229-38.

31. McIntyre RS, Soczynska JK, Konarski JZ, Woldeyohannes HO, Law CW, Miranda A, Fulgis D, Kennedy SH: Should depressive syndromes be reclassified as "metabolic syndrome type II"? Ann Clin Psychiatry 2007, 19:257-64.

32. McClung CA: Circadian genes, rhythms and the biology of mood disorders. Pharmacol Ther 2007, 114:222-32.

33. Partonen T, Lonnqvist J: Seasonal affective disorder. Lancet 1998, 352:1369-74.

34. Spanel R, Pendas A, Abarca C, Zoibig K, Sanchis-Segura C, Magnus MC, Lascorz J, Depner M, Holzberg D, Soyka M, Schreiber S, Matsuda F, Lathrop M, Schumann G, Albrecht U: The clock gene Per2 influences the glucotamergic system and modulates alcohol consumption. Nat Med 2005, 11:335-42.

35. Lamia KA, Storch KF, Weitz CJ: Physiological significance of a peripheral tissue circadian clock. Proc Natl Acad Sci USA 2008, 105:15172-7.

36. Um HJ, Yang S, Yamazaki S, Kang H, Viollet B, Foretz M, Chung JH: Activation of 5’-AMP-activated kinase with diabetes drug metformin induces casein kinase Ipsilon (CKIepsilon)-dependent degradation of clock protein mPer2. J Biol Chem 2007, 282:794-8.

37. Mersch PP, Vastenburg NC, Meesters Y, Bouhuys AL, Beersma DG, Hoofdakker RH van den, den Boer JA: The reliability and validity of the Seasonal Pattern Assessment Questionnaire: a comparison between patient groups. J Affect Disord 2004, 80:209-19.