Dissecting daily and circadian expression rhythms of clock-controlled genes in human blood

Rhythmic gene expression in human blood

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Abstract

The identification and investigation of novel clock-controlled genes (CCGs) has been conducted thus far mainly in model organisms such as nocturnal rodents, with limited information in humans. Here, we aimed to characterize daily and circadian expression rhythms of CCGs in human peripheral blood during a sleep/sleep deprivation (S/SD) study and a constant routine (CR) study. Blood expression levels of 9 candidate CCGs (*SREBF1, TRIB1, USF1, THRA1, SIRT1, STAT3, CAPRIN1, MKNK2,* and *ROCK2*), were measured across 48 h in 12 participants in the S/SD study, and across 33 h in 12 participants in the CR study. Statistically significant rhythms in expression were observed for *STAT3, SREBF1*, *TRIB1*, and *THRA1* in samples from both the S/SD and the CR studies, indicating that their rhythmicity is driven by the endogenous clock. The *MKNK2* gene was significantly rhythmic in the S/SD but not the CR study, which implies it's exogenously-driven rhythmic expression. Additionally, we confirmed the circadian expression of *PER1*, *PER3*, and *REV-ERBα* in the CR study samples, while *BMAL1* and *HSPA1B* were not significantly rhythmic in the CR samples; all five genes previously showed significant expression in the S/SD study samples. Overall, our results demonstrate that rhythmic expression patterns of clock and selected clock-controlled genes in human blood cells are in part determined by exogenous factors (sleep and fasting state) and in part by the endogenous circadian timing system. Knowledge of the exogenous and endogenous regulation of gene expression rhythms is needed prior to the selection of potential candidate marker genes for future applications in medical and forensic settings.

Introduction

Daily lives of all organisms, including humans, are governed by the endogenous circadian timing system. Circadian clocks are present in virtually every cell and exert their functions via a transcriptional-translational autoregulatory feedback loop composed of genes such as *PER1, PER2, PER3, CRY1, CRY2, BMAL1, CLOCK* and their protein products (Lowrey and Takahashi 2004; Lowrey and Takahashi 2011). A number of studies have reported that these core clock genes are rhythmically expressed in human peripheral tissues, such as skin tissue culture and oral mucosa (Bjarnason et al., 2001), adipose tissue explants (Gómez-Santos et al., 2009), and peripheral blood mononuclear cells (PBMC) (Takata et al., 2002; Boivin et al. 2003; Archer et al., 2008; Ackermann et al., 2013). However to date, the identification of novel clock-controlled genes (CCGs) has been conducted mainly in nocturnal rodents (Ripperger et al., 2000; Bozek et al., 2009; Zhang et al., 2009).

These CCGs, despite being regulated by the core clock elements listed above, are not part of the clock's mechanism, but are thought to be the means by which the clock adapts the body's physiological and metabolic processes to recurring environmental changes (Duffield 2003; Lamont et al., 2007). The CCGs encode a diverse group of molecules, such as ion channels, metabolic enzymes or transcription factors (Lamont et al., 2007). However, even though the expression patterns of the core clock genes have been experimentally confirmed in humans (Bjarnason et al., 2001; Takata et al., 2002; Gómez-Santos et al., 2009; Ackermann et al., 2013), the information regarding clock-related or clock-controlled genes in humans is limited. Transcriptome studies report that approximately 3 – 10% of genes in a given mammalian tissue

are rhythmic (Cermakian and Boivin 2009); nevertheless, the overlap between various tissues can be very small.

More recently, human studies have started investigating the functions of CCGs and the external factors influencing their expression to better understand the mechanisms linking the circadian clock, sleep and diseases such as cardiovascular disease (Takeda and Maemura 2011; Portaluppi et al., 2012), cancer (Sahar and Sassone-Corsi 2009; Savvidis and Koutsilieris 2012), sleep disorders (Archer et al., 2003; Lu and Zee 2006; Sack et al., 2007), hypertension (Scheer et al.,2009), diabetes, obesity (Laposky et al., 2008; Scheer et al., 2009), and metabolic syndrome (Turek et al., 2005; Maury et al., 2010). Knowledge about the expression of CCGs in human blood, however, remains scarce.

In this study, the temporal expression patterns of 9 CCGs were assessed in human peripheral blood samples collected during sleep/sleep deprivation (S/SD) and constant routine (CR) studies: *Sterol Regulatory Element-Binding Transcription Factor 1* (*SREBF1*) (Bozek et al., 2009; Zhang et al., 2009), *Signal Transducer and Activator of Transcription 3* (*STAT3*) (Bozek et al., 2009; Hughes et al., 2009), *Tribbles Homolog 1* (*TRIB1*) (Ollila et al., 2012), *Upstream Transcription Factor 1* (*USF1*) (Shoulders and Naoumova 2004; Shimomura et al., 2013), *MAP Kinase Interacting Serine/Threonine Kinase 2* (*MKNK2*) (Chudova et al., 2009), *Thyroid Hormone Receptor Alpha* (*THRA1*) (Zandieh Doulabi et al., 2004; Zhu and Cheng 2010; Vollmers et al., 2012), *Sirtuin 1* (*SIRT1*) (Rodgers et al., 2005; Longo and Kennedy 2006; Asher et al., 2008; Nakahata et al., 2008; Nakahata et al., 2009), *Cell Cycle Associated Protein 1* (*CAPRIN1*) (Panda et al., 2002) and *Rho-Associated, Coiled-Coil Containing Protein Kinase 2* (*ROCK2*) (Saito et al., 2013). These genes were selected as representative CCGs, rather than to determine any mechanistic pathways. Instead, our selection of candidate genes was motivated by

findings from a number of rodent studies, which have shown that these genes either exhibited daily expression patterns (Zandieh Doulabi et al 2004; Chudova et al., 2009; Vollmers et al., 2012), or that they were directly (Ollila et al., 2012; Shimomura et al., 2013) or indirectly (Panda et al., 2002; Asher et al., 2008; Saito et al., 2013) linked to the circadian timing system and/or sleep/wake processing, while knowledge on their expression in human blood was mostly absent.

Using two different study protocols, we aimed to determine whether or not the selected candidate genes are expressed in a rhythmic manner in human blood, and if so, to distinguish the genes that exhibit daily 24 h rhythmicity (S/SD study) from those that show circadian rhythmicity (CR study). Additionally, in the CR samples, we analysed the expression of four core clock genes, *PER1*, *PER3*, *REV-ERBα* and *BMAL1*, and the *HSPA1B* heat shock gene, previously observed to be significantly rhythmic in the S/SD study (Ackermann et al., 2013), to assess their circadian rhythmicity**.**

Materials and methods

Clinical laboratory study

Two studies, the sleep/sleep deprivation (S/SD) study and constant routine (CR) study, were conducted at the Surrey Clinical Research Centre (CRC) at the University of Surrey (UK). All procedures were conducted in accordance with the Declaration of Helsinki and a favourable opinion was obtained from the University of Surrey Ethics Committee. Written and oral informed consent was obtained from the participants prior to any procedures being performed and they were allowed to withdraw from the study at any time. All subject information was coded and held in strictest confidence according to the Data Protection Act (UK, 1998).

Eligibility criteria for the S/SD study have been previously described in detail (Ackermann et al. 2012). The eligibility of the subjects for the CR study was determined by completion of validated sleep questionnaires (PSQI $\leq$ 5, Beck Depression Inventory < 10, Epworth Sleepiness Scale < 10, Horne-Östberg and Munich Chronotype questionnaire, extreme chronotypes were ineligible), medical and physical assessments and analysis of blood and urine screening samples. Inclusion criteria included: age between 18 and 35 years; completion and fulfilment of the defined criteria of pre-study questionnaires; taking the combined oral contraceptive pill if female and being in the active phase of the menstrual cycle (i.e. taking the hormone pills) during the in-laboratory session; passing a medical assessment; consent to contacting the candidate's GP for confirming the candidate's medical history; agreement to refrain from alcohol, caffeine, exercise and bright light for 72 hours before and during the in-laboratory session; agreement to eat standardised meals for the 48 hours prior to the laboratory session; refraining from taking any over-the-counter (including non-steroidal anti-inflammatory

drugs) or prescribed medication (apart from oral contraceptives) for a washout period of seven days prior to the laboratory session; reporting a habitual, regular sleep-wake cycle for the month preceding screening that involves going to bed between 22:00 and 01:00 h, and getting up between 06:00 and 09:00 h with 6 – 9 h in bed; agreement to keep a regular sleep/wake schedule for the duration of the study; wearing Actiwatches (AWL) and completing written sleep diaries for the duration of the study. Exclusion criteria included significant medical history or taking specific medication. Participants were excluded if they had a history of ever suffering from systemic, psychiatric or neurological disease or drug and alcohol abuse; have taken regular medication that affects melatonin synthesis or circadian rhythms (antihypertensive drugs, non-steroidal anti-inflammatory drugs, hypnotic drugs, benzodiazepines, antidepressants, antipsychotic drugs, barbiturates, antiepileptic drugs) in the last six months; have donated over 400 ml of blood within 3 months (90 days) of screening for the study; work night shifts or have travelled across more than two time zones within one month of and throughout the study; are a smoker or have been a smoker in the 6 months prior to their screening visit; are a vegetarian or have other dietary restrictions as this can impact metabolism; drink > 21 units of alcohol per week if male and > 14 units per week if female; have a body mass index (BMI) < 19 or > 30 kg/m^2 or a total body weight < 50 kg as assessed at the screening visit; have a positive drugs of abuse urine screen at screening or upon entry into the laboratory session; have a positive cotinine urine screen at screening or upon entry into the laboratory session; have a positive alcohol breath test at screening or upon entry into the laboratory session; have abnormal blood biochemistry and/or haematology as deemed significant by the study physician; are positive for HIV or Hepatitis B or C; have a clinically significant allergy e.g. to food stuffs such as shellfish, peanuts; are pregnant; would be considered to be unsafe to participate as determined by the

medical investigator; have received any investigational drug and/or participated in any clinical trial within 3 months of the screening assessment.

For 7 days before the in-laboratory session for both studies, participants maintained a regular sleep/wake schedule aligned with their habitual sleep patterns. For the S/SD study, participants maintained a 23:00 - 07:00 h schedule; for the CR study the participants were asked to select an 8 h sleep period going to bed between 22:00 – 01:00 h and waking up between 06:00 – 09:00 h. Compliance for both studies was confirmed by using activity/light monitors (Actiwatch, CamNtech, Cambridge, UK), sleep logs, and time-stamped voicemail. During the final 72 h of this baseline period, the participants were required to refrain from consuming alcohol, caffeine and taking any medication. This baseline period ensured that the participants beginning the clinical study were not sleep deprived, and that their circadian phase was stabilized. A detailed schematic representation of the S/SD and CR study protocols is shown in Figure 1.

Sleep/sleep deprivation study (S/SD). A detailed description of the S/SD study protocol has been reported elsewhere (Ackermann et al., 2012; Ackermann et al., 2013). In brief, the participants (15 healthy, young males aged 24 ± 5 (years ± standard deviation) participated in a 66 h in-laboratory session, which included 3 night periods: adaptation (N1) and baseline (N2) nights with normal sleep, and a sleep deprivation night 3 (N3), when the participants remained awake and supine in dim light conditions (<5 lux in the direction of gaze). Environmental light and posture were controlled before and after a sleep episode as samples were also being taken for measurement of plasma melatonin, which is highly influenced by such factors (Deacon and Arendt 1994, Zeitzer et al. 2000). The participants were aware of clock time during the duration

of the S/SD study. Blood samples were collected every hour via a catheter. Samples from 12 participants (mean age ± standard deviation = 23 ± 5 yr) at two hourly intervals (25 samples per participant) from 12:00 h on Day 2 (D2) until 12:00 h on Day 4 (D4) were selected for analysis.

Constant routine (CR) study. Healthy subjects between 18 – 35 years of age participated in the CR study. After the baseline-at-home period the participants were admitted into the laboratory, where abstinence from alcohol, nicotine and drugs of abuse was confirmed. The in-laboratory session included an adaptation night with habitual sleep times followed by continual wakefulness until 23:00 h on Day 3. Electroencephalography monitoring occurred from 12:00 h on Day 2 until 23:00 h on Day 3 to ensure the subjects remained awake throughout the CR protocol. The participants were subjected to strictly controlled constant routine conditions, including dim lighting (<5 lux in the direction of gaze), semi-recumbent posture, hourly intake of isocaloric snacks with 100 ml of water. They were not aware of clock time during the study period. Hourly blood samples were collected via an intravenous catheter. For the current gene expression study, 2 hourly samples (from 15:00 h on Day 2 until 23:00 h on Day 3; 17 samples per participant) were collected into PAXgene RNA tubes (Qiagen, Crawley, UK) from 12 participants (6 males, mean age ± SD, 25 ± 6 yr; and 6 females, mean age ± SD, 23 ± 3 yr). These participants were selected based on the quality of their extracted RNA, as assessed using the RIN (RNA Integrity Number) with values ≥ 7.6. Four of the females were on 30 µg ethinylestradiol and 150 µg progestin; one was on 0 µg ethinylestradiol and 75 µg progestin; and one on 30 µg ethinylestradiol and 3000 µg progestin.

Melatonin concentration and dim light melatonin onset (DLMO) assessment

Radioimmunoassay analysis was performed on plasma samples to measure melatonin concentration (Stockgrand Ltd., University of Surrey, Surrey, UK) as described (Fraser et al., 1983; Sletten et al., 2009). The data were used to calculate dim light melatonin onset (DLMO), using a defined 25% threshold, for each individual for both the sleep (night 2; N2) and sleep deprivation (night 3, N3), as described previously (Sletten et al., 2009; Ackermann et al., 2012). The calculated DLMO was used to phase-adjust the gene expression data, for determination of the 24 h rhythmicity, amplitude and acrophase with the non-linear mixed model method.

RNA isolation

Blood samples were stored at -80^{o}C prior to RNA extraction during both studies (S/SD and CR). PAXgene Blood RNA Kit 50 (PreAnalytiX, Hombrechtikon, Switzerland) was used for RNA extraction from CR study blood samples, and the PAXgene 96 Blood RNA Kit (PreAnalytiX, Hombrechtikon, Switzerland) was used to isolate RNA from the S/SD study blood samples (Ackermann et al., 2013). According to the manufacturer, the differences in the kit protocols (binding columns and centrifuge vs 96-well plate and vacuum pump) do not affect the RNA yield and quality. Both extraction procedures were performed according to the enclosed manufacturer's protocols. Nanodrop ND-2000 (NanoDrop Technologies, Wilmington, DE, USA) was used to measure RNA concentration in the extracted samples, and the quality was assessed with Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany). Total RNA samples were kept at -80^{o}C until assayed.

Reverse transcription (RT) reaction

The RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Benelux, Amsterdam, Netherlands) was used for cDNA synthesis, following the manufacturer's protocol for First Strand cDNA Synthesis. Random hexamer primers were used and the optional denaturation step was included. The reaction was performed on MJ Research Thermal Cycler PTC-200 (GMI, Minnesota, USA) with the following program: 5 min at 25^{o}C, 60 min at 42^{o}C and 5 min at 70^{o}C. The cDNA was kept at -20^{o}C until assayed.

Real time quantitative PCR reaction

cDNA samples were diluted to a final concentration of 2.5 ng/µl (based on RNA input) and used in subsequent real time quantitative PCR (qPCR) reactions, with a final volume of 10 µl. Each reaction contained 2 µl diluted cDNA, 5 µl LightCycler480 SYBR Green I Kit (Roche Diagnostics, Mannheim, Germany), 1 µl of appropriate forward and reverse primer mix (3 µM) and 2 µl nuclease-free water. Negative controls with nuclease-free water instead of cDNA were included in each run.

Expression of *SREBF1*, *TRIB1*, *USF1*, *MKNK2*, *THRA1*, *SIRT1*, *STAT3*, *CAPRIN1*, *ROCK2*, and *ACTB* genes was analysed in the S/SD study samples (in total 300 samples, 25 samples per subject, n=12) and in CR samples (in total 204 samples, 17 samples per subject, n=12). Additionally, in the CR study samples expression of *PER1*, *PER3*, *REV-ERBα*, *BMAL1* and *HSPA1B* was assessed, to compare with the daily expression profiles reported previously in our S/SD study samples (Ackermann et al., 2013).

ACTB was chosen as the reference gene, based on the results from the same sample set (S/SD study) where five different housekeeping genes (*GAPDH*, *ACTB*, *HPRT*, *PPIB* and *UBC*) were tested and compared against each other both alone and in combinations (Ackermann et al.,

2013). As confirmation, a single cosinor test was performed on the *ACTB* expression data (z-scored, averaged across individuals) from the S/SD and CR studies. The expression levels of *ACTB* during the CR and S/SD were not significantly rhythmic (p-value$_{CR}$ = 0.40; p-value$_{S/SD}$ = 0.39). All primers were acquired from Metabion (Martinsried, Germany) and their efficiency as well as target specificity was tested prior to their use in the experiments. Primer-BLAST was used for primer design, the option of spanning an exon-exon junction included. Sequences of *PER1*, *PER3*, *REV-ERBα*, *BMAL1*, *HSPA1B* and *ACTB* primers were taken from previously published studies (Archer et al., 2008; Kimura et al., 2011; Visser et al., 2011; Ackermann et al., 2013). The sequences of all the primers used are presented in Table S1 (Supplementary material).

All real time qPCR reactions were run in triplicate on a Light Cycler 480 II platform (Roche Diagnostics) in 384-well plates. The reaction protocol consisted of denaturation at 95^{o}C for 10 min and 45 cycles of denaturation (95^{o}C, 10 s), annealing (60^{o}C, 10 s) and extension (72^{o}C, 10 s), followed by a melting curve step with continuous data acquisition from 65^{o}C to 97^{o}C.

Real time qPCR data analysis

The second derivative maximum method, implemented in the Light Cycler 480 software (Roche Diagnostics), followed by the delta-delta-cycle-threshold (ΔΔCT) method (Livak and Schmittgen 2001) was used to quantify relative gene expression in the S/SD and CR samples. Afterwards, *ACTB* normalized, relative gene expression values were z-scored (per individual) and three different analyses of gene expression were conducted.

Statistical analyses

ANOVA: A two-way, repeated measures ANOVA with a Bonferroni correction was performed to determine the significance of changes in gene expression levels between the two different conditions (sleep vs sleep deprivation) and the time of day, and their interaction (time of day*condition) for the S/SD study data without considering a circadian rhythm model. Subsets for analysis were determined as follows: 1) 12 hour periods using samples collected from 00:00 h on Day 3 to 12:00 h on Day 3 versus samples from 00:00 h on Day 4 to 12:00 h on Day 4, to assess expression changes covering the sleep and sleep deprivation periods, and 2) 24 hour periods using samples from 14:00 h to 12:00 h (first 24 h) versus samples from 14:00 h to 12:00 h (second 24 h), to examine the expression changes between the two 24 h days. The first time point (12:00 h) was omitted in order to obtain the same sample numbers in both comparisons. To account for missing samples (< 1.5%) linear interpolation based on non z-scored, *ACTB* normalized expression data from the same subject and gene was applied to simulate gene expression levels. Determination of the expression changes across time for genes tested in the CR study samples was performed using a one-way ANOVA with Bonferroni correction.

Single individual cosinor analysis: To ascertain whether the changes in gene expression levels over time followed a 24 h sinusoidal pattern in each individual, single cosinor analysis was performed separately for S/SD samples divided into two subsets ("sleep" and "sleep deprivation"). Each of the subsets included one of the following conditions, either normal wake/sleep (first 24 h - samples from 12:00 h on Day 2 to 12:00 h on Day 3 – "sleep" subset) or sleep deprivation (second 24 h - samples from 12:00 h on Day 3 to 12:00 h on Day 4 – "sleep

deprivation" subset). Single cosinor analysis for the CR sample set was performed for the 33 h period comprising the whole set of samples (from 15:00 h on Day 2 to 23:00 h on Day 3).

Estimation of amplitude and acrophase with a non-linear mixed model: To estimate the amplitude and acrophase for each gene across all individuals participating in the S/SD and CR studies, a non-linear mixed model was used. For these analyses, the difference between an individual's DLMO and the average DLMO (for all individuals) was calculated. This value was used to obtain DLMO-corrected amplitude and acrophase values. To ascertain whether the changes in gene expression levels over time followed a 24 h sinusoidal pattern, a cosinor analysis was performed as done previously (Ackermann et al., 2013):

$$\text{normalized z} - \text{score} = \alpha + \beta \cdot \cos(2\pi \cdot \frac{TP-t}{24}) \qquad (1)$$

Where β is the amplitude, t is the acrophase and α is the independent term.

To avoid multiple solutions due to the periodicity of the cosine function and to reduce the amount of correlation between β and t, a variable transformation of (1) was applied:

$$normalized\ z - score = \alpha + e^{\beta'} \cos\left(2\pi \frac{\left(TP - 24\frac{e^{t'}}{1+e^{t'}}\right)}{24}\right) \qquad (2)$$

Repeated measures for each individual in the estimation of α, β and t were incorporated as a random effect in the model. A non-linear mixed model (Davidian et al., 1995; Lindstrom et al., 1990) with fixed and random effects was implemented using the nlmer function from the R package lme4 (Bates et al., 2015a; Bates et al., 2015b) on z-scored, *ACTB* normalized data for both studies.

The effect of sleep deprivation on β and t was included as a fixed effect in (2) by comparing it against the sleep condition:

$$normalized\ z-score = \alpha + e^{\beta' + \gamma * C} \cos\left(2\pi \frac{\left(TP - 24\frac{e^{t'+\theta*C}}{1+e^{t'+\theta*C}}\right)}{24}\right) \quad (3)$$

Where *C* takes 1 in sleep deprivation, 0 otherwise.

The statistical significance of differences of amplitude and acrophase depending on sleep condition were estimated by comparing the likelihood of the nested models (2) and (3) by means of ANOVA using the anova() command from R.

The most statistically supported model was then compared with the nested null model:

$$normalized\ z-score = \alpha \quad (4)$$

using the same ANOVA framework.

After multiple testing correction (Bonferroni) was applied, the new significance level for ANOVA performed in the S/SD study samples was set at p-value < 0.006 and in the CR study samples at p-value < 0.004. For the non-linear mixed model test the new significance level for analyses in the S/SD and CR study samples was set at p-value < 0.004. For the single cosinor tests the significance level was set at p-value < 0.05. In all the tests, non-statistical values that were obtained are designated as n.s.

Results

Daily rhythms in gene expression levels in the S/SD study samples

With the ANOVA, we found that *MKNK2* showed a statistically significant time of day variation during the first and second 24 h of the S/SD study (p-value <0.006; after Bonferroni correction), as well as during two shorter time periods, one comprising the sleep night with half a day afterwards (from 00:00 h to 12:00 h on Day 3) and the other comprising the sleep deprivation night and half a day afterwards (from 00:00 h to 12:00 h on Day 4) (p-value <0.006; after Bonferroni correction). Analysis of *MKNK2* with the non-linear mixed model revealed that the cosinor model was better than compared to the null model, and the obtained estimates of acrophase and amplitude were statistically significant after Bonferroni correction ($p < 0.004$) (Table 1). The single cosinor method showed that *MKNK2* was rhythmic in 5 of the 12 individuals tested (42%) during the sleep condition and in 4 (33%) individuals during the sleep deprivation condition (Table 2).

The genes *SREBF1, STAT3* and *TRIB1* exhibited significant time of day variation in expression during the first and second 24 h of the S/SD study, as well as during the shorter time periods (ANOVA; $p < 0.006$; after Bonferroni correction). Non-linear mixed model analysis showed that the acrophase and amplitude estimates were statistically significant during the S/SD study (Table 1). Single cosinor analysis found that *SREBF1* expression was significantly rhythmic in 2 (17%) and 4 (33%) individuals, and *TRIB1* in 7 (58%) and 4 (33%) individuals during sleep and sleep deprivation conditions, respectively (Table 2). *STAT3* was rhythmic in 3 (25%) individuals during sleep and in 3 (25%) individuals during sleep deprivation.

THRA1 showed significant time of day variation during the first and second 24 h of the study, as well as during the shorter time periods (ANOVA; $p < 0.006$; after Bonferroni correction). ANOVA also revealed a statistically significant interaction between time of day and sleep condition for *THRA1* ($p_{THRA1} = 0.006$; after Bonferroni correction). Furthermore, an overall increase in *THRA1* expression levels during sleep deprivation, based on total sum of z-scores (-27.36 during sleep vs 22.88 during sleep deprivation) was observed. Not all of the tests of the non-linear mixed model analysis could be performed for *THRA1*, because of the limitations of the algorithm regarding the starting values of the parameters. Thus, we could not compare the second and third nested models of the non-linear mixed model method (see Materials and Methods) together. Because of this, the effect of condition on the gene expression could not be assessed. When only applying the second model, however, which assumes that condition does not influence the expression of *THRA1*, we obtained statistically significant acrophase and amplitude estimates during the S/SD study (Table 1). Single cosinor analysis showed that *THRA1* expression was significantly rhythmic in 9 (82%) and 6 (55%) individuals during the sleep and sleep deprivation conditions, respectively (Table 2).

For *SIRT1*, *ROCK2* and *CAPRIN1,* ANOVA revealed a statistically non-significant time of day variation in expression, for all the time periods analysed. The amplitude and peak estimates, as obtained with the non-linear mixed model method, were statistically significant for the three genes (Table 1). The single cosinor method revealed that *SIRT1* was significantly rhythmic in 5 (45%) and 2 (18%) individuals, *CAPRIN1* was significantly rhythmic in 3 (25%) and 5 (42%) individuals during sleep and sleep deprivation, respectively, while *ROCK2* was significantly rhythmic in 3 (25%) individuals during sleep, and in 3 individuals (25%) during sleep deprivation (Table 2).

USF1 did not exhibit a significant time of day expression for any of the tested periods; the estimates obtained with the non-linear mixed model method were also not significant (Table 1). Single cosinor analysis showed *USF1* to be rhythmic in only 2 (17%) individuals during sleep, and in none during sleep deprivation (Table 2).

Additionally, the amplitude and acrophase parameters were estimated by means of the non-linear mixed model method for the clock genes *PER1*, *PER3*, *BMAL1* and *REV-ERBα*, as well as a heat shock gene *HSPA1B*, for which the expression data were generated in our previous study (Ackermann et al. 2013) in the same S/SD study samples. The non-linear mixed model method supported the cosinor model better than the nested null model for *PER1*, *PER3*, *BMAL1* and *HSPA1B* during the S/SD study. The *REV-ERBα* results were incomplete (the same situation as with *THRA1* occurred, where the second and third nested models of the non-linear mixed model method could not be compared), and thus assuming no effect of condition on the data, the non-linear mixed model method showed that the amplitude and peak estimates were statistically significant in the S/SD study data (Table 1). Averaged gene expression levels in different conditions (sleep vs sleep deprivation vs constant routine) are presented in Figure 2 and in Supplementary figure S1. Individual expression profiles of all the genes analysed in the S/SD study are presented in Supplementary Material (Figure S2).

Circadian rhythms in gene expression levels in the CR study samples

MKNK2 had a statistically significant circadian variation of expression (as shown with one-way ANOVA; $p < 0.004$; after Bonferroni correction), but statistically not significant estimates for amplitude and acrophase, calculated with the non-linear mixed model analysis

(Table 3). The single cosinor method also showed that *MKNK2* was significantly rhythmic in only 1 (8%) individual (Table 4).

SREBF1 also had a statistically significant circadian variation of expression, as shown by the ANOVA; the non-linear mixed model method showed, that both amplitude and peak estimates of *SREBF1* were significant (Table 3). However, with single cosinor analysis only 2 individuals showed statistically significant *SREBF1* expression (Table 4).

The genes *STAT3*, *TRIB1* and *THRA1* also showed significant circadian variation in expression by ANOVA. Both amplitude and acrophase estimates obtained with the non-linear mixed model were also statistically significant (Table 3). However, single cosinor analysis showed that *STAT3* expression was significant in 3 (25%) individuals and *TRIB1* expression was significantly rhythmic in only 1 (8%) individual (Table 4). By contrast, expression of *THRA1* was significantly rhythmic in 7 (58%) individuals (Table 4).

SIRT1 and *ROCK2* had statistically non-significant circadian variation in expression (ANOVA), and non-significant acrophase and amplitude estimates (Table 3). Single cosinor analysis showed that *SIRT1* was significantly rhythmic in only 1 (8%) individual and *ROCK2* was rhythmic in 3 (25%) individuals (Table 4).

Likewise, *CAPRIN1* had no significant circadian variation in expression (ANOVA), however, the non-linear mixed model results revealed that the amplitude and acrophase estimates were statistically significant (Table 3). *CAPRIN1* was significantly rhythmic in 4 (33%) individuals according to the single cosinor analysis (Table 4).

The results of ANOVA and non-linear mixed model analyses (Table 3) for *USF1* were all statistically non-significant, no circadian variation in expression being observed. In addition, no

significant rhythms in any of the study participants were detected with single cosinor analysis (Table 4).

The four clock genes *BMAL1*, *PER1*, *PER3* and *REV-ERBα* and the heat shock protein gene *HSPA1B*, previously studied in the S/SD study samples (Ackermann et al. 2013), were also tested in the CR study samples. The ANOVA and non-linear mixed model method results were statistically significant for the genes *PER1*, *PER3* and *REV-ERBα* (Table 3). The expression of *PER1*, *PER3* and *REV-ERBα* was significantly rhythmic in 10 (83%), 11 (92%) and 11 (92%) individuals, respectively, as shown by the single cosinor analysis (Table 4). In the CR study samples, the genes *HSPA1B* and *BMAL1* had statistically significant time of day variation (ANOVA), but the non-linear mixed model revealed that both estimates were not statistically significant (Table 3). In addition, *HSPA1B* expression was significantly rhythmic in only 3 (25%) individuals, while *BMAL1* was significantly rhythmic in only 2 (17%) individuals (Table 4). Averaged gene expression levels in different conditions (sleep vs sleep deprivation vs constant routine) are presented in Figure 2 and in Supplementary figure S1. Individual expression profiles of all the genes analysed in the CR study are presented in Supplementary Material (Figure S3).

Discussion

The expression patterns of 9 candidate clock-controlled genes (*SREBF1, TRIB1, USF1, THRA1, SIRT1, STAT3, CAPRIN1, MKNK2* and *ROCK2*) were determined in human PBCs from blood samples collected during controlled S/SD and CR studies. A comparison of average expression levels of selected genes during S/SD and CR is presented in Figure 2, and in Supplementary Material (Figure S1), and individual expression profiles of genes analysed in S/SD and CR studies are presented in Supplementary Figures S2 and S3, respectively.

Overall, we found that at the group level the clock controlled genes *SREBF1, STAT3, THRA1* and *TRIB1* exhibited statistically significant circadian rhythms in expression in human PBCs under CR conditions; furthermore results of the non-linear mixed model method suggest that the expression and rhythmicity of these genes were unaffected by sleep deprivation (S/SD).

To our knowledge this is the first study reporting expression patterns of *TRIB1, USF1, THRA1, SIRT1, STAT3, CAPRIN1, MKNK2* and *ROCK2* in human blood samples collected during two different laboratory protocols designed to distinguish daily from circadian rhythmicity. Out of the candidate gene set investigated, only *SREBF1* gene expression has been previously measured during sleep deprivation in a study by Arnardottir et al., 2014. This study, however, included participants, who were behaviourally resistant and sensitive to sleep deprivation, which does not reflect the normal human situation studied here. Our non-linear mixed model analysis revealed that on group level *THRA1, TRIB1, MKNK2, SREBF1,* and *STAT3* exhibited significant daily rhythmicity during the S/SD study. Furthermore the expression of *SREBF1, STAT3, THRA1* and *TRIB1* was also significantly rhythmic in the CR study. The S/SD study design included timed meals, light/dark and wake/sleep conditions and these

exogenous factors likely influence the daily rhythmic expression of above mentioned genes. Results of the non-linear mixed model analysis for *MKNK2*, *STAT3, SREBF1, THRA1* and *TRIB1* during the S/SD study, imply that the sleep condition (i.e. sleep or sleep deprivation) does not influence the rhythmic expression of these genes to a large extent. However, there was a decrease in the number of subjects with significant rhythms during SD for *THRA1, TRIB1, STAT3* and *MKNK2*, and an increase in the number of subjects with significant rhythms in *SREBF1* expression during SD, as estimated by single cosinor analysis (Table 2). Therefore, even though at the group level the non-linear mixed model did not indicate any statistically significant influence of the condition on the expression of these genes, results from the single cosinor analysis suggest some effect of 24 h wakefulness and increased sleep pressure on the expression of these genes in individual subjects.

In a recent study, Arnardottir et al. (2014) found that on average the expression of *SREBF1* decreased during SD in subjects selected based on their behavioural resistance or sensitivity to sleep deprivation. We also observed that in healthy individuals of our S/SD study, the overall expression of *SREBF1* decreased during the SD condition (total sum of z-scores, 24.08 in S vs. -24.44 in SD). However, we also found an increase in the number of subjects with significant rhythms (Table 2) during sleep deprivation. In animal studies it has been suggested that the SREBP1 protein can play a role in restricted feeding-induced phase shifting of the circadian clock (Zhang et al., 2009).

We have shown that *THRA1* had a significant daily rhythm during the S/SD study (Tables 1 and 2). Literature reports regarding rhythmicity of *THRA1* transcript in mice or rats are conflicting (Zandieh Doulabi et al 2004; Vollmers et al., 2012). In one study the authors found that the transcript does not oscillate in the mouse liver (Vollmers et al., 2012), however, in

another study, *THRA1* mRNA has been shown to be rhythmic in rat liver (Zandieh Doulabi et al., 2004). Moreover, the findings implied that the amplitude of *THRA1* mRNA might be modified by restricted feeding.

The *TRIB1* gene encodes a highly conserved pseudokinase protein that functions as an adaptor in signalling processes in the cell. Our findings on *TRIB1* support the work of Ollila et al. (2012) who proposed *TRIB1* as a link between sleep and lipid metabolism regulation in humans, and suggested that sleep duration and lipid metabolism may in part be controlled by the same genes in humans. On an individual level, our data indicate that *TRIB1* expression, exhibiting 24 h rhythmicity in the S/SD study, is likely dependent on the sleep/wake state, since the number of individuals with significant rhythms decreased by 25% during total sleep deprivation (Table 2), suggesting an effect of increasing sleep pressure on *TRIB1* expression.

Previously, we reported changes in the expression of four clock genes *PER1*, *PER3*, *BMAL1* and *REV-ERBα,* and a heat-shock gene *HSPA1B* during sleep and SD (Ackermann et al., 2013) by means of a non-linear curve fitting analysis. In the current study, we reanalysed the expression data of the aforementioned genes by means of a non-linear mixed model analysis which, in addition to non-linearity, models the random effects of repeated measures from different individuals. This analysis has shown that, on a group level, there was no statistically significant effect of condition on these genes. However, a decrease in the number of subjects with significant rhythmicity during the SD condition (Ackermann et al., 2013) was found with a single cosinor for *PER1*, *PER3*, *BMAL1*, *REV-ERBα,* and *HSPA1B*. A similar effect of SD was recently demonstrated for the human metabolome in the same S/SD study, with less rhythmic metabolites observed during 24 h of wakefulness (Davies et al., 2014).

We have found that *PER1*, *PER3* and *REV-ERBα* genes were rhythmic in CR conditions, consistent with the results from previously reported studies (Takata et al., 2002; Archer et al., 2008), thus confirming their status as core clock genes in PBCs. The results obtained for *BMAL1*, however, were not so straightforward. In the S/SD study, *BMAL1* expression was not influenced by the sleep condition (Table 1), however, single cosinor analysis showed a 30% decrease in number of subjects with significant rhythms during the sleep condition (Ackermann et al., 2013). However, during the CR study only 2 of the 12 subjects had significant circadian rhythms in *BMAL1* expression. Amplitude and acrophase estimates for *BMAL1*, calculated using the non-linear mixed model, were not statistically significant in CR, although both estimates were rhythmic in S/SD. James et al. (2007) reported large inter-individual variability in *BMAL1* expression in PBMCs during CR conditions. Other reports (Teboul et al., 2005; Kusanagi et al., 2008) also noted much larger inter-individual variation in the expression of *BMAL1* during CR conditions compared to other clock genes. These results are in agreement with the data obtained for *BMAL1* in our CR study, where only 17% of subjects displayed significant circadian rhythmicity. Further studies are needed to better understand the time-wise expression changes of *BMAL1* and its large inter-individual variation. For the *HSPA1B* gene a statistically significant effect of time of day on gene expression was found with ANOVA, and statistically significant rhythms were detected in 3 individuals (single cosinor analysis) during the CR study.

Despite the strengths of the CR protocol to minimize the exogenous factors that may confound assessment of circadian phase, our CR study is not without caveats. The group of individuals participating in the CR study comprised of equal numbers of young males (n=6) and females (n=6). Thus the differences between individuals observed, might be due to sex differences and the fact that the female participants were required to take combination oral

contraceptive pills to minimize any possible variations in response due to different phases of the menstrual cycle. The overall small sample size of the analysed group did not allow statistical testing of the effect of sex on gene expression to be performed, but should be investigated in future studies. One possible explanation for the observed discrepancies between the single cosinor and the non-linear mixed models is the lack of statistical power of the non-linear mixed model for detecting S/SD differences. In particular, the non-linear mixed model can be considered as over-parameterized given that it considered five parameters, which were estimated from repeated measures on only 12 individuals.

Many of the PBCs are known to be involved in immunity, and some of the genes we analysed have also been implied to play a direct or indirect role in various immune processes. *TRIB1*, which functions as an adaptor in signalling pathways in the cells, has been identified as a myeloid oncogene and implied in human leukaemia as well as in non-neoplastic disorders (Yokoyama and Nakamura 2011, Yokoyama et al., 2010). Inhibition of *ROCK2* causes a decrease in the ability of T-cells to secrete proinflammatory cytokines IL-17 and IL-21, thus implicating a role for *ROCK2* in their regulation (Zanin-Zhorov et al., 2014). We observed an increase in *ROCK2* expression during sleep deprivation (total sum of z-scores, -21.7 in S vs. 19.58 in SD), which might cause an increase in proinflammatory cytokine secretion promoting systemic inflammation. *SREBF1* gene encodes a protein (SREBP1c) that regulates genes required for glucose metabolism and fatty acid and lipid production (Bozek et al. 2009). Thus, regulation of intracellular lipid metabolism is critical for proper lymphocyte growth and function. Furthermore, *SREBF1* has been demonstrated to play an important role in acquisition of specific metabolic programs by T lymphocytes, required for their clonal expansion, which is necessary for effective adaptive immunity (Kidani et al., 2013). In our study, the observed

decrease in *SREBF1* during sleep deprivation (total sum of z-scores, 24.08 in S vs. -24.44 in SD), suggests a suppression of the gene's expression during SD, which might be related to a decrease in T lymphocyte expansion and compromised adaptive immunity responses. More detailed studies, incorporating cytokine and cell measurements, however, are needed to determine the actual involvement of the mentioned genes in immune responses during sleep deprivation.

Few studies have investigated the effect of total sleep deprivation on gene expression in humans (Cirelli et al., 2004; James et al., 2007; Ackermann et al., 2013; Möller-Levet et al., 2013, Arnardottir et al., 2014). Direct comparisons between the studies are very difficult because of the differences in the SD protocols. For example, timing or composition of meals can influence the expression of metabolism-related genes, as meal composition and timing influence gene expression (Leonardson et al., 2010) and entrain the peripheral clocks, leading to phase shifts and even to uncoupling between the central and peripheral oscillators in mammals (Kräuchi et al., 2002, Hirao et al., 2010, Schoeller et al., 1997). Other differences include study participant selection criteria, as for example in the Arnardottir et al. (2014) study, where participants were selected on the basis of their resistance to sleep deprivation from a preselected group of twin-pairs; as well as the applied methodology (i.e., microarrays or transcriptome sequencing) (James et al., 2007; Möller-Levet et al., 2013).

In summary, we have been able to characterize and differentiate both the daily and circadian rhythms of a number of genes related to circadian timing, sleep and metabolism in human PBCs and assess changes in their expression and rhythmicity during sleep, sleep deprivation and constant routine conditions. Our data provide valuable high resolution baseline information about clock-controlled genes, including their daily and circadian expression patterns in human blood cells and the effect of sleep status on their rhythmic expression. Our results will

be beneficial for future research on the molecular mechanisms linking circadian timing and sleep/wake processing, as well as in future studies investigating clock and clock-controlled genes as potential candidate marker genes for medical and forensic applications.

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Tables

Table 1. Results of the non-linear mixed model curve fitting analysis for S/SD study samples.

Gene	Condition	Amplitude (DLMO adjusted)	Acrophase (decimal time) (DLMO adjusted)
BMAL1†	*S/SD*	0.53	17:46
CAPRIN1	*S/SD*	0.52	23:87
HSPA1B†	*S/SD*	0.55	16:57
MKNK2	*S/SD*	0.69	15:53
PER1†	*S/SD*	0.71	08:12
PER3†	*S/SD*	0.79	04:42
REV-ERBα†	*S/SD*	0.61	00:38
ROCK2	*S/SD*	0.49	01:20
SIRT1	*S/SD*	0.61	01:66
SREBF1	*S/SD*	0.55	23:22
STAT3	*S/SD*	0.68	15:84
THRA1	*S/SD*	0.80	01:24
TRIB1	*S/SD*	0.69	14:95
USF1	*S/SD*	0.29	14:54

*Presented are average amplitude and acrophase estimates for two conditions (sleep and sleep deprivation) after individual DLMO adjustment. †indicates genes previously tested by Ackermann et al., 2013.

Table 2. Results of the single cosinor analysis for genes tested in the S/SD study samples.

Gene	Sleep	Sleep Deprivation	Average amplitude	Average acrophase (decimal time)
BMAL1†	5	2	0.98	17:10
CAPRIN1	3	5	0.99	23:72
HSPA1B†	4	1	0.99	17:27
MKNK2	5	4	0.96	15:95
PER1†	3	1	0.89	09:11
PER3†	6	5	1.06	04:14
REV-ERBα†	6	4	0.97	00:70
ROCK2	3	3	0.87	01:31
SIRT1	5	2	0.93	01:11
SREBF1	2	4	0.90	23:27
STAT3	3	3	0.91	16:25
THRA1	9	6	1.02	01:52
TRIB1	7	4	0.99	13:66
USF1	2	0	0.90	06:34

*Presented in the Table are the numbers of subjects (out of 12, except for *THRA1* and *SIRT1* - out of 11, and *PER1,3*, *REV-ERBα*, *BMAL1* and *HSPA1B* out of 10) with significant rhythms ($p < 0.05$) per condition for each tested gene and the average acrophases and amplitudes in those subjects.† indicates genes previously tested by Ackermann et al., 2013.

Table 3. Results of the non-linear mixed model curve fitting analysis for the CR study samples.

Gene	Amplitude (DLMO adjusted)	Acrophase (decimal time) (DLMO adjusted)
BMAL1	0.31	17:24
CAPRIN1	0.49	01:06
HSPA1B	0.35	15:43
MKNK2	0.37	15:31
PER1	0.98	07:61
PER3	1.14	03:52
REV-ERBα	1.02	01:09
ROCK2	0.47	23:81
SIRT1	0.41	17:12
SREBF1	0.50	00:56
STAT3	0.59	17:60
THRA1	0.79	01:51
TRIB1	0.52	13:00
USF1	0.14	14:00

*Presented are relative amplitude and acrophase estimates (in decimal time) after individual DLMO adjustment.

Table 4. Results of the single cosinor analysis for genes tested in the CR study samples.

Gene	**Nr of subjects**	**Amplitude**	**Acrophase (decimal time)**
BMAL1	2	0.92	16:45
CAPRIN1	4	0.98	23:86
HSPA1B	3	0.89	20:80
MKNK2	1	0.96	18:42
PER1	10	1.10	08:07
PER3	11	1.20	03:99
REV-ERBα	11	1.12	01:53
ROCK2	3	0.98	23:08
SIRT1	1	0.97	21:27
SREBF1	2	1.01	00:55
STAT3	3	1.06	18:27
THRA1	7	1.03	01:68
TRIB1	1	0.96	16:15
USF1	0	NA	NA

*Presented in the Table are the numbers of subjects out of 12 with significant rhythms ($p < 0.05$) for each tested gene and the averaged acrophases (in decimal time) and amplitudes in those subjects.

Figure legends

Figure 1. The sleep/sleep deprivation (S/SD) and constant routine (CR) study protocols. The S/SD study scheme is modified from Figure 1 from Ackermann et al., 2012.

Figure 2. Plots of mean, z-scored, *ACTB*-normalized expression levels of selected genes (*TRIB1*, *THRA1*, *SREBF1*, *SIRT1*, *REV-ERBα*, *PER3*, *MKNK2* and *BMAL1*) tested in the subjects from the S/SD and CR studies, three different conditions (sleep; sleep deprivation; constant routine) overlaid together. Black - the first 24 h of the S/SD study including the sleep night; red - the second 24 h of the S/SD study including the sleep deprivation night; green – the CR study, 33 h, no sleep permitted. The time axis is presented as time (h) relative to individual DLMO.

Supplementary Figure S1. Plots of mean, z-scored, *ACTB*-normalized expression levels of genes tested in the subjects from the S/SD and CR studies, three different conditions (sleep; sleep deprivation; constant routine) overlaid together. Black - the first 24 h of the S/SD study including the sleep night; red - the second 24 h of the S/SD study including the sleep deprivation night; green – the CR study, 33 h, no sleep permitted. The time axis is presented as time (h) relative to individual DLMO.

Supplementary Figure S2. Individual profiles of expression levels (coloured lines) of 14 genes (*USF1*, *TRIB1*, *THRA1*, *STAT3*, *SREBF1*, *SIRT1*, *ROCK2*, *REV-ERBα*, *PER3*, *PER1*, *MKNK2*, *HSPA1B*, *CAPRIN1* and *BMAL1*) presented as *ACTB*-normalized z-scores during the two 24 h periods of the S/SD study, including the sleep night (N2) and the sleep deprivation night (N3)..

Underlined genes had significant acrophase and amplitude parameters, as calculated using the non-linear mixed model. Gene name in red indicates genes with incomplete results from the non-linear mixed model method (see Results and Discussion sections). The boxes underneath the graphs represent the conditions during the study. White box: 100 lux, free movement, awake; grey box: < 5 lux, semirecumbent position, awake; black box: 0 lux, supine position, asleep.

Supplementary Figure S3. Individual expression profiles (coloured lines) of 14 genes (*USF1, TRIB1, THRA1, STAT3, SREBF1, SIRT1, ROCK2, REV-ERBα, PER3, PER1, MKNK2, HSPA1B, CAPRIN1* and *BMAL1*) presented as *ACTB*-normalized z-scores during the 33 h period of the CR study. Underlined genes had significant acrophase and amplitude parameters, as calculated using the non-linear mixed model. The boxes underneath the graphs represent the conditions during the study. Grey box: < 5 lux, semirecumbent position, awake.