Circadian Forensics: Estimating blood trace deposition time using rhythmic biomarkers

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Circadiane forensics:
Het schatten van het tijdstip van afzetting van bloedsporen met behulp van ritmische biomarkers

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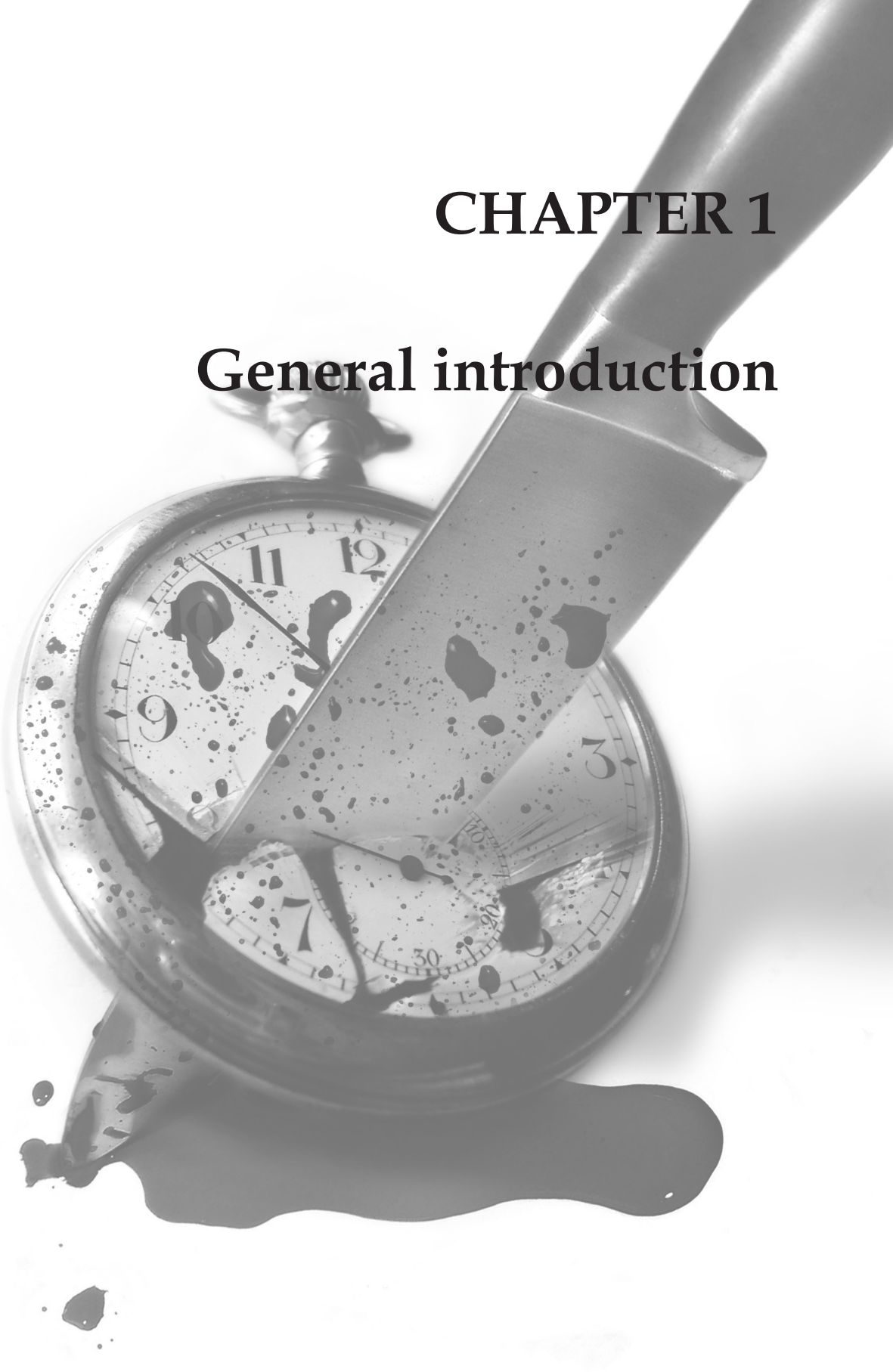
CHAPTER 1

General introduction



Circadian rhythms

The daily lives of almost all organisms are governed by the presence of circadian rhythms, which are a natural mechanism for adjusting behaviour, metabolism, and physiology to cope with the temporal changes of the environment (*Edery 2000*). They were first identified in the XVIII century, while observing the opening and closing of the Mimosa plant leaves, which would persist even in the absence of light; a finding attributed to external influences. This was the first evidence for the existence of an internal biological clock (*de Mairan 1729*). In the following years circadian rhythms were found in most of existing life forms, from single cell to multicellular organisms, proving they are conserved throughout evolution (*Dunlap 1999; Panda et al., 2002*).

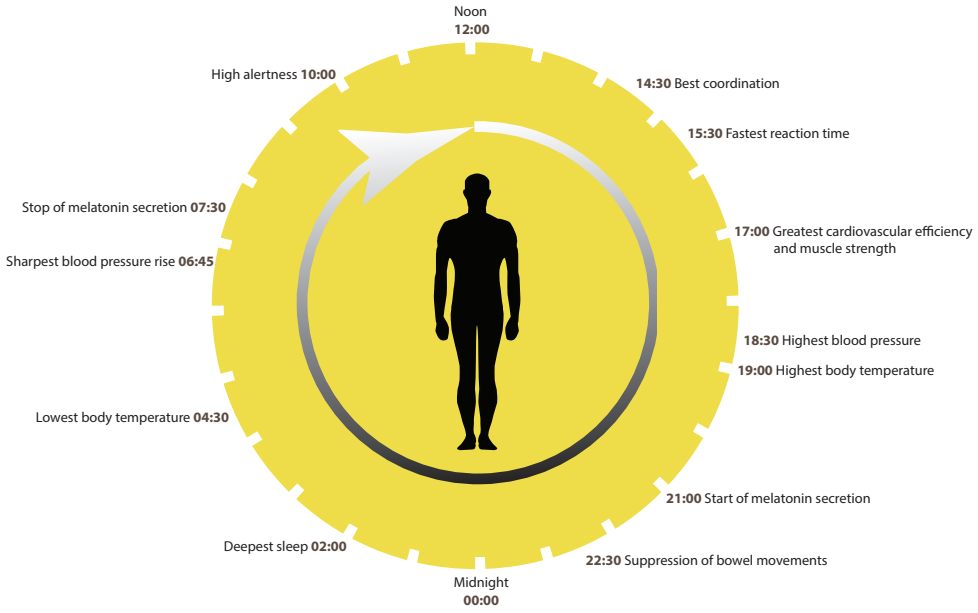


Figure 1. Circadian rhythms are reflected in various physiological as well as behavioural processes in humans (adapted from School of Biological Sciences, Royal Holloway University of London).

Circadian rhythms can be characterized by three inherent properties. First of all, they are self-sustaining or free-running, persisting in the absence of any external time cues (i.e. light/dark cycle) with a period of approximately 24 h (*Kuhlman et al., 2007*). Secondly, they are susceptible to resetting (entrainment), by external time cues (zeitgebers) such as the daily light/dark cycle, temperature or food (*Johnson et al., 2003*). Lastly, the circadian rhythms are temperature compensated, meaning that their period length is constant over a wide range of physiologically relevant temperatures (*Izumo et al., 2003; Tsuchiya et al., 2003*). All those features contribute to a robust, yet susceptible to fine-tuning, circadian system.

In mammals the circadian systems have a hierarchical organization (*Dibner et al., 2010*). They are comprised of the "master clock" located in the suprachiasmatic nuclei (SCN) in the hypothalamus, that synchronizes the phase of other clocks called the peripheral clocks, present in practically all tissues outside the SCN (*Dibner et al., 2010; Lowrey & Takahashi 2004*). However, all circadian systems begin with the molecular circadian oscillators present in almost all individual cells, composed of a set of genes and their protein products that generate the circadian rhythms through changes in their expression.

The molecular oscillators – clock mechanism

The autonomous molecular oscillators are comprised of a set of genes and proteins that form autoregulatory transcriptional feedback loops (*Lowrey & Takahashi 2004; Mohawk et al., 2012*). They drive the transcription and translation of circadian clock genes, which rhythmically activate or repress their own expression with a period of approximately 24 hours. This transcription network in mammals is organized into the "positive limb" and "negative limb". The *Clock* (Clock locomotor output cycles kaput) and *Bmal1* (Brain and muscle ARNT like protein 1) genes encode bHLH-PAS

(basic helix-loop-helix) proteins CLOCK and BMAL1 that together form a heterodimer complex, which constitutes the "positive limb" of the feedback circuit. The CLOCK/BMAL1 heterodimer binds to specific DNA elements, called E-boxes (5'-CACGTG-3') and E'-boxes (5'-CACGTT-3') localized in promoters of target genes and initiates their transcription. Amongst the activated genes are the members of the "negative limb" of the feedback circuit, the *Period* (*Per: Per1, Per2, Per3*) and *Cryptochrome* genes (*Cry: Cry1* and *Cry2*) (*Lowrey & Takahashi 2004*). The protein products of these genes, namely PER and CRY, dimerize and together inhibit the transcriptional activity of the CLOCK/BMAL1 complex.

The degradation of the PER and CRY proteins terminates the repression phase, and is required to start a new cycle of transcription. The rate of PER and CRY degradation is crucial for setting the length of the period of the clock (*Galego & Virshup 2007*). The PER proteins are phosphorylated by the casein kinases CK1ε and CK1δ, which target the proteins for ubiquitination by the βTrCP (β-transducing-repeat-containing protein) and degradation by the 26S proteasome (*Mohawk et al., 2012*). The degradation of the CRY proteins goes via different path: the FBXL3 (F-box/LRR-repeat protein 3) protein polyubiquitinates the CRY proteins, targeting them for proteosomal degradation (*Siepka et al., 2007*). However, targeting the different CRY proteins for polyubiquitination requires unique kinases: CRY1 is phosphorylated by AMPK1 (*Lamia et al., 2009*), while CRY2 phosphorylation involves a DYRK1/GSK-3β cascade (*Kurabayashi et al., 2010*).

The CLOCK/BMAL1 heterodimer also activates the transcription of a second feedback loop, which acts in coordination with the main loop described above. The second loop involves CLOCK/BMAL1 acting on the E-box elements of the orphan-receptor genes Rev-Erbα/β and RORα/β. Their protein products, REV-ERB and ROR, competitively bind to the Retinoic acid-related Orphan receptor Response Elements (RORE) binding sites located within the Bmal1 promoter, where the ROR proteins initiate Bmal1 transcription, while the REV-ERB proteins inhibit it (*Lowrey & Takahashi 2004;*

Mohawk et al., 2012). Originally, this loop was thought to be an accessory loop; however, a study by *Cho et al. (2012)* has shown that the Rev-Erbα/β are necessary for normal period regulation of circadian behavioural rhythmicity. A potential transcription loop composed of PAR bZip genes with D-box elements in their promoters, although not required for clock function, is thought to account for period's precision and to make the core oscillations more robust.

The central (master) clock

The "master clock" or "master pacemaker" is located in the suprachiasmatic nuclei (SCN), which is a small, bilateral structure of approximately 100 000 neurons (in humans), situated in the ventral hypothalamus, above the optic chiasm (*van der Pol 1980; Moore 1983*). The SCN receives direct light input from the melanopsin-containing retinal ganglion cells, called intrinsically photosensitive retinal ganglion cells (ipRGCs) via the retinohypothalamic tract (RHT) (*Do & Yau 2010*). It serves as a point where the whole population of SCN neurons couple together and oscillate in a coherent manner. *Welsh et al. (1995)* have first described that at a single cell level the neurons in the SCN show a broad range of cell-autonomous circadian period lengths, from 22 to 30 hours; a finding later confirmed by other labs (*Liu et al., 1997; Ko et al., 2010*). The intercellular coupling of the whole population of the SCN neurons results in a much more precise, averaged period that is reflected as the locomotor activity rhythm. However, the heterogeneity in period lengths of single neurons presents two important features of the SCN – phase lability and plasticity – that allow for period adjustment in different photoperiod (daily changes in light intensities) conditions. Besides providing a uniform period length, the intercellular coupling has another role, which is ensuring the robustness of the oscillations of the SCN oscillatory system, since single neuron cells do not oscillate in a robust manner (*Webb et al., 2009; Mohawk et al., 2012*). Furthermore, the robustness of the SCN oscillations is highly important, since

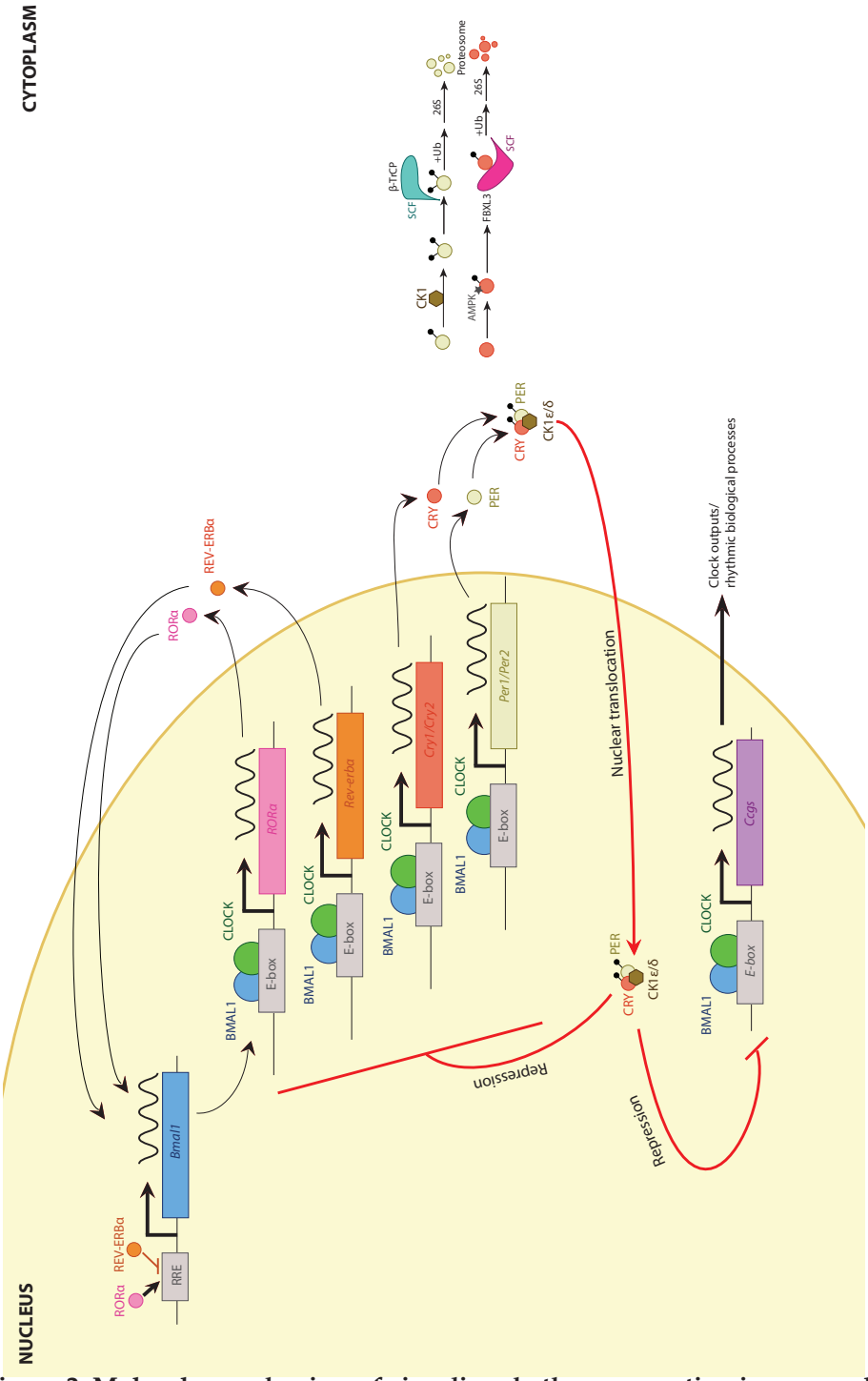


Figure 2. Molecular mechanism of circadian rhythms generation in mammals (adapted from *Mohawk et al., 2012*).

it allows for the SCN to remain in the appropriate phase in the presence of physiological rhythm perturbations such as restricted food availability, that can uncouple the behaviour from a natural light/dark cycle. SCN is also responsible for synchronizing phases among various peripheral/non-SCN tissues by neuronal and humoral signals (reviews by *Takahashi et al., 2008; Mohawk et al., 2012*).

Initially, it was thought that the SCN was the only structure capable of generating circadian rhythms. Since biological clocks were discovered in unicellular organisms, it seemed reasonable that clock mechanisms can be found in single cells of multicellular organisms. But the discovery of the SCN in higher organisms (reviewed in *Klein et al., 1991*) caused most investigators to believe in differences in clock organization between bacteria and metazoans. In 1998 *Balsalobre et al.,* have shown that a serum shock induces circadian expression of genes in cultured rat fibroblast cells. In the next years, reports showing presence of circadian clocks in liver, kidneys, heart and muscle (*Yamazaki et al., 2000; Balsalobre 2002; Yoo et al., 2004*) were published, thus proving that non-SCN clocks (known as peripheral clocks or oscillators) are present in virtually all tissues and organs of higher organisms.

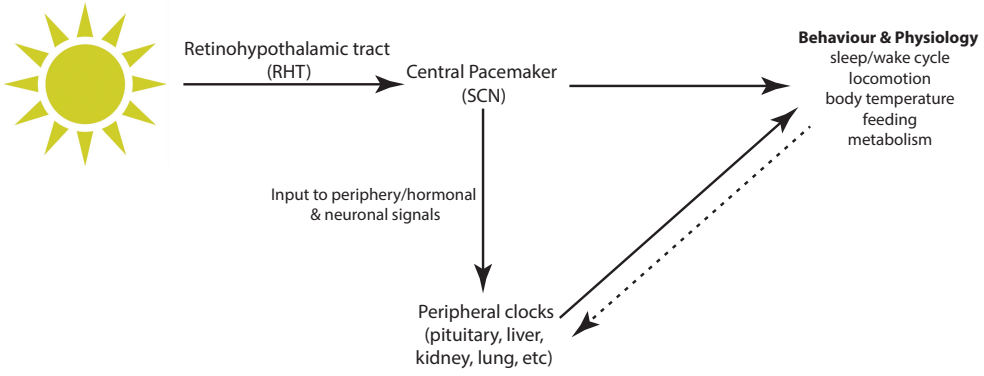


Figure 3. The organization of circadian systems in mammals. The suprachiasmatic nucleus (SCN) receives photic signal from the retina via the retinohypothalamic tract (RHT). The oscillations of the coupled SCN neurons are entrained and neural/humoral signals are released, which then reach the peripheral clocks, and result in physiological and behavioural outputs.

The peripheral clocks

Previously described molecular oscillators, comprising the autoregulatory transcriptional feedback loops, are present in the SCN neurons and in practically all mammalian tissues (*Yamazaki et al., 2000; Balsalobre 2002*). Besides the set of core clock genes, most tissues express up to thousands of rhythmic genes, which vary greatly depending on the tissue type (*Cermakian & Boivin 2009*). These genes are called clock-controlled genes, as their rhythmic expression is driven by the core clock genes. Genome-wide analyses have shown that from 2 to 10% of the total genome is transcribed in a circadian manner in various mouse tissues (*Cermakian & Boivin 2009; Hughes et al., 2009*); however, most of the rhythmic genes vary in-between the tissues. Circadian gene expression is tissue-specific, and set to best accommodate the tissue's respective function throughout a circadian cycle. Clock-controlled genes in different tissues are involved in various pathways depending on the tissue type and its function/role. Most of those genes are dependent on signals from the environment, on the rhythmic expression of core clock genes, such as *Bmal1*, or on systemic signals (*Panda et al., 2002; Hughes et al., 2009*). The systemic signals can drive and entrain gene expression rhythms and physiology of the peripheral oscillators; however, their nature is still being researched. Currently known cues include feeding, humoral factors, or body temperature fluctuations.

Sleep/wake cycle, metabolism, immunity and their connection with the circadian systems

Many behavioural and physiological processes are driven by the underlying molecular pathways, regulated by diverse external and systemic cues. One obvious example of a clock regulated process is the sleep/wake cycle. In a famous experiment conducted by *Aschoff (1965)*, human volunteers spent 3 to

4 weeks in isolation, devoid of any external indicators of periodicity, otherwise keeping a regular, daily routine (*Aschoff 1965*). During the course of the experiment, the subjects woke up later and later with each day, which led to a gradual increase of their sleep/wake cycle length, from 24 to approximately 28 hours.

However, upon returning to „natural" daily conditions with light, social interaction etc., present, the subjects' sleep/wake cycle length was restored to approximately 24 hours (*Aschoff 1965*). It was thus shown that in humans the sleep/wake cycle follows the individuals' endogenous clock, but is also susceptible to entrainment by external cues (zeitgebers). This study, as well as others conducted in subsequent years, have found that the behavioural and physiological rhythms are not only a manifestation of the internal circadian clock, but are capable of feeding back to the clock themselves, modifying the phase, amplitude or even period of the endogenous rhythms.

Another process that has been found to be tightly linked with circadian rhythms is metabolism. In recent years, this connection has become evident, as more metabolic disorders, like obesity, diabetes, hypertension, and cardiovascular disease, have been linked with disturbances of the inherent biological rhythms (*Maury et al., 2009; Bellet & Sassone-Corsi 2010; Froy 2010; Eckel-Mahan & Sassone-Corsi 2013; Kalsbeek et al., 2014*). This is especially apparent in shift workers, who due to a desynchrony between the internal clock and the external environment with its time cues, often have increased body mass, elevated blood glucose levels, and heightened risk of developing hypertension and cardiovascular disease, compared to their counterparts working conventional hours (*Sack et al., 2007; Scheer et al., 2009; Arendt 2010*). However, the connection between the circadian clock and metabolic homeostasis is much more complex. Rodent studies show that feeding/food has the capacity to act as a zeitgeber, and that modifying the natural feeding patterns, can cause a phase shift of gene expression in the liver; however, in case of arrhythmic model animals, it also has the potential to restore the temporal

rhythms in the periphery (*Edmonds & Adler 1976; Damiola et al., 2000; Escobar et al., 2009; Mohawk et al., 2012*). Therefore, feeding can be both a circadian output, and a time cue able to synchronize in particularly the peripheral tissue.

The immune functions in humans and the circadian clock have also been shown to be closely linked (*Born et al., 1997; Keller et al., 2009; Lange et al., 2010; Ackermann et al., 2012*). The immune system enables the organism to adapt to specific aspects of the environment, in particular to defend itself against diseases caused by various pathogenic agents. Many parameters and functions of the immune system, like the proliferation of lymphocytes, levels of cytokines or circulating white blood cells, are dependent on the time of day (*Kawate et al., 1981; Young et al., 1995; Esquifino et al., 1996*). Moreover, certain diseases display intensification of symptoms at specific time of the day, i.e. asthma symptoms worsen overnight, while stroke and myocardial infarction are most prevalent in the early morning hours (*Marler et al., 1989; Elliott 2001; Mortola 2003*). Those observations, together with in vitro experiments led to the concept of chronotherapy – correlating treatment or medicine administration with daily circadian rhythmicity, i.e. at a time when the efficacy of the drug would be at its highest, while its potential toxicity/side effects would be at their lowest.

The connection between biological rhythms and various aspects of human physiology can be utilized to improve many aspects of human life. As mentioned in the previous paragraphs, recognizing the dependence of toxicity and efficiency of chemical compounds on time of day led to correlating drug treatment times with the body's internal time, so that the side effects of the drug are reduced. Furthermore, the connection that was found between shift work, metabolic disorders, and circadian rhythms is now taken into consideration in human health care, to increase awareness and to develop new strategies to impede the epidemic of these ailments.

Circadian biology can also be applied in other areas of life and research, and this thesis is going to focus on utilizing rhythmic molecules in forensic trace deposition time estimations, as outlined in the following subchapter.

Circadian biology meets forensic biology

One of the commonly encountered issues in criminal investigations, that forensic researchers have devoted themselves to solve, is time. Whether it is determining the time of death, or age of biological traces found at the crime scene, none of the current methods can match in precision and reliability to DNA-based human identification method, which is viewed as the golden standard in forensic science (*Lynch 2003*).

In forensic science, time can be regarded as either the time of death of an individual, usually assessed by analysing the post-mortem processes happening in the body, or as the age of human biological stains found at crime scenes, e.g., the time that has passed since the discovered biological material had been left at the crime scene (*Kayser & de Knijff 2011; Bremmer et al., 2012*). However, a third aspect of addressing the time is the time of day or night at which the biological evidence was left at the crime scene, and it is called trace deposition time. This perspective sheds light on new opportunities, and possible leads for the investigation, such as testing a person's alibi directly from evidence material. Knowing when during the day or night the biological material, e.g., blood, that was left at the crime scene could aid in either confirming or rejecting the alibi claims of a potential suspect, and could highlight the involvement of yet unknown suspects. In fact, in recent years few studies have applied circadian biomarkers to forensic questions, mostly regarding the time of death (*Mikami et al., 1994; Ackermann et al., 2010; Kimura et al., 2011; Odriozola et al., 2013*).

The first time circadian biology was applied for forensic purposes was in 1994. In this study, pineal melatonin levels were assessed to estimate the time of death (*Mikami et al., 1994*). The concentration of hormone melatonin displays robust 24 hour rhythms that reach the highest value around 3 o'clock in the morning. Moreover, melatonin is widely used as a marker of circadian phase in various research studies as well as in clinical settings (*Arendt 2005; Benolucif et al., 2008; Hofstra & Weerd 2008*). Melatonin, alongside another circadian hormone called cortisol, has been proposed as a marker for trace deposition timing in a proof-of-principle study in 2010 by *Ackermann et al.* This study has shown that oscillations of the two circadian hormones not only persist in blood and saliva, as previous studies had established, but that melatonin and (less so) cortisol are stable over time of sample storage. Marker stability is a crucial prerequisite for forensic applications, where samples are usually collected days, weeks or months after deposition. Furthermore, by determining the detection sensitivity of the markers in limited sample volumes that are typically encountered during criminal investigations, the study has shown that it is feasible to use the ELISA-based tool in forensic settings. With all the possible advantages of the described approach, the authors also discussed the potential caveats of the proposed method. One important aspect to consider is the influence of external cues on the secretion of the circadian hormones used. In particular, secretion of melatonin is heavily suppressed by light (*Lewy et al., 1980*), and cortisol, also known as the stress hormone, can have disturbed levels in individuals under chronic stress (*Ockenfels et al., 1995; Chrousos 2009*) or suffering from PTSD (posttraumatic stress disorder) or addiction (*Lovallo 2006; Fries et al., 2009*).

In 2011 *Kimura* and his colleagues investigated the usefulness of known human circadian genes *BMAL1*, *PER2*, and *REV-ERBa* for estimating the time of death of individuals. The expression of those genes was tested in various tissues, i.e. kidney, liver and heart, collected within 72 hours post-mortem. The authors reported ranges of ratios of *PER2/BMAL1* and *REV-ERBa/BMAL1* as indicative of a

time frame during which the individual died. Due to irregularities in inter-individual expression levels of *PER2*, the authors recommended using the *REV-ERBa/BMAL1* ratio for forensic time of death estimations.

A common feature of all the presented studies, is the small amount of markers tested and thus the limited discriminatory power of the obtained results. As such, using only melatonin, or melatonin and cortisol together, or the *REV-ERBa/BMAL1* ratio, allows to distinguish only between the day and night (*Ackerman et al., 2010; Kimura et al., 2011*). A point that is emphasized in all the studies is the need for more biomarkers – rhythmic, robust, reliable, easily detectable, and quantifiable in forensic samples. Because the circadian rhythms are ubiquitous in almost every aspect of human biology, also on a molecular level, the possibility of identifying novel, rhythmic biomarkers is very high (*Kayser & de Knijff 2011*).

Scope of this thesis

The main objectives of criminal investigation are to determine the who, the how and the when of the crime. Who was the perpetrator that committed the criminal offense; how did the crime happen; and when or at what time did the crime happen. Currently, there are methods which allow the researchers and investigators to answer the first two questions, the who and the how. The when, however, is still in need of developing reliable and precise tests. Research presented in this thesis aimed at unravelling one of the aspect of the when – what time during the day or night a biological trace was left at the crime scene – by applying the insights from circadian biology to an open forensic problem.

Chapter 2, describes the study determining the utility of miRNA markers miR-142-5p and miR-541, which were previously proposed for forensic time of death determination (*Odriozola et al., 2013*), for blood stain deposition timing. Contrary, we did not find these particular miRNA markers suitable for estimating blood trace deposition time. **Chapter 3** presents the assessment of expression patterns of genes, that were implied to be rhythmic mostly in animal models or in various human peripheral tissues, in human blood from healthy volunteers subjected to sleep laboratory protocols. In this study, the rhythmicity of several clock-controlled genes in humans was established, and the most informative ones were subsequently tested to determine their potential to predict time of day for forensic purposes, as reported in **chapter 4**. In **chapter 5**, a study on the rhythmicity of metabolites tested in human blood samples is presented. In this project metabolites with robust, 24 hour rhythms were identified, and their suitability for blood trace deposition timing was determined. Finally, **chapter 6** provides a general discussion on the research presented in **chapters 2 – 5**, including elaboration on ongoing as well as future studies on this subject.

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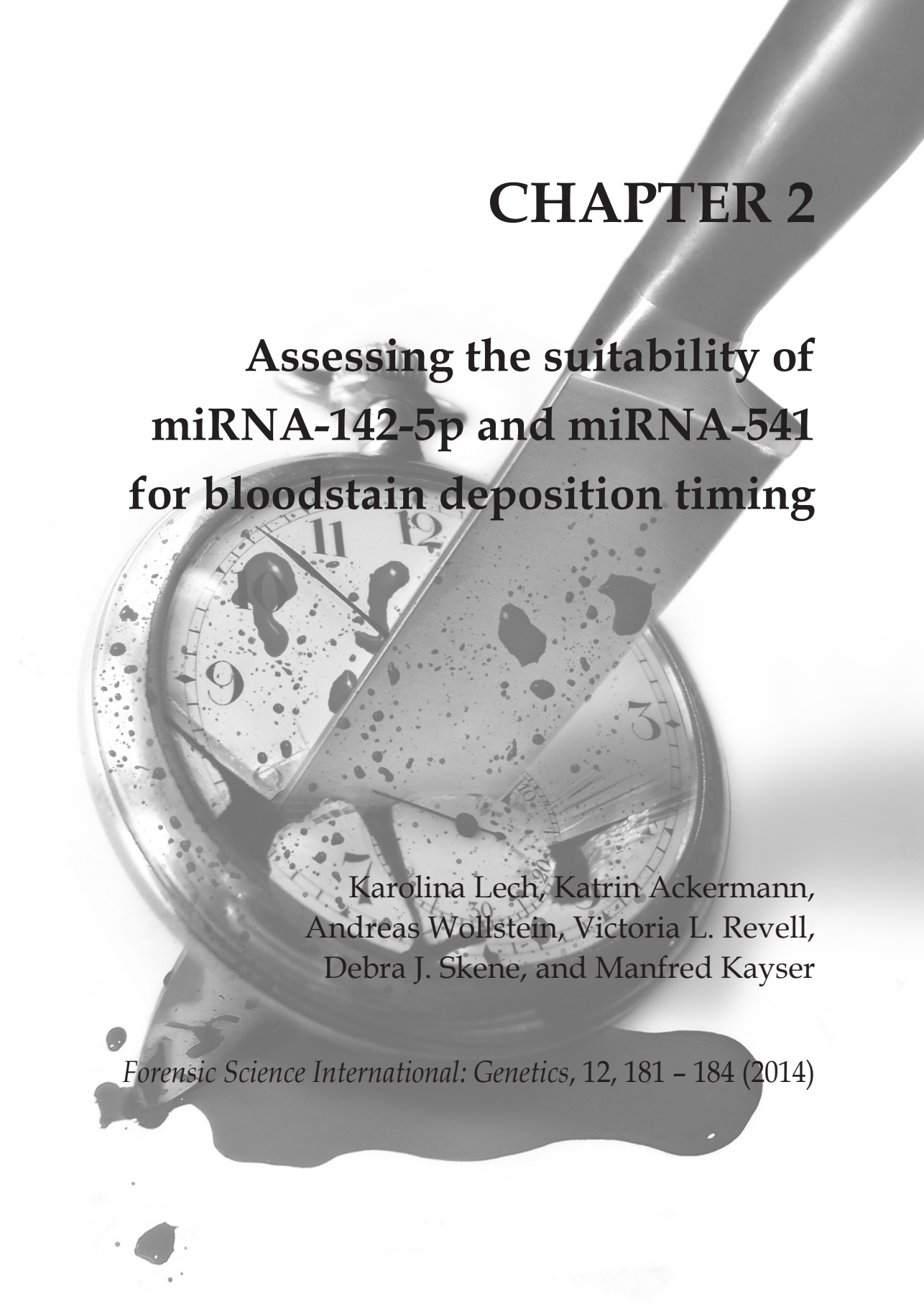

CHAPTER 2

Assessing the suitability of miRNA-142-5p and miRNA-541 for bloodstain deposition timing

Karolina Lech, Katrin Ackermann, Andreas Wollstein, Victoria L. Revell, Debra J. Skene, and Manfred Kayser

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Abstract

A recent proof-of-concept pilot study proposed using microRNA (miRNA) markers for time of death determination. The markers - miRNA-142-5p and miRNA-541, were reported to show considerable expression differences in vitreous humour between individuals who died during the day or night. Here, we investigated whether these miRNA markers show the same diurnal expression pattern in blood, which would make them useful for estimating bloodstain deposition time, to allow molecular alibi testing for forensic casework. We analysed venous blood samples collected from 12 healthy individuals every 4 hours during the 24 hour day/night period under controlled sleep-laboratory conditions. MiRNA-142-5p normalised against miRNA-222 showed no statistically significant expression differences between blood samples collected during day time and night time (one-way ANOVA $p=0.81$), and also no statistically significant rhythmicity during the 24 hour day/night period (cosine fit for all individuals $p>0.05$, averaged data $p=0.932$). MicroRNA-541 amplification in blood was above the 34-cycle threshold applied in the study, indicating too low quantities for obtaining reliable data. Overall, we conclude that the two miRNA markers previously suggested for time of death determination in vitreous humour are not suitable for estimating the deposition time of forensic bloodstains. Future studies may find out if miRNA markers with significant diurnal expression patterns can be identified and how useful they would be for forensic trace deposition timing.

Introduction

Circadian rhythms are entrainable, endogenously generated oscillations with a period of approximately 24 hours (h), present in most organisms, including humans. In recent years it has been shown that every cell contains its own molecular clock composed of a network of oscillating mRNAs and their protein products (*Takahashi et al., 2008; Mohawk et al., 2012*). Numerous biological processes exhibit significant daily variations including activity and behaviour, heart rate, hormone secretions, or sleep-wake cycle amongst others (*Lowrey & Takahashi 2004; Partch et al., 2014*). Knowledge about the underlying molecular pathways of these processes and their components could provide a plethora of potential rhythmic biomarkers also for forensic applications. In principle, such rhythmic biomarkers can be useful for molecular time estimations in two forensically relevant ways: estimating the time of death and estimating the deposition time of biological traces found at a crime scene. The latter would provide a molecular alibi test allowing DNA-identified sample donors to be linked with criminal acts, which is highly appreciated for crime investigation (*Kayser & de Knijff 2011*).

The first approach to estimate trace deposition time by incorporating a chronobiological aspect into the forensic field was published in 2010 (*Ackermann et al., 2010*). In this proof-of-principle study, the 24 h profiles of two circadian hormones, melatonin and cortisol, were reproduced in small blood and saliva samples collected around the clock. The feasibility of applying this approach in a forensic setting was also shown - it requires only minute amounts of samples and utilizes commercial assays together with basic laboratory equipment. The time frame estimated with those two hormones is, however, limited to either late night (melatonin) or early morning hours (cortisol) (*Ackermann et al., 2010*), and both hormones are known to be influenced by external factors *(Ackermann et al., 2010*). Hence, the exploration for additional biomarkers with rhythmic changes in concentration that eventually would prove

invaluable for increasing the precision of biological trace deposition timing in forensic applications, is encouraged.

Determining the time of death with biochemical markers, i.e. melatonin, has been described earlier (*Mikami et al. 1994 ; Ackermann et al., 2006*), and recently a proof-of-principle study proposed utilization of microRNAs (miRNAs) for that purpose (*Odriozola et al. 2013*). MicroRNAs are of great interest for various forensic applications (*Kayser & de Knijff 2011*), due to their small size, tissue-specific expression and higher resistance to degradation compared to mRNA, and were previously proposed as suitable markers for forensic body fluid identification (*Bartel 2004; Sood et al., 2006; Zubakov et al., 2010*). In recent years various microRNAs have been implied in the regulation of circadian clock (*Cheng et al., 2007; Chen et al., 2013; Mehta et al., 2013*), and some of them were shown to exhibit diurnal expression changes in diverse tissues, such as liver or suprachiasmatic nucleus (SCN) (*Cheng et al., 2007; Mehta et al., 2013*).

In their study (*Odriozola et al., 2013*), the authors assessed the miRNA expression profile of vitreous humour, and discovered that miRNA-142-5p and miRNA-541 exhibited considerable variations in their expression levels between individuals who died during day- or night time, when normalised against the reference marker miRNA-222. They also showed that there was no correlation between miRNAs expression and the time elapsed after death, suggesting in-vitro stability of the markers for at least 24 h, which is an important prerequisite for forensic applications.

In the present study, we investigated whether these two miRNA markers exhibit the same diurnal changes in expression levels in other types of forensically relevant biological samples, in this case blood, and if they do, to what degree their combined use together with melatonin and cortisol could increase the precision of estimating blood trace deposition time. For this purpose, we determined the temporal profiles of miRNA-142-5p and miRNA-541 in blood samples from 12 individuals collected every 4 hours during a 24 h day/night cycle under controlled wake/sleep laboratory conditions.

Materials and methods

Biological samples

Detailed characterization of sample collection as well as the sleep laboratory protocol used, is described elsewhere (*Ackermann et al., 2012*). In short, two-hourly blood samples were collected from 15 healthy male volunteers aged 19 – 35 years (n = 15; mean age ± SD = 24 ± 5 years) participating in the 66 hour-long study (Clinical Research Centre, University of Surrey, UK) after various medical assessments and completion of sleep questionnaires. The subjects underwent a week of regular sleep-wake routine, before being admitted into the sleep laboratory. The sleep laboratory protocol design controlled for food intake and lighting conditions and included adaptation, baseline and sleep deprivation nights.

For the current study, 7 four-hourly blood samples (spanning a course of 24 h) per each participant (n = 12) were included. Three subjects were omitted from analysis due to having either significantly shifted melatonin profiles or an abnormal white blood cells (WBC) count (*Ackermann et al., 2012, 2013*). The analysed samples were collected during the baseline night (N2) at 22:00, 02:00, 06:00 h (n=36) during which the participants were not subjected to sleep deprivation (normal sleep) and during a wake period (D3) mimicking a normal day, at 10:00, 14:00, 18:00 and 22:00 h (n=48).

RNA Isolation and quantitation

Blood samples were collected in EDTA tubes and stored at -80^{o}C until use. Total RNA was extracted from 400 µl of whole blood using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol with minor modifications, such that the samples after homogenization were incubated on ice for 30 minutes and centrifuged at 4^{o}C, speed 13 000 g for 5 minutes, and one volume of 70% ethanol (Merck, Darmstadt, Germany) was used

for precipitation. Purity of each sample was assessed with NanoDrop ND-2000 (NanoDrop Technologies, Wilmington, DE, USA) and the quality and quantity were determined using the Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany). Total RNA samples were stored at -80°C until assayed.

Reverse Transcription Reaction

Reverse transcription (RT) reactions were performed in a final volume of 15 µl, using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). Each reaction contained 100 ng of total RNA, 6 µl TaqMan MicroRNA Assay RT Primer Pool (containing miRNA-142-5p, miRNA-222, miRNA-541), 0.2 µl of 100mM dNTPs, 3 µl of MultiScribe Reverse Transcriptase enzyme (50 U/µL), 1.5 µl of reverse transcription buffer (10x) and 0.19 µl of RNase Inhibitor (20 U/µL). The RT primer pool was prepared according to the manufacturer's instructions concerning custom primer pool preparation (Applied Biosystems, Life Technologies, User Bulletin_4465407) with the exception of using nuclease-free water instead of 1x TE buffer. Negative controls containing distilled water instead of RNA were included. RT reactions were performed on MJ Research Thermal Cycler PTC-200 (GMI, Minnesota, USA) with the following program: 16°C for 30 minutes, 42°C for 30 minutes, 85°C for 5 minutes and 4°C on hold. Samples were kept at -20°C until assayed.

RT-qPCR reaction

MiRNA-142-5p and miRNA-222 expression levels were tested in 84 samples (12 subjects, 7 time points per subject). MiRNA-541 expression was tested in a subset of 14 samples (2 subjects, 7 time points per subject). The RT reaction contained 0.5 µl of appropriate TaqMan MicroRNA Assay (Applied Biosystems, Life Technologies), 2 µl of two times diluted RT product, 5µl of TaqMan Fast Universal PCR Master Mix (2x) NoAmpErase UNG (Applied Biosystems, Life

Technologies) and 2.5 µl of nuclease-free water in total volume of 10 µl. No-template controls (NTC) with distilled water instead of RT reaction product were included. All reactions were run in triplicate in 384-well plate format on Roche LightCycler480 (Roche Diagnostics, Mannheim, Germany). The program consisted of a 10 minutes pre-incubation step in 95°C followed by 45 cycles of incubation and extension at 95°C for 15 seconds and at 60°C for 60 seconds, respectively, and finished with 30 seconds at 40°C.

Data Analysis

Relative quantification of microRNA amplification was performed with the second derivative maximum method (Roche Diagnostics) followed by the delta cycle threshold (CT) method (*Livak & Schmittgen 2001*). All samples with CT values over 34 were considered as not expressing the tested microRNAs. MiRNA-222 was used as the reference gene for the calculations, because it was shown to be amplified stably and consistently in vitreous humour samples and was identified as the best single candidate reference gene by the Genorm program (*Odriozola et al., 2013*). Additionally, initial testing of miRNA-222 with the NormFinder (*Andersen et al., 2004*) in a subset of blood samples, revealed it to be a good candidate for normalisation purposes. To exclude the possibility of time-of-day dependent rhythmicity, normaliser levels were further analysed with non-linear curve fitting and single cosinor methods.

The expression levels for miRNA-142-5p were estimated as fold change (FC), defined by the equation $FC=2^{dCT}$. Assessment of rhythmicity was performed with three independent methods. First, a one-way ANOVA analysis was implemented to determine the difference of normalised miRNA-142-5p expression levels between samples collected during the day and night. Secondly, the relative expression values for each subject were analysed with the single cosinor test with a 24 h period to determine the presence or absence of diurnal expression pattern (*Ackermann et al., 2013*). The analysis was performed for each subject

separately, as well as for averaged expression data from all subjects.

Next, non-linear curve fitting was performed using a cosine function with a 24 h period on z-scored, miRNA-222-normalised expression data of miRNA-142-5p for all individuals simultaneously, as described elsewhere (*Minors & Waterhouse 1988*), to obtain the estimates for amplitude and peak and the significance of fit. The equation for non-linear curve fitting is presented as follows:

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

Where TP is the time point, a_i is the term for individual i, β is the amplitude and t is the peak time of the cosine function.

Results

The expression levels of miRNA-142-5p (in the range of 31 – 33.6 CT) and miRNA-222 (between 22 – 25 CT) during the 24 h day/night cycle were tested in total RNA isolated from 84 blood samples of 12 subjects collected at 7 time points every 4 hours, and for miRNA-541 in a subset of 14 samples from 2 participants. Testing of miRNA-541 was not performed in all subjects due to initial data analysis indicating amplification of this particular miRNA above 34 CT (on average 37 CT), which was the detection threshold applied in this study. This result indicates that the level of miRNA-541 found in these blood samples was too low for reliable data generation. Before normalisation we determined the significance of fit of a cosine curve on miRNA-222 data, in order to exclude the possibility of using a rhythmic normaliser in our analysis. With the single cosinor test, p-values >0.05 were obtained in 11 out of 12 individuals, and no significant time-of-day variation was found using non-linear curve fitting for both amplitude (p=0.61) and peak (p=0.35) estimates.

Calculated fold-changes of miRNA-142-5p, normalised against miRNA-222, throughout the 24 h period are presented in **Figure 1**. No significant differences between daytime and night time expression levels were detected (one-way ANOVA p=0.81). Furthermore, rhythmicity assessment using the cosinor method revealed no statistically significant results for any subject individually (all p-values >0.05) and for data averaged across individuals (p=0.932). The expression of miRNA-142-5p for all subjects and all time-wise samples simultaneously was additionally assessed with the non-linear curve fitting method and the results are presented in **Figure 2**.

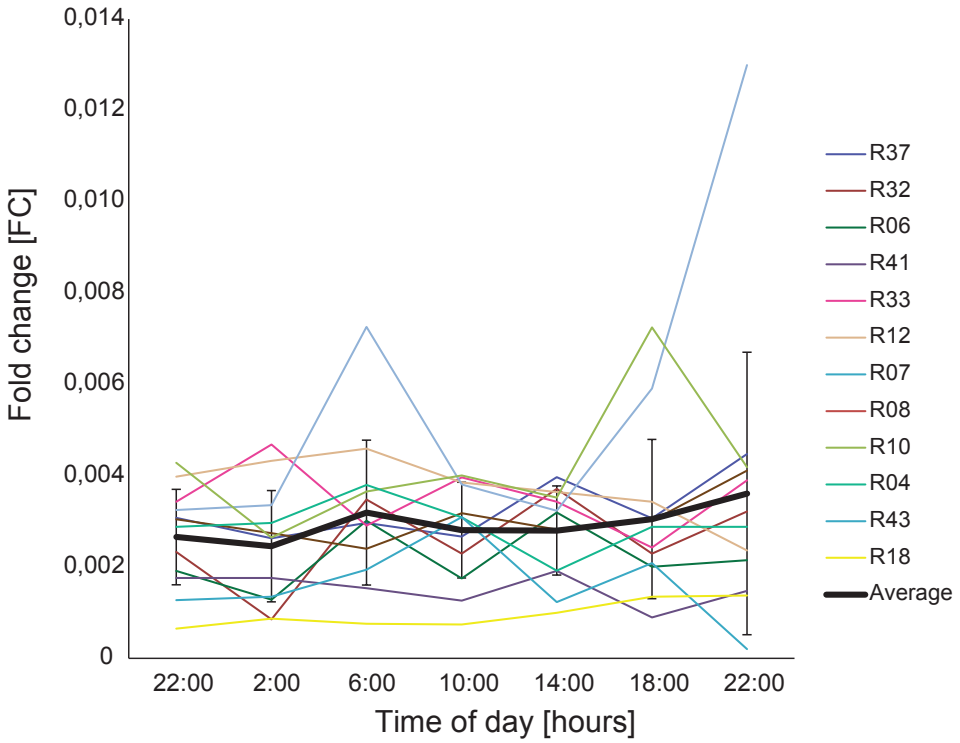

Figure 1. MiRNA-142-5p levels, normalised against miRNA-222, in 12 healthy individuals depicted in coloured lines presented as fold change (FC) versus blood collection time within one 24 h day/night cycle. The black line represents the average fold change across all individuals with the standard deviation bars. The high FC value of R07 at 22:00 h is probably due to partial coagulation of this particular blood sample.

The estimates for peak and amplitude obtained with this method are not statistically significant (estimate$_{amplitude}$ =-0.05; p$_{amplitude}$ =0.74; estimate$_{peak}$ =-16.67; p$_{peak}$ =0.15). All the applied methods showed that miRNA-142-5p levels do not display any rhythmic changes in the blood samples we analysed.

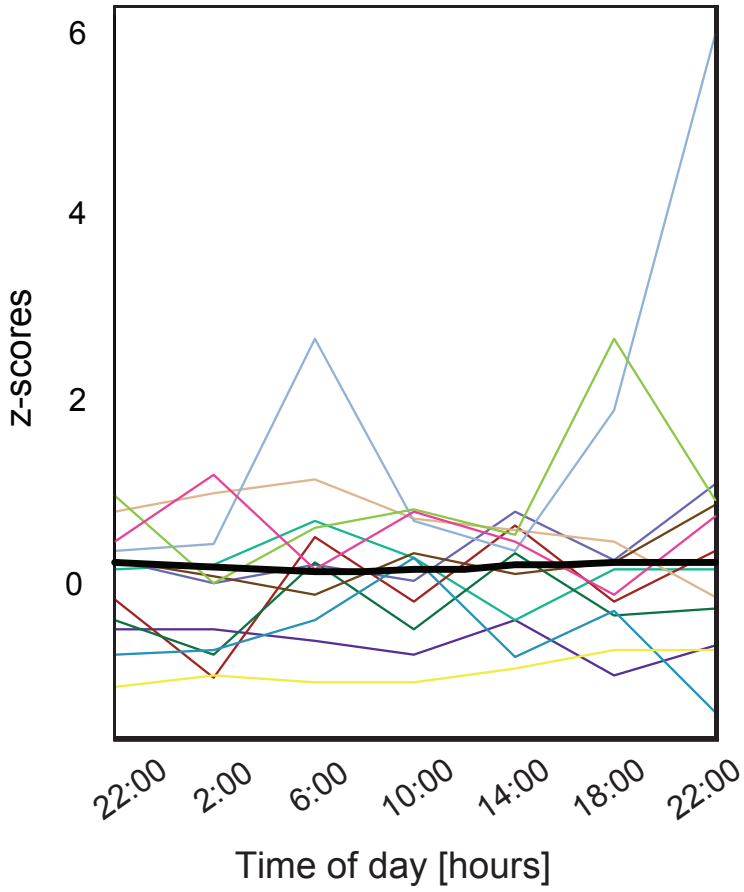

Figure 2. Z-scored, miR-222 normalised levels of miRNA-142-5p in each of the 12 individuals depicted in coloured lines with a superimposed cosine curve as calculated with the non-linear curve fitting method (*Andersen et al., 2004*) depicted in black.

Discussion

In our study we aimed at establishing the 24 h day/night expression profiles of miRNA-142-5p and miRNA-541 in blood, because both markers were recently proposed to be suitable for time of death determination, due to observed diurnal differences in their expression levels in vitreous humour (*Odriozola et al., 2013*). In addition, previous microarray data suggested oscillation of miRNA-142-5p in murine liver, however this finding was not confirmed by RT-qPCR (*Na et al., 2009*) as it typically is recommended in order to avoid technical artefacts. These findings motivated our study for testing the suitability of both microRNA markers to estimate bloodstain deposition time for future forensic applications.

Our data for miRNA-142-5p suggest that this miRNA marker is found in blood in reliably measurable quantities, but also that its expression in this sample type does not oscillate in regard to day- and night time. Although our data do not allow us to ultimately conclude whether the changes in miRNA-142-5p expression that were reported in vitreous humour are driven by the endogenous clock, or are a response to external stimuli or both, we suggest further testing on miRNA-142-5p expression in vitreous humour and other human tissues before this marker is applied for time of death determination. Because of its non-rhythmic profile in blood, we do not regard miRNA-142-5p as suitable for any forensic time estimation from blood, including trace deposition timing as well as death timing.

Our results for miRNA-541 suggest that it is found in blood at levels too low to provide reliable results, at least when using RT-qPCR, which is currently the standard method for single-marker expression analysis. MiRNA-541 is a member of the microRNA 379-410 cluster of brain-specific microRNAs, and was shown to be abundantly expressed in distant axonal neuron cells (*Zhang et al., 2011*). Hence, the low levels detected in blood samples in our study, may not be too surprising and may possibly be a consequence of the

tissue-specific expression of miRNA-541. We conclude that, due to low quantities of miRNA-541 found in blood, it is not suitable for any forensic time estimation from blood including trace deposition timing as well as death timing.

Although we could not confirm the previous findings from vitreous humour in blood, we would like to emphasize the suitability of our protocol and samples to validate or de-novo identify oscillating biomarkers potentially useful for trace deposition timing as well as for time of death determination. The choice of sample type, i.e. blood was based on its suitability in light of both the forensic and chronobiological aspects. Blood represents the forensic trace encountered commonly at crime scenes, typically allowing the occurrence of violent crime to be confirmed, and is usually available with dead bodies. The blood samples we used in our analysis were collected around the clock, spanning a full 24 h day/night period and allowing assessment of temporal expression of a possibly rhythmic biomarker. Collection of these samples occurred under strictly controlled conditions in a dedicated sleep laboratory specialized for human chronobiology research, to assure elimination or minimization of exogenous factors that could confound the rhythm of the internal clock. In particular, such a laboratory setting allows blood sample collection during undisturbed sleep, which is otherwise impossible. Additionally, our study design entailed stringent criteria of selection of participants – exclusion of extreme chronotypes, restriction of the influence of sex and age on the phase of the clock (*Dijk et al., 2000; Duffy & Czeisler 2002*) etc. The conditions such as lighting intensity, meal composition and timing were controlled, yet still resembled natural everyday settings, which is important when confronted with the real crime case scenarios.

The ultimate goal of our study was to combine the time estimates obtained with melatonin and cortisol together with miRNA-142-5p and miRNA-541 expression data to increase the precision of bloodstain deposition timing. However, since miRNA-142-5p levels in analysed blood samples were found to be stable during the 24 h day/night period, and miRNA-541 was not

present in quantities high enough to allow reliable detection with the method used, we could not consider these miRNAs as markers useful for our aim. Hence, until new suitable biomarkers are identified in future studies, our previously suggested melatonin and cortisol system (*Ackermann et al., 2010*) remains the only available molecular approach for a person's alibi testing directly from crime scene stains.

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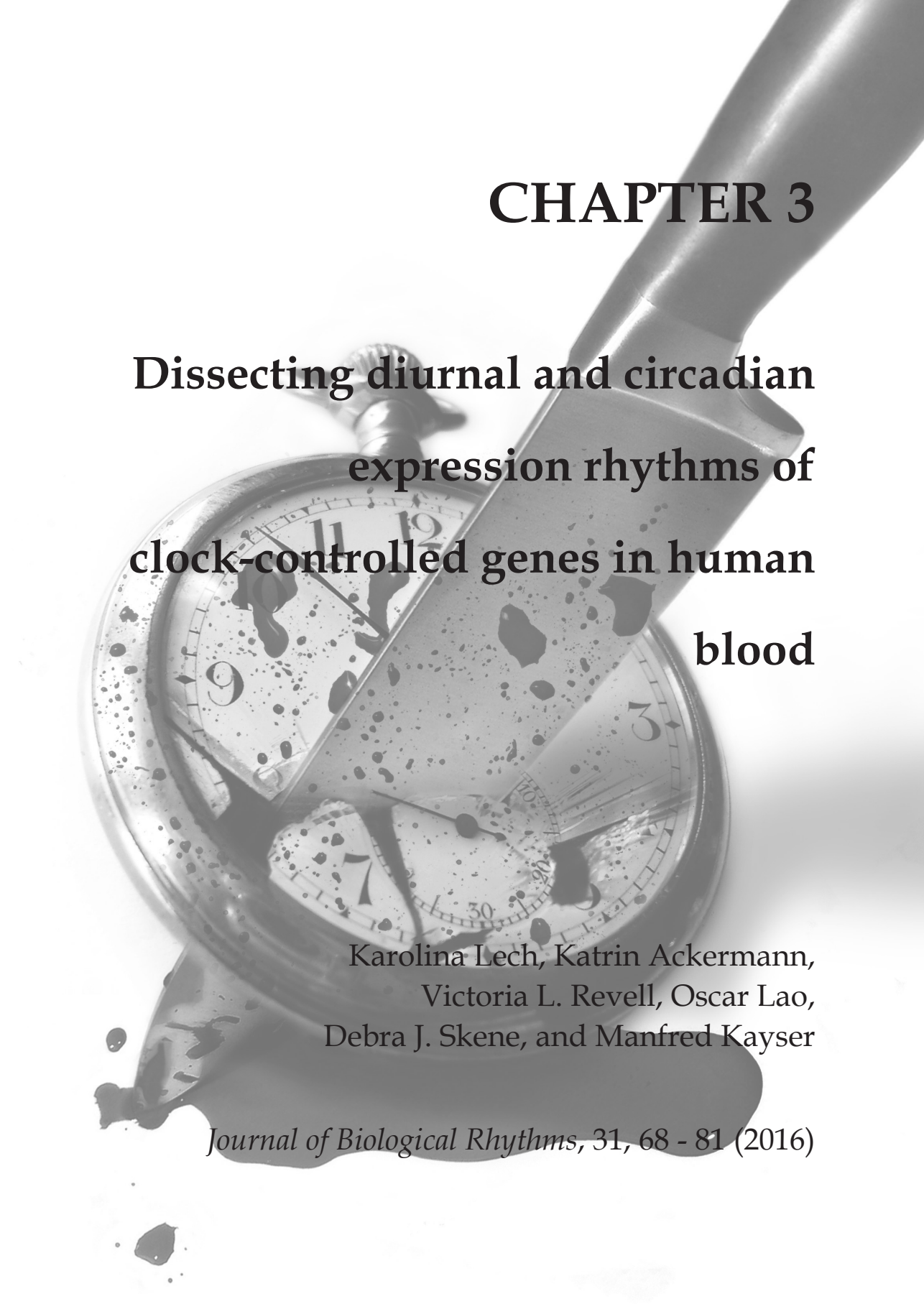

CHAPTER 3

Dissecting diurnal and circadian expression rhythms of clock-controlled genes 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 hours in 12 participants in the S/SD study, and across 33 hours 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-ERBa* 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 & Takahashi 2004, 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 & 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 & Maemura 2011;*

Portaluppi et al., 2012), cancer (*Sahar & Sassone-Corsi 2009; Savvidis & Koutsilieris 2012*), sleep disorders (*Archer et al., 2003; Lu & 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 & 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 & Cheng 2010; Vollmers et al., 2012*), sirtuin 1 (*SIRT1*) (*Rodgers et al., 2005; Longo & 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 hour (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-ERBa* 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 ≤ 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 h before and during the in-laboratory session; agreement to

eat standardised meals for the 48 h 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, 2013*). In brief, the participants (15 healthy, young males (mean age ± standard deviation) aged 24 ± 5) 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 & 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.

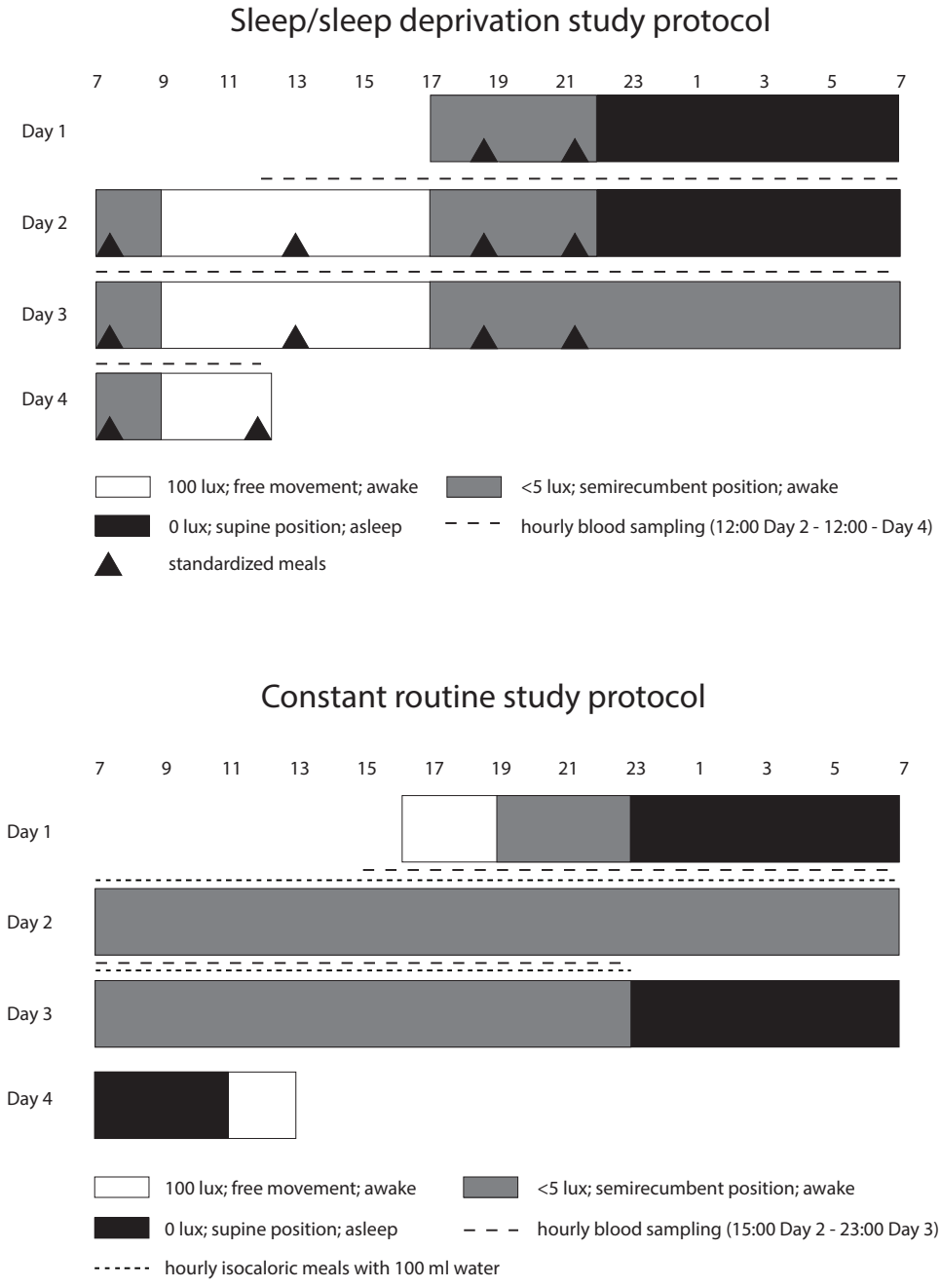

Figure 1. The sleep/sleep deprivation (S/SD) and constant routine (CR) study protocols. The S/SD study scheme is modified from Figure 1 from *Ackermann et al., 2010*.

Samples from 12 participants (mean age ± standard deviation = 23 ± 5 years) 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°C, 60 min at 42°C and 5 min at 70°C. The cDNA was kept at -20°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-ERBa*, *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 ($p\text{-value}_{CR}$ =0.40; $p\text{-value}_{S/SD}$ =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-ERBa*, *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°C for 10 min and 45 cycles of denaturation (95°C, 10 s), annealing (60°C, 10 s) and extension (72°C, 10 s), followed by a melting curve step with continuous data acquisition from 65°C to 97°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 & Schmittgen 2001*) was used to quantify relative gene expression in the S/SD and CR samples. Afterwards, *ACTB*-normalised, 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*-normalised 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)*:

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

Where β is the amplitude, t is the acrophase and a 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 a, 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*-normalised 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:

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

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**).

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-ERBa†	*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 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-ERBa†	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-ERBa, 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.*

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 (25%) individuals 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-ERBa*, 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-ERBa* 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**). 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 3**). 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-ERBa* 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-ERBa* (**Table 3**). The expression of *PER1*, *PER3* and *REV-ERBa* was significantly rhythmic in 10 (83%), 11 (92%) and

11 (92%) individuals, respectively, as shown by the single cosinor analysis.

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**). Expression profiles of all the genes analysed in the CR study are presented in **Supplementary material** (**Figure S3**).

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-ERBa†	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-ERBa†	*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 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.*

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**). 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

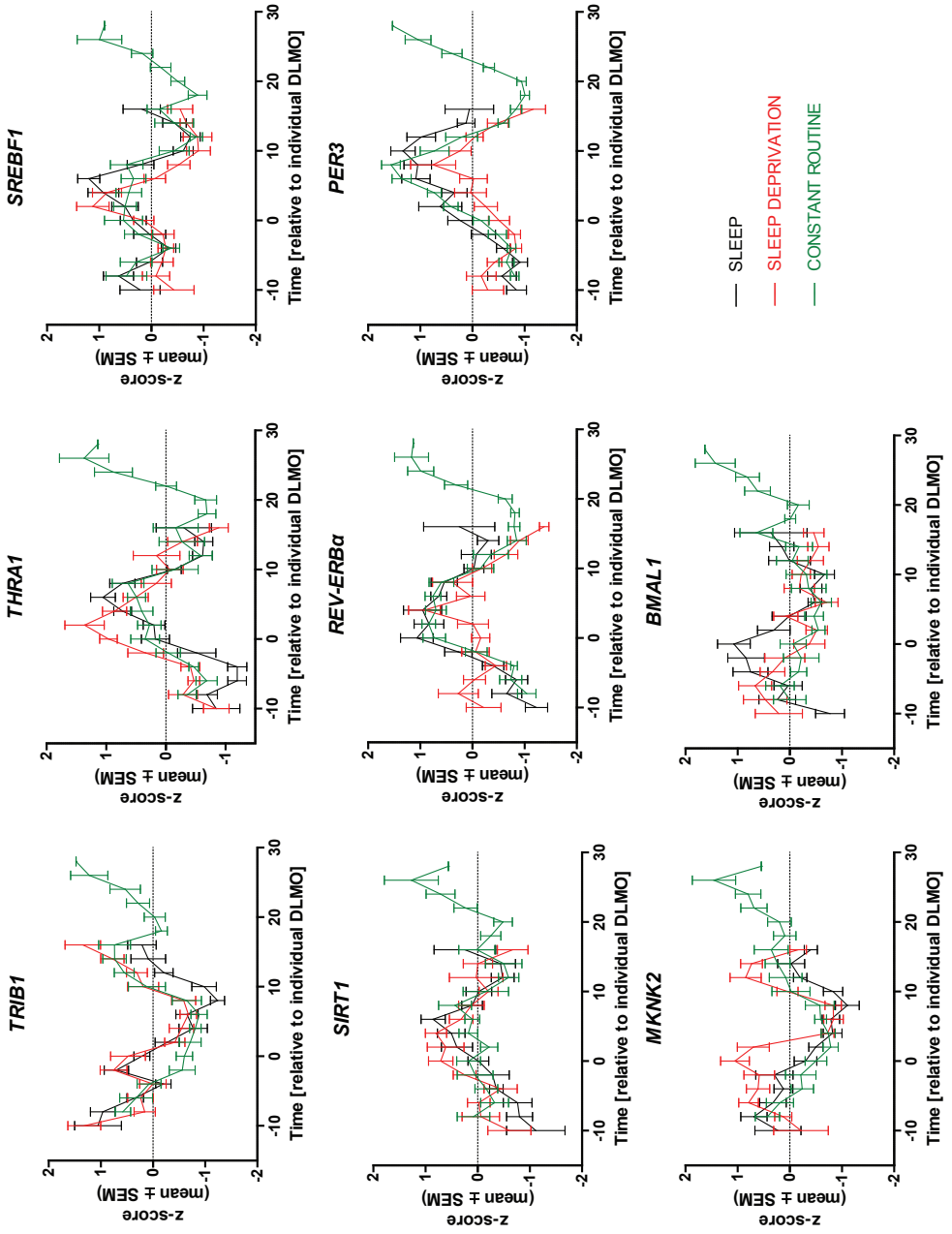

Figure 2. Plots of mean, z-scored, *ACTB*-normalised expression levels of selected genes (*TRIB1*, *THRA1*, *SREBF1*, *SIRT1*, *REV-ERBa*, *PER3*, *MKNK2* and *BMAL1*) tested in the subjects from the S/SD and CR studies, three different conditions (sleep; sleep deprivation; constant routine) overlaid 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 premitted. The time axis is presented as time (h) relative to individual DLMO.

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-ERBa,* 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-ERBa,* 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-ERBa* 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 the 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 minimise 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 & 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 (*Schoeller et al., 1997; Kräuchi et al., 2002; Hirao et al., 2010*). 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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Supplementary material

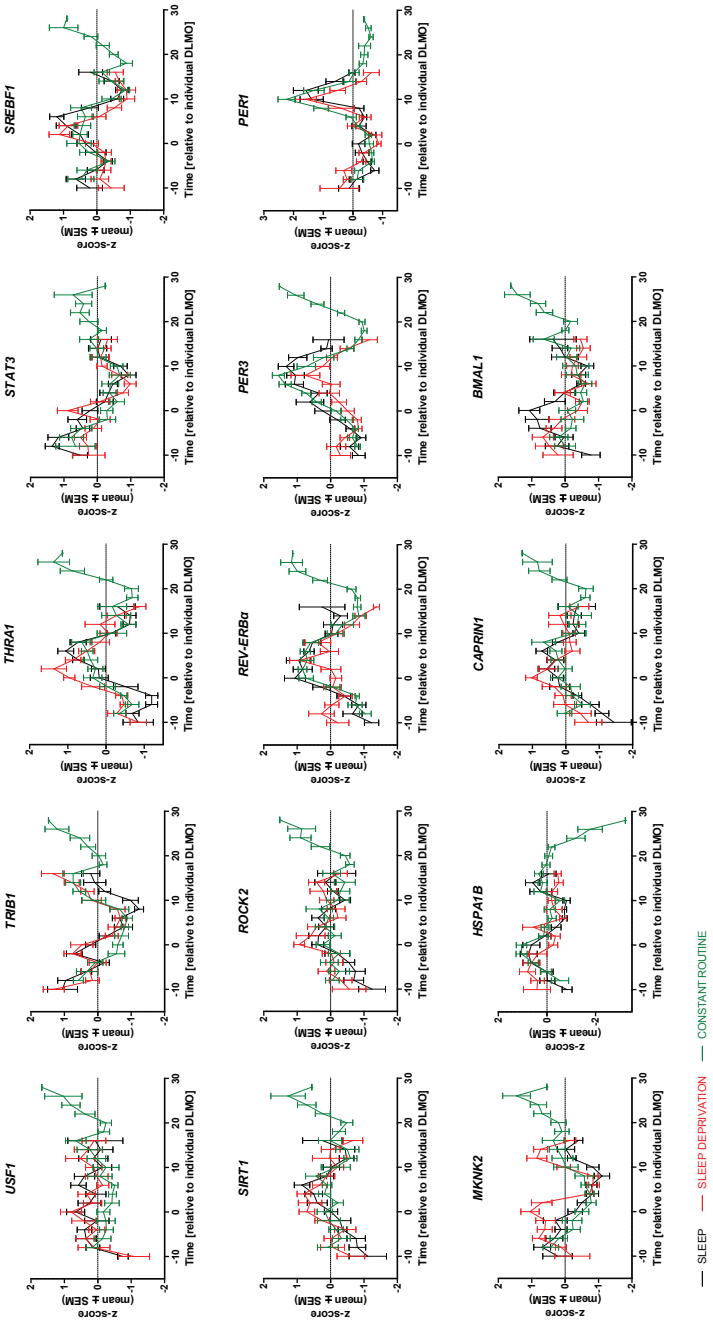
Supplementary Table 1. Sequences and product sizes of the primers used.

	Primer name	Sequence (5'- 3')	Product size
1	**ACTB_FP**	TGACCCAGATCATGTTTGAG	75bp
	ACTB_RP	CGTACAGGGATAGCACAG	
2	**CAPRIN1_FP**	AGTTGAAACGGTTGAGGTGGT	87bp
	CAPRIN1_RP	GGAGTCAAAGAGTGGGGCTC	
3	**SIRT1_FP**	AGTAGGCGGCTTGATGGTAA	70bp
	SIRT1_RP	CCTCAGCGCCATGGAAAATG	
4	**SREBF1_FP**	GAGCGAGCACTGAACTGTGT	74bp
	SREBF1_RP	TCCGAGAATTCCTTGTCCCC	
5	**ROCK2_FP**	ACGACTTGGGAGAAATGGGG	103bp
	ROCK2_RP	TACTACAGGAGCTGCCGTTTC	
6	**MKNK2_FP**	ATAACAAAGGCATCGCCCAC	130bp
	MKNK2_RP	GAGCAGTCCCCGTTGAGTTT	
7	**USF1_FP**	TGCTTCGACAACAGGTGGAA	123bp
	USF1_RP	AGCCCCTGAATCCCCATAGT	
8	**THRA1_FP**	TGGCCATGGACTTGGTTCTA	71bp
	THRA1_RP	CTCCCGGTTCTGCTCAATCA	
9	**STAT3_FP**	AGGATGGCCCAATGGAATCA	142bp
	STAT3_RP	CCGCATATGCCCAATCTTGA	
10	**TRIB1_FP**	GCTGCAAGGTGTTTCCCATTA	83bp
	TRIB1_RP	GCCAGTAATGTTGCTGTGCG	
11	**BMAL1_FP**	GCCTACTATCAGGCCAGGCTCA	149bp
	BMAL1_RP	AGCCATTGCTGCCTCATCATTAC	
12	**HSPA1B_FP**	CTGTACCAGGGTGCCGGTGGT	148bp
	HSPA1B_RP	AGTCCCAACAGTCCACCTCAAAGAC	
13	**REV-ERBα_FP**	GCGGCGATCGCAACCTCTAGT	115bp
	REV-ERBα_RP	GTAGGTGATGACGCCACCTGTGTT	
14	**PER1_FP**	GGACACTCCTGCGACCAGGTACTG	126bp
	PER1_RP	GGCAGAGAGGCCACCACGGAT	
15	**PER3_FP**	GGTCGGGCATAAGCCAATG	143bp
	PER3_RP	GTGTTTAAATTCTTCCGAGGTCAAA	

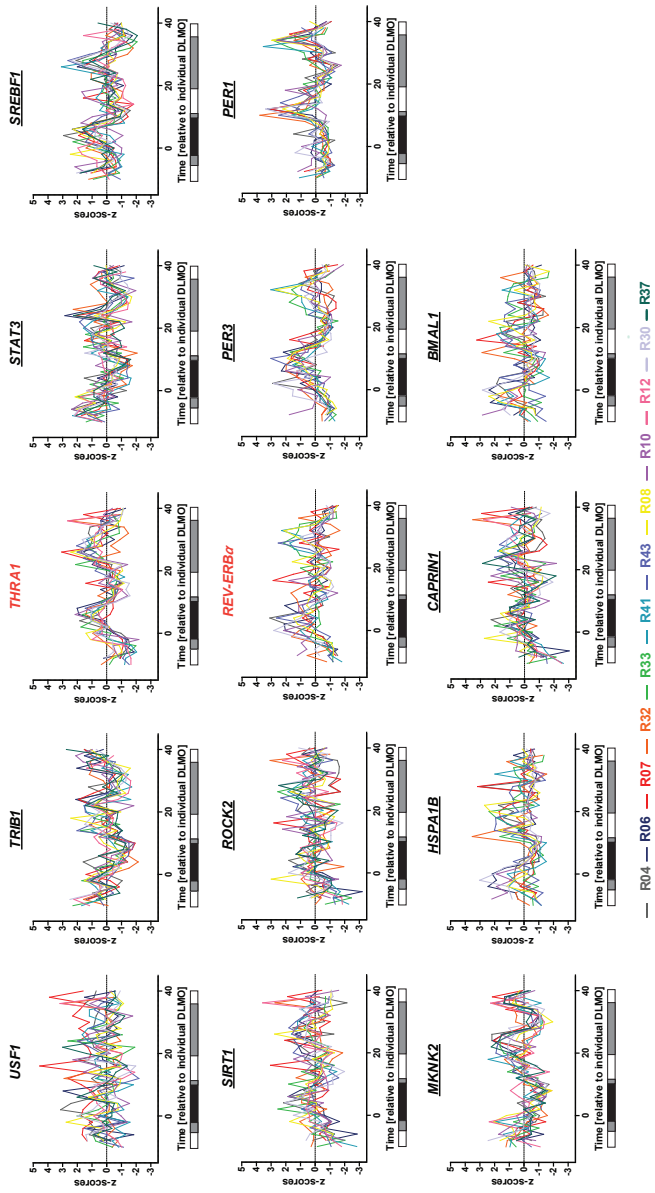
Supplementary Table 2. Diurnal preference (chronotypes), as assessed by the Horne-Östberg test, and the times in and out of bed (h) of the individuals of the CR study.

Subject Code	H-O	Bedtime [h]	Waketime [h]
901m	63 MM	23:00	07:00
903m	56 N	23:00	07:00
904m	45 N	23:00	07:00
906f	45 N	00:00	08:00
908f	54 N	23:30	07:30
909m	55 N	00:00	08:00
915f	51 N	23:00	07:00
916f	49 N	00:00	08:00
917f	56 N	00:00	08:00
918f	56 N	00:30	08:30
919m	67 MM	22:30	06:30
921f	62 MM	23:00	07:00

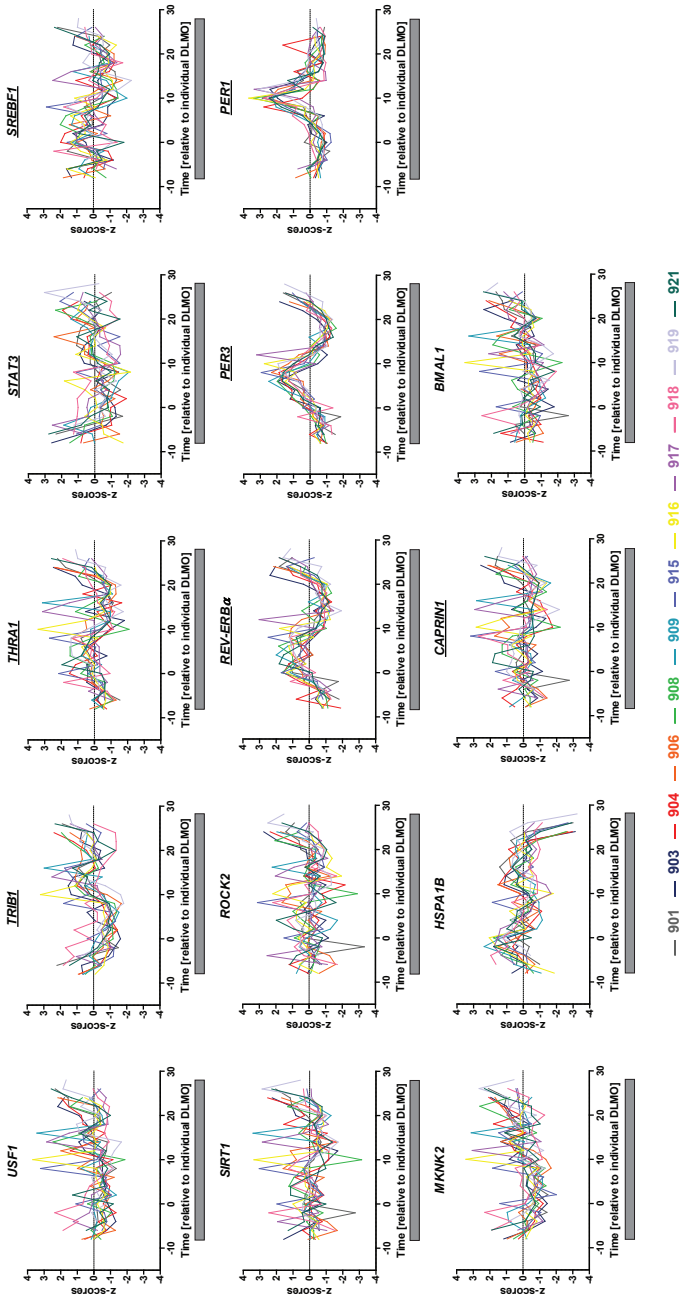
Depending on the score, the subjects are considered as: Definitely Morning Types (DM) 70-86, Moderately Morning Types (MM) 59-69, Neither Type (N) 42-58, Moderately Evening Types (ME) 31-41 and Definitely Evening Type (DE) 16-30.



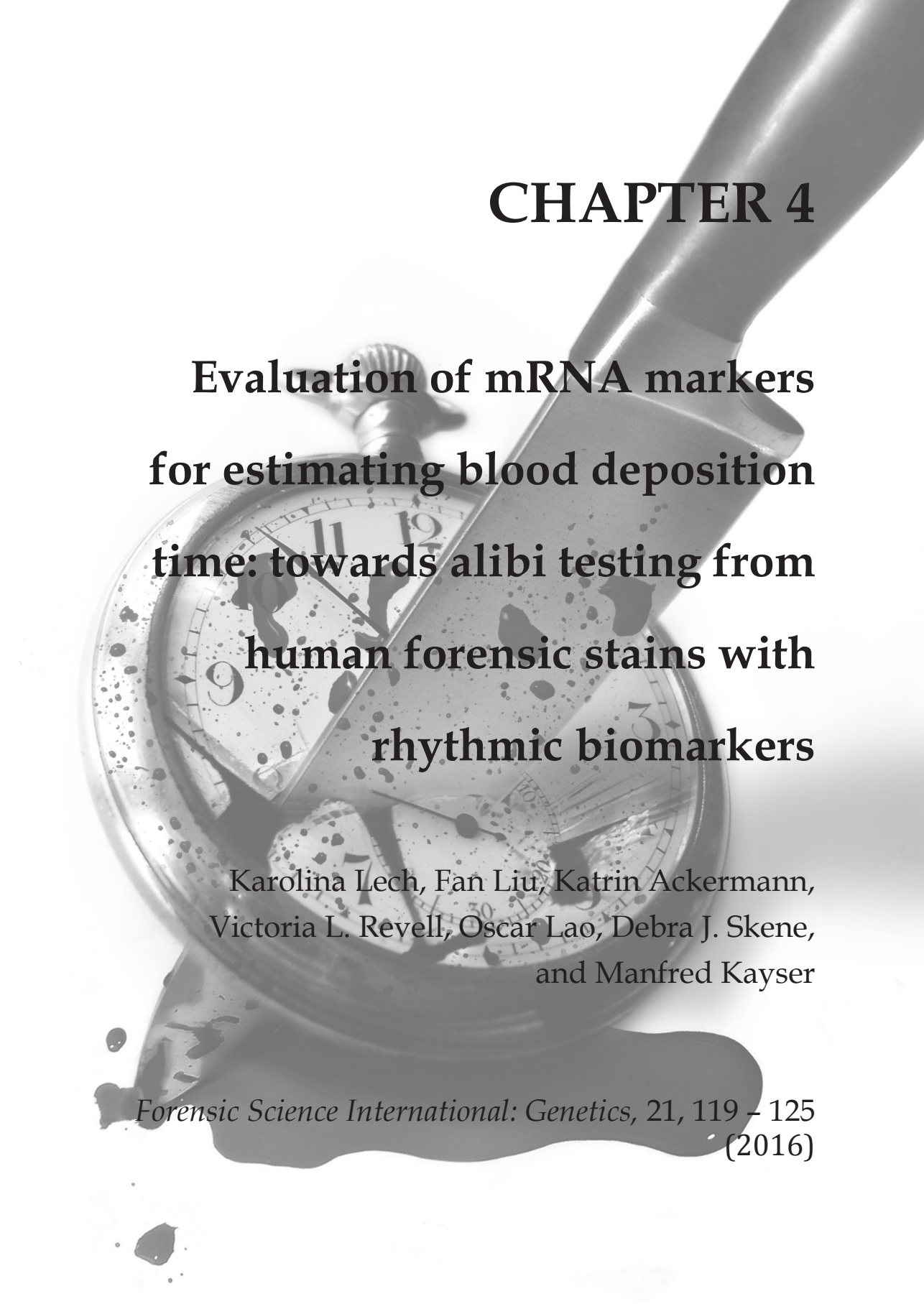
Supplementary Figure S1. Plots of mean, z-scored, *ACTB*-normalised expression levels of genes tested in the subjects from the S/SD and CR studies, three different conditions (sleep; sleep deprivation; constant routine) overlaid together. Black – the first 24 h of the S/SD study including the sleep night; 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-ERBa, PER3, PER1, MKNK2, HSPA1B, CAPRIN1* and *BMAL1*) presented as *ACTB*-normalised 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 profiles of expression levels (coloured lines) of 14 genes (*USF1, TRIB1, THRA1, STAT3, SREBF1, SIRT1, ROCK2, REV-ERBa, PER3, PER1, MKNK2, HSPA1B, CAPRIN1* and *BMAL1*) presented as *ACTB*-normalised 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.



CHAPTER 4

Evaluation of mRNA markers for estimating blood deposition time: towards alibi testing from human forensic stains with rhythmic biomarkers

Karolina Lech, Fan Liu, Katrin Ackermann, Victoria L. Revell, Oscar Lao, Debra J. Skene, and Manfred Kayser

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Abstract

Determining the time a biological trace was left at a scene of crime reflects a crucial aspect of forensic investigations as - if possible - it would permit testing the sample donor's alibi directly from the trace evidence, helping to link (or not) the DNA-identified sample donor with the crime event. However, reliable and robust methodology is lacking thus far. In this study, we assessed the suitability of mRNA for the purpose of estimating blood deposition time, and its added value relative to melatonin and cortisol, two circadian hormones we previously introduced for this purpose. By analysing 21 candidate mRNA markers in blood samples from 12 individuals collected around the clock at 2 h intervals for 36 h under real-life, controlled conditions, we identified 11 mRNAs with statistically significant expression rhythms. We then used these 11 significantly rhythmic mRNA markers, with and without melatonin and cortisol also analysed in these samples, to establish statistical models for predicting day/night time categories. We found that although in general mRNA-based estimation of time categories was less accurate than hormone-based estimation, the use of three mRNA markers *HSPA1B*, *MKNK2* and *PER3* together with melatonin and cortisol generally enhanced the time prediction accuracy relative to the use of the two hormones alone. Our data best support a model that by using these five molecular biomarkers estimates three time categories, i.e. night/early morning, morning/noon, and afternoon/evening with prediction accuracies expressed as AUC values of 0.88, 0.88, and 0.95, respectively. For the first time, we demonstrate the value of mRNA for blood deposition timing and introduce a statistical model for estimating day/night time categories based on molecular biomarkers, which shall be further validated with additional samples in the future. Moreover, our work provides new leads for molecular approaches on time of death estimation using the significantly rhythmic mRNA markers established here.

Introduction

The crucial question regarding the time when a human trace was left at a scene of crime is frequently encountered during criminal investigations, alongside individual identification of the trace donor. While the principle, the markers, the methodology, and the interpretation of DNA-based individual identification are all well established and routinely applied to forensic casework around the world, estimation of trace deposition time lacks reliable and robust techniques as of yet. If possible however, it would permit testing the sample donor's alibi directly from analysing the trace evidence, which would allow linking (or not) the DNA-identified sample donor with the crime event, and thus is of importance for solving a forensic case. Furthermore, molecular alibi testing from crime scene traces could provide information useful for finding unknown sample donors.

Currently, research on estimating trace deposition time is mainly focused on determining the age of a human forensic stain, i.e. how much time has passed since the human material was left at the scene of a crime. Reflectance spectroscopy and biochemical methods have been suggested for estimating the age of blood stains (*Arany & Ohtani 2011; Bremmer et al., 2011, 2012*), as well as the principle of differential, time-dependent RNA degradation (*Bauer et al., 2003; Hampson et al., 2011*). A second aspect of trace timing, i.e. determining when during the 24 h day/night cycle the human material was left at the crime scene, was introduced in the last years by employing knowledge of circadian biology (*Ackermann et al., 2010*).

Circadian rhythms are endogenous oscillations with an approximately 24 h period that govern the daily lives of most organisms, humans included. These intrinsic rhythms are generated by an autoregulatory negative-feedback loop that is formed by a set of core clock genes, such as circadian locomotor output cycles kaput (*CLOCK*), brain and muscle ARNT-like 1 (*BMAL1*), cryptochrome (*CRY*) and period (*PER*), together with their respective proteins

(*Yagita et al., 2001; Okamura et al., 2004; Hughes et al., 2009; Mohawk et al., 2012*). These oscillations, through regulation of expression of other genes, so called clock-controlled genes (CCGs), are reflected in various biological processes like hormone secretion, metabolic reactions, behaviour, and many others (*Kaller et al., 2009; Mohawk et al., 2012; Eckel-Mahan & Sassone-Corsi 2013; Kalsbeek et al., 2014*). It was shown that approximately 3-10% of all mRNAs in a respective tissue exhibit diurnal variations in expression, and therefore can be considered as CCGs (*Panda et al., 2002; Storch et al., 2002; Cermakian & Boivin 2009*). Thus, the ubiquitous nature of these rhythms presents a vast amount of potential molecular biomarkers, which in principle are suitable for application in forensic trace deposition timing as well as time of death estimation under certain prerequisites (*Kayser & de Knijff 2011*).

The first study describing the application of the circadian hormone melatonin for forensic time estimation, i.e. in determining the time of death, was published in 1994 (*Mikami et al., 1994*), and over the next years more studies focusing on time estimations with rhythmic biomarkers were reported (*Minami et al., 2009; Ackermann et al., 2010; Kimura et al., 2011; Kasukawa et al., 2012*). Estimating time of death is similar to estimating trace deposition time, and assessment of both can in principle be done with rhythmic biomarkers, provided that they are stable under post-mortem/post-deposition conditions. In 2010, we reported a proof-of-concept study on the use of two circadian hormones, melatonin and cortisol, for determining the deposition time of blood and saliva (*Ackermann et al., 2010*), describing for the first time trace deposition timing from the chronobiological perspective. The proposed approach and methodology demonstrated the feasibility of reproducing circadian profiles of two hormones in blood and saliva samples, and highlighted the advantages for forensic applications. For instance, this method requires a small sample volume typically encountered in forensic casework, and the assays, as well as the laboratory equipment, are commercially available. Furthermore, no or only limited signs of in-vitro time-wise degradation, as prerequisite for using these markers for

trace deposition timing, were observed for these hormones. However, the day/night time range that can be estimated with melatonin and cortisol alone is limited (*Ackermann et al., 2010*). Moreover, the effect of external and internal factors on both of the circadian hormones is recognised and, if disregarded, can be a cause of difficulties in result interpretation under particular circumstances (*Ackermann et al., 2010*). For example, melatonin secretion is inhibited by exposure to light, in a dose-dependent manner (*Lewy et al., 1980; McIntyre et al., 1992*). In normal subjects melatonin suppression starts between 200-400 lux (equivalent to ordinary fluorescent light), however upon light removal, melatonin concentration returns to normal night time levels (*Lewy et al., 1980*). In another study, it has been shown that acute suppression of melatonin secretion occurs after exposure to intensive light (600 lux or higher) for an hour (*McIntyre et al., 1992*). Furthermore, a disruption in melatonin's circadian pattern has been noted in subjects suffering from mental disorders such as major depressive disorder (*Frazer et al., 1986*). Cortisol levels have been shown to be disrupted in individuals suffering from addiction, chronic stress, or posttraumatic stress disorder (PTSD) (*Ockenfels et al., 1995; Chrousos 2005; Lovallo 2006; Fries et al., 2009*). Because of such factors influencing these two circadian hormones, and due to the limited time resolution they provided when being applied to trace deposition timing, additional rhythmic molecular biomarkers are required for increasing the accuracy as well as the reliability of molecular means for trace deposition timing.

In recent years, RNA profiling for forensic purposes has become more enthusiastically explored, and many studies describe its utility, especially for forensic tissue and body fluid identification (*Juusola & Ballantyne 2003, 2007; Zubakov et al., 2008, 2009; Lindenbergh et al., 2012; Sijen 2014*) and, less so, for post-mortem interval determination (*Bauer et al., 2003; Sampaio-Silva et al., 2013*). In 2011 and 2012, applications of rhythmic mRNA and microRNA markers for the time of death determination were reported (*Kimura et al., 2011; Odriozola et al., 2013*). *Kimura et al. (2011)* analysed the expression levels of three circadian genes – *BMAL1, PER2* and *REV-ERBa* (also

known as *NR1D1* – nuclear receptor subfamily 1, group D, member 1) – in kidney, liver and heart samples obtained from forensic autopsy material. Based on gene expression values, the authors constructed a range of ratios used for time of death estimation. *Odriozola et al. (2013)* reported two microRNAs, miR-541 and miR-142-5p, with diurnal variations in their abundance in vitreous humour samples from deceased individuals, further proposed as suitable candidate markers for time of death estimation. Recently, we demonstrated that these two microRNA markers are not suitable for blood trace deposition timing (*Lech et al., 2014*). In this study, miR-541 was shown to be present in very low levels in blood, not allowing for meaningful conclusions, whereas miR-142-5p was not rhythmic in the tested blood samples (*Lech et al., 2014*). Among other reasons, these findings may be explained by possible tissue specificity of miR-541 and miR-142-5p expression, which should be further explored.

Because in all those previous studies only a small number of biomarkers were tested, this ultimately limited the precision and significance of the obtained time estimates (*Ackermann et al., 2010; Kimura et al., 2011; Odriozola et al., 2013*). Expectedly, a larger set of biomarkers is needed, in order to achieve reliable and narrow time predictions, suitable for forensic applications. In the present study, we provide the first attempt for assessing the suitability of mRNA markers for estimating blood deposition time. Recently (*Ackermann et al., 2013; Lech et al., 2016*), we analysed the expression of 12 well-known clock and clock-related genes (*Lech et al., 2016*) and of 9 candidate clock-controlled genes (*Ackermann et al., 2013*) and measured the concentration of melatonin and cortisol in blood samples drawn from 12 individuals around the clock at 2 h intervals for 48 h under controlled conditions of a sleep/sleep deprivation (S/SD) study protocol, and under a separate constant routine study protocol (CR) (*Ackermann et al., 2012, 2013; Lech et al., 2016*). Data analysis in these previous biologically-motivated studies focused on identifying diurnal and circadian genes, understanding their biological function, and assessing the influence of sleep and

no-sleep on gene expression.

In the present forensically-motivated study, we used the raw expression data of the 21 genes as well as melatonin and cortisol from the 2 hourly collected samples during the first 36 h of the S/SD study. Here, we did not consider data from the samples collected during the one night of sleep deprivation in the S/SD study and did not use any data from the samples collected in the CR study. Both sampling scenarios represent non-natural conditions not relevant for the present study, where we like to simulate real-life conditions as much as possible for selecting time predictive mRNA markers for future forensic applications. Based on these data, we selected the statistically significant rhythmic mRNA markers, and performed multinomial logistic regression modelling with and without melatonin and cortisol for predicting day/night time categories.

Materials and Methods

Gene expression data

The gene expression data used in this study is part of a larger data set we previously generated from the blood samples collected during the Sleep/Sleep Deprivation Study (S/SD) conducted at Surrey Clinical Research Centre (CRC) at the University of Surrey, UK. Full details of the study protocol, eligibility criteria, and the data acquisition procedure were reported elsewhere (*Ackermann et al., 2012, 2013; Lech et al., 2016*). However, for the present analysis we used 18 two-hourly blood samples per each of 12 male participants (mean age ± SD = 23 ± 5 years), i.e. 216 samples in total. These samples spanned the first 36 h of the S/SD study (from 12:00 h Day 2 to 22:00 h Day 3), excluding the sleep deprivation condition (00:00 h Day 3 to 12:00 h Day 4). The reason for selecting this sample set was that while conditions such as lighting intensity, food intake, posture and physical activity etc., were controlled

throughout the study, the in-laboratory day/night layout still resembled that of real life (*Ackermann et al., 2012, 2013; Lech et al., 2016*). We also did not consider the data previously generated from the samples collected under the constant routine (CR) protocol (*Ackermann et al., 2013*), as they do not represent real-life conditions. For blood sample collection procedure, RNA extraction method, cDNA synthesis, qPCR data and subsequent analyses we refer to the Method sections of the two previous articles (*Ackermann et al., 2013; Lech et al., 2016*). Expression data from the following mRNA markers were used: brain and muscle ARNT-like 1 (*BMAL1*) (*Fukuya et al., 2007; Archer et al., 2008; Ackermann et al. 2013*), circadian locomotor output cycles kaput (*CLOCK*) (*Kusanagi et al., 2008; Ackermann et al., 2013*), cryptochrome 1 (*CRY1*) (*Kusanagi et al., 2008; Ackermann et al., 2013*), cryptochrome 2 (*CRY2*) (*Kusanagi et al., 2008; Ackermann et al., 2013*), D site of albumin promoter (albumin D-box) binding protein (*DBP*) (*Ackermann et al. 2013*), deleted in esophageal cancer 1 (*DEC1*) (*Boivin et al., 2003; Kusanagi et al., 2008; Ackermann et al., 2013*), heat shock 70kDa protein 1B (*HSPA1B*) (*Ackermann et al., 2013*), period 1 (*PER1*) (*Boivin et al., 2003; Fukuya et al., 2007; Kusanagi et al., 2008; Chudova et al., 2009; Ackermann et al., 2013*), period 2 (*PER2*) (*Boivin et al., 2003; Fukuya et al., 2007; Archer et al., 2008; Kusanagi et al., 2008; Ackermann et al., 2013*), period 3 (*PER3*) (*Boivin et al., 2003; Archer et al., 2008; Kusanagi et al., 2008; Ackermann et al., 2013*), nuclear receptor subfamily 1, group D, member 1 (*REV-ERBA*) (*Ackermann et al., 2013*), RAR-related orphan receptor alpha (*RORA*) (*Ackermann et al., 2013*), cell cycle associated protein 1 (*CAPRIN1*) (*Panda et al., 2002; Lech et al., 2016*), MAP kinase interacting serine/threonine kinase 2 (*MKNK2*) (*Chudova et al., 2009; Lech et al., 2016*), rho-associated, coiled-coil containing protein kinase 2 (*ROCK2*) (*Saito et al., 2013; Lech et al., 2016*), sirtuin 1 (*SIRT1*) (*Asher et al., 2008; Lech et al., 2016*), sterol regulatory element-binding transcription factor 1 (*SREBF1*) (*Bozek et al., 2009; Lech et al., 2016*), signal transducer and activator of transcription 3 (*STAT3*) (*Bozek et al., 2009; Hughes et al., 2009; Lech et al., 2016*), thyroid hormone receptor alpha (*THRA1*) (*Zandieh Doulabi et al., 2004; Lech et al., 2016*), tribbles homolog 1 (*TRIB1*) (*Ollila*

et al., 2012; Lech et al., 2016), upstream transcription factor 1 (*USF1*) *Shimomura et al., 2013; Lech et al., 2016*), and actin beta (*ACTB*), used here as the reference gene. Melatonin and cortisol concentration measurements, obtained in the same samples (*Ackermann et al., 2012, 2013*) were used as well.

In short, gene expression data were analysed with the delta-delta-cycle-threshold (ΔΔCT) method (*Livak & Schmittgen 2001*), and afterwards with the single cosinor and non-linear curve fitting (nlcf) and non-linear mixed model (nlm) methods, to determine the presence of 24 h rhythmicity, as described previously (*Ackermann et al., 2012, 2013; Lech et al., 2016*). Selection of genes for time category prediction was based on the statistically significant outcomes from the nlcf, nlm, and single cosinor methods. The requirements to be met were statistically significant outcomes of either the nlcf (for 2 out of 3 conditions: sleep, sleep deprivation and collapsed) or nlm methods, and presence of statistically significant rhythms in at least 25% of tested individuals (single cosinor method). Afterwards, based on the mean peak time estimates (obtained via either nlcf or nlm method) of the selected genes were used for establishing the most suitable time categories that were subsequently used in the prediction modelling.

Model building and time predictions

Prediction models were constructed based on multinomial logistic regression, where the *ACTB*-normalised expression levels of the genes and the concentration values of the hormones were considered as the predictors, and the multinomial time categories as the response variable, similar as described elsewhere (*Liu et al., 2009*). Note that for model building the expression levels of gene markers and hormone measurements were not z-scored, because the z-scoring will not be possible for evidentiary samples in future forensic application. Multinomial logistic regression was used to predict the probabilities of different possible outcomes of a categorically distributed dependent variable, given a set of independent variables,

as previously applied for prediction of eye and hair colour categories based on SNP genotypes (*Liu et al., 2009; Walsh et al., 2013*). Besides logistic regression, there is an array of well-established statistics or machine-learning techniques for prediction modelling, such as linear discriminant analysis (*Webb 2003*) and support vector machines (*Burges 1998*). It has been shown that different methods often perform similarly in the work of eye colour prediction (*Liu et al., 2009*). We chose multinomial logistic regression here for its simplicity (only regression betas needed), portability (compatible to all statistical platforms), and robustness (without the fundamental assumption on normality of explanatory variables). The most suitable time categories used in the prediction modelling were selected by considering the mean peak time estimates of the selected genes and hormones. We then tested different combinations of molecular predictors: hormones alone, genes alone, and hormones and genes together. Selection of the final set of molecular predictors was based on their contribution to the prediction accuracy, and mRNA markers with an insignificant effect were removed from the models, which were then rebuilt.

Due to the relatively small sample size, the performances of the prediction models were evaluated using the leaving-one-out cross-validation (LOOCV) approach (*Golub et al., 1999*), i.e. building the prediction model from 215 observations and predicting the day/night category for the remaining observation. This procedure was then repeated 216 times (one time for each observation). The area under the receiver operating characteristic (ROC) curves, the AUC, was measured. Its values range from 0.5, representing random prediction to 1.0, representing perfect prediction. The concordance between the predicted and observed categories was categorized into 4 groups: true positives (TP), true negatives (TN), false positives (FP) and false negatives (FN). We derived 4 accuracy parameters: sensitivity = TP/(TP+FN)*100, specificity = TN/(TN+FP)*100, positive predictive value (PPV) = TP/(TP+FP)*100, and negative predictive value (NPV) = TN/(TN+FN)*100. Note that, although the 216 observations were not completely independent from each other, the prediction results are unlikely to be biased, since all prediction results were cross

validated. Because the variability (such as confidence intervals) cannot be directly derived from LOOCV results, we conducted a permutation analysis to estimate the variability of our accuracy estimates under the null hypothesis, i.e. assuming no relationship between predictors and time categories, by randomly reshuffling the dependent variable (time categories, k=1000). For each shuffling, we derived the accuracy estimates based on the above described multinomial logistic regression and LOOCV. The resultant average values and 5%-95% quantiles (**Table S1**) provide unbiased estimates about the variability of our accuracy estimates under the null that is specific to our data set.

Results and Discussion

Choosing mRNA predictors of blood deposition time

From the 21 genes tested for day/night rhythms in expression (**Table 1**), we identified 11 genes, i.e. *BMAL1*, *CAPRIN1*, *HSPA1B*, *MKNK2*, *PER1*, *PER3*, *ROCK2*, *SIRT1*, *STAT3*, *THRA1* and *TRIB1* that showed statistically significant results according to either the nlcf or nlm method, and at the same time had statistically significant rhythms in at least 25% of the individuals according to the cosinor method (**Table 1**). Their expression patterns are presented in **Figure 1**.

Next, we assigned the 11 significantly rhythmic mRNA markers, as well as melatonin and cortisol, to day/night time categories based on their mean expression/concentration peak time estimates. We found that peak times of several of the 13 molecular biomarkers were overlapping. In particular, expression of *PER1* and cortisol concentration were highest in the same time range, i.e. between 08:00 h and 10:00 h, whereas expression of *ROCK2*, *SIRT1*, and *THRA1* as well as melatonin concentration were highest between 01:00 h and 03:00 h.

The expression of *PER3* was highest around 04:00 h, and the expression of *BMAL1*, *HSPA1B*, *TRIB1*, *MKNK2,* and *STAT3* were highest in the afternoon between 15:30 h and 17:00 h. Consequently, we established three time categories of peak expression/concentration of these 13 biomarkers, i.e. night/early morning (23:00 – 06:59), morning/noon (07:00 – 14:59) and afternoon/evening (15:00 – 22:59), together comprising one complete day/night cycle.

Table 1. Results of the single cosinor, non-linear curve fitting (nlcf) or non-linear mixed model methods for all 21 genes/mRNA markers tested.

Gene/mRNA marker	Single Cosinor (% of significant subjects)	Non-Linear Curve Fitting/Non-Linear Mixed Model
BMAL1	50	significant
CAPRIN1	25	significant
CLOCK	42	significant
CRY1	25	not significant
CRY2	42	not significant
DBP	25	not significant
DEC1	0	not significant
HSPA1B	42	significant
MKNK2	42	significant
PER1	25	significant
PER2	25	not significant
PER3	67	significant
REV-ERBa	58	not significant
ROCK2	25	significant
RORa	8	not significant
SIRT1	42	significant
SREBF1	17	not significant
STAT3	25	significant
THRA1	75	significant
TRIB1	58	significant
USF1	17	not significant

**Presented are the percentages of subjects with significant rhythms in expression (as calculated with the single cosinor) and outcomes of the nlcf or nlm method). Underlined are the significantly rhythmic genes selected for prediction modelling.*

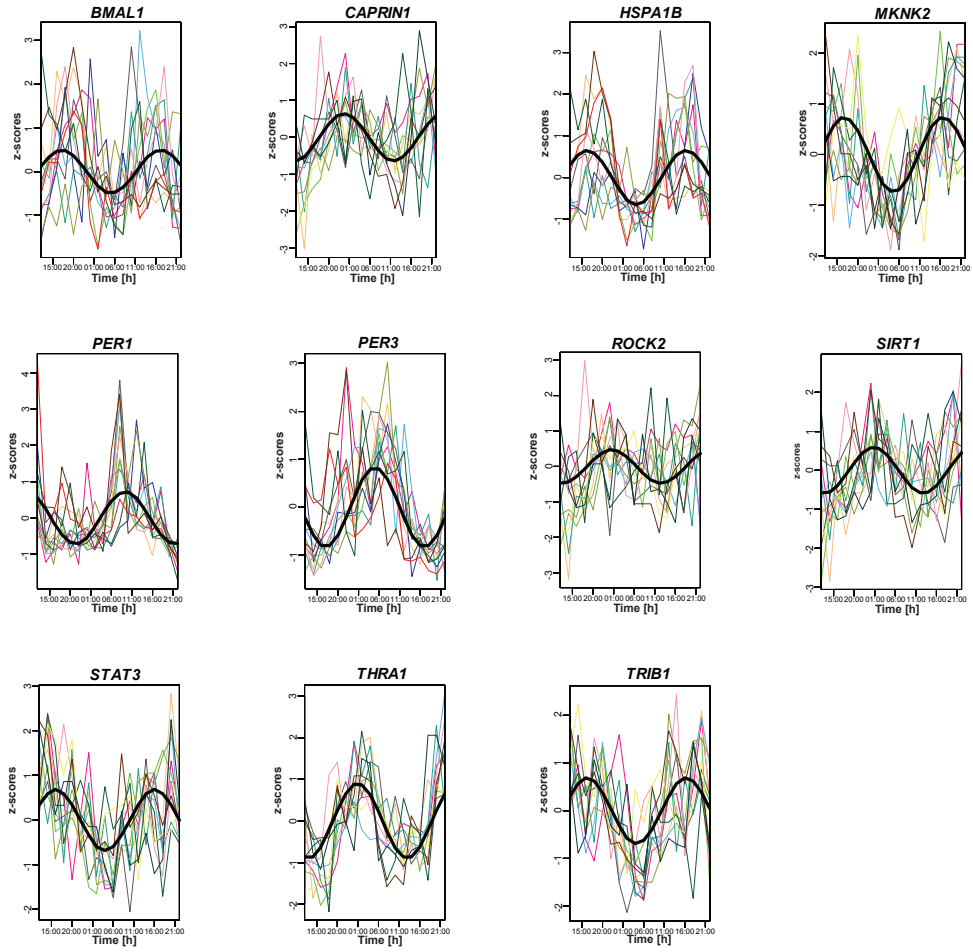

Figure 1. Individual profiles of 11 genes/mRNA markers with significantly rhythmic expression patterns in 2-hourly collected blood samples from 12 male individuals over a period of 36 h under controlled real-life conditions. Data are presented as *ACTB*-normalised z-scores, with individual-based colour coding. The black line represents the superimposed mean cosine curve, as calculated by the nlcf method.

Modelling blood deposition time using molecular biomarkers

We then performed statistical prediction modelling of these three day/night time categories using the 11 mRNA markers (*BMAL1*, *CAPRIN1*, *HSPA1B*, *MKNK2*, *PER1*, *PER3*, *ROCK2*, *SIRT1*, *STAT3*, *THRA1* and *TRIB1*) and the 2 hormone markers (melatonin and cortisol) in different combinations (see **Methods**) to establish the most accurate and robust model, while minimizing the number

of biomarkers involved. The latter was done by keeping the final forensic application in mind, where typically the amount of biological material is limited, and thus is the number of molecular tests possible before the evidence material is exhausted. We tested the genes with and without the hormones, as well as the hormones alone to work out the relationship between these two types of molecular biomarkers in the time prediction modelling.

We noticed that some of the mRNA markers used for model building did not contribute significantly to the prediction accuracy obtained with the models. From this, we concluded that their effect was "masked" by other, more robust markers included in the respective model. The markers with negligible independent input into the prediction accuracy were consequently excluded from the respective models to keep the number of markers at the minimum, which were then rebuilt with only considering the independently contributing molecular predictors.

In the model considering all 11 significantly rhythmic mRNA markers, 5 were excluded due to redundant predictive effects, while 6 mRNA markers with independent predictive effects were identified, i.e. *HSPA1B, MKNK2, PER1, PER3, THRA1,* and *TRIB1*. The model based on these 6 independent mRNA predictors achieved AUC values of 0.75 for morning/noon, 0.80 for afternoon/evening, and 0.93 for night/early morning (**Table 2**). A model considering only melatonin and cortisol provided lower AUC for the night/early morning (0.85) but somewhat higher AUCs for the afternoon/evening (0.83) and morning/noon (0.85) time categories compared with the model based on 6 mRNA markers (**Table 2**). These results indicate that at least for the night/early morning category the use of mRNA markers is largely beneficial. The results for the hormone-based model were not surprising, as the two hormones are characterized by robust, truly circadian patterns of secretion with sharp, distinct peaks and high amplitude (*Weitzman et al., 1971; Arendt et al., 2006; Ackermann et al., 2013*) and melatonin is an established marker of circadian phase, also used in sleep-related studies (*Ackermann et al., 2013; Möller-Levet et al., 2013*). It is clear that all AUC values from

all models are far beyond the corresponding 95% upper boundaries (max AUC_{NULL} = 0.58) from a permutation analysis under the null hypothesis assuming no relationship between predictors and time category (**Table S1**). Additionally, regression parameters from the final models are provided in **Table S2** in the **Supplementary material**.

Because of the higher AUCs for one but the lower AUCs for the other time categories as achieved with the genes and the hormones separately, we then combined all 11 significant mRNA markers and the 2 hormone markers in a prediction model and tested for redundant biomarker effects. As a result, 8 (*BMAL1, CAPRIN1, PER1, ROCK2, SIRT1, STAT3, THRA1* and *TRIB1*) out of the 11 mRNA markers were removed from the model.

The final model comprising of five biomarkers (*MKNK2*, *HSPA1B*, *PER3*, melatonin and cortisol) predicted the night/early morning category with particularly high accuracy AUC of 0.95, while the other two time categories were both predicted with an AUC of 0.88 (**Table 2**). Compared with the model based solely on the two circadian hormones, and with the model based solely on mRNA markers, the combined hormone/mRNA model achieved higher accuracies for all three predicted time categories (**Table 2**). Hence, the use of mRNA markers, particularly *HSPA1B, MKNK2* and *PER3*, did overall increase the day/night time prediction accuracy, compared to those achieved with hormones only, underlining the beneficial use of mRNA markers for blood deposition timing.

Table 2. Results of time prediction modelling using multinomial logistic regression.

melatonin & cortisol					
Predicted time category	***AUC***	***sens***	***spec***	***ppv***	***npv***
morning/noon	0.85	0.66	0.85	0.68	0.84
afternoon/evening	0.83	0.81	0.69	0.68	0.82
night/early morning	0.85	0.46	0.95	0.71	0.86
HSPA1B, PER1, PER3, TRIB1, THRA1, MKNK2					
Predicted time category	***AUC***	***sens***	***spec***	***ppv***	***npv***
morning/noon	0.75	0.51	0.90	0.71	0.79
afternoon/evening	0.80	0.83	0.69	0.68	0.84
night/early morning	0.93	0.78	0.94	0.79	0.93
MKNK2, HSPA1B, PER3, melatonin & cortisol					
Predicted time category	***AUC***	***sens***	***spec***	***ppv***	***npv***
morning/noon	0.88	0.73	0.91	0.80	0.87
afternoon/evening	0.88	0.79	0.79	0.75	0.83
night/early morning	0.95	0.75	0.93	0.75	0.93

AUC – area under the receiver operating characteristic (ROC) curves; PPV – positive predictive value; NPV – negative predictive value, spec – specificity; sens – sensitivity.

Future forensic application considerations

We chose to conduct our trace timing analyses in blood mainly because bloodstains are amongst the most commonly found biological evidence on scenes of violent crimes. It is important to keep in mind that without additional testing in other forensically relevant tissues, the results we present here are to be regarded as specific for blood.

Even though blood has been proposed as sort of a "gate to access and analyse the transcriptome of various organs" (*Kohane & Valtchinov 2012*) it should be noted that our findings would need to be properly revalidated in other forensically relevant tissue types, such as saliva, semen, skin, vaginal secretion, and menstrual blood, before applying them to such forensic traces. Besides expanding molecular trace deposition timing to other forensically relevant tissue types than blood, additional testing in various human tissues would also be advantageous in determining whether the mRNA markers proposed here are also informative for time of death estimations, as previously suggested for melatonin (*Mikami et al., 1994*).

Another crucial aspect that shall be tested carefully in future studies is the time-wise stability of the proposed mRNA markers. Since blood stains are found at various crime scenes, they are exposed to a multitude of variable conditions, i.e. high/low humidity, drought, temperature changes, but are also located on different types of surfaces. All these factors can possibly influence the stability of the mRNA, which should be tested for the specific mRNA markers proposed here. Many mRNA markers previously suggested for forensic tissue identification purposes have shown strong time-wise stability (*Zubakov et al., 2008, 2009*), which has to be demonstrated for the candidate mRNA markers we suggest here for blood timing before they can be introduced to molecular alibi testing in forensic case-work.

Finally, our results were established from 12 male individuals, and additional samples shall be analysed in the future to further validate our prediction model.

Conclusions

In this study, we investigated whether mRNA provides a suitable resource for establishing biomarkers to estimate blood deposition time. We demonstrated that particular mRNA markers have added value for blood deposition timing over the previously established

two circadian hormones melatonin and cortisol. We introduced a prediction model comprising three mRNA markers, *HSPA1B*, *MKNK2* and *PER3*, together with two circadian hormones melatonin and cortisol, provides improved prediction accuracy of three day/night categories compared to those achieved with a model based on the two hormones alone or mRNA markers alone. To our knowledge this is the first study assessing the suitability of mRNA markers for trace deposition timing and the first time a statistical model for estimating blood deposition time with molecular biomarkers is presented. To achieve a more detailed level of time category prediction than revealed here, additional rhythmic biomarkers with different peak times will be needed. Future studies should focus on identifying them, and eventually incorporating them together with the markers used here to develop a final prediction model. Moreover, our work provides new leads for future studies on time of death investigation using the significantly rhythmic mRNA markers established here, which represents a second aspect of forensic time estimation in need of improved biomarkers, methodology, and technology.

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Supplementary material

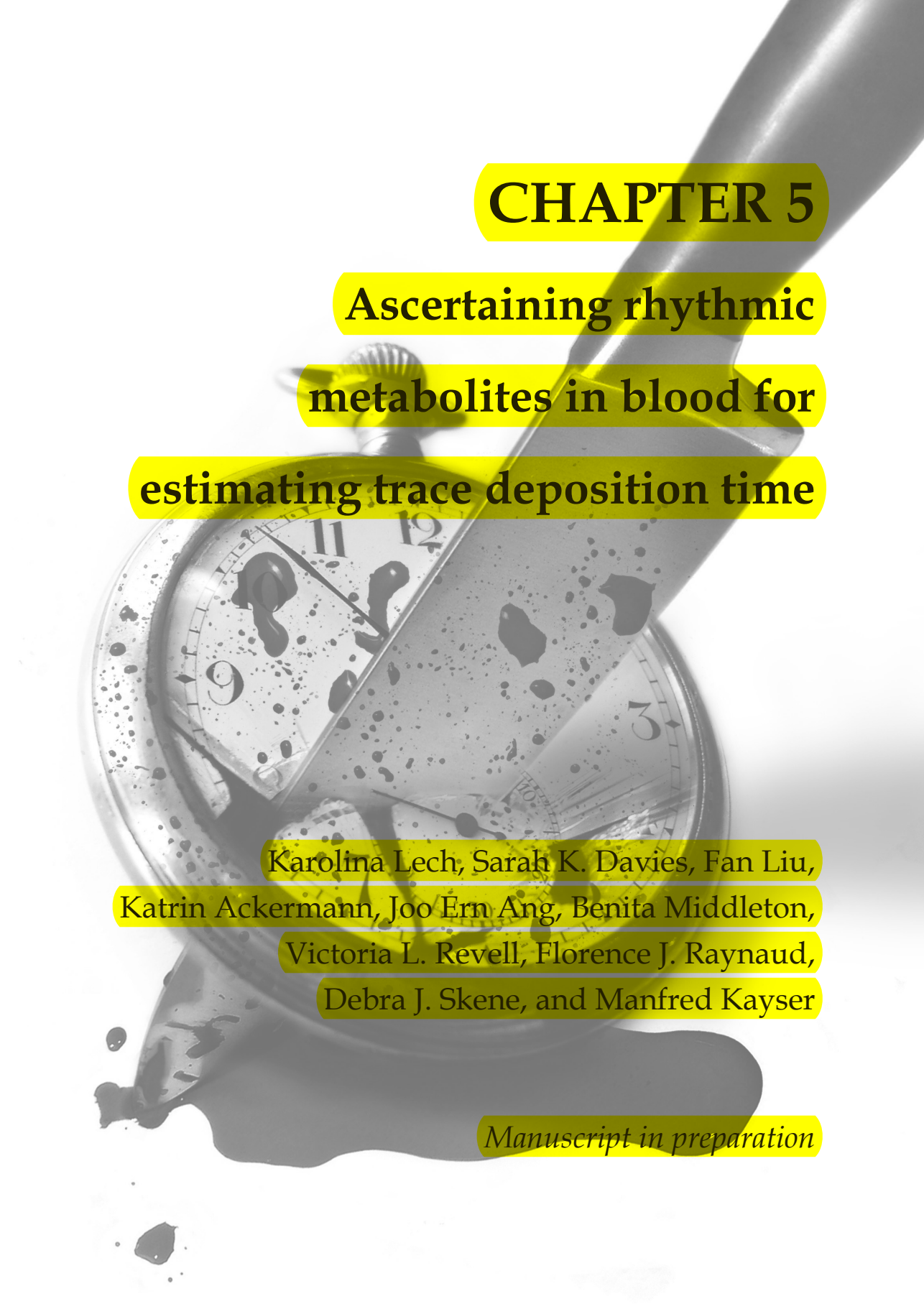
Table S1. Accuracy estimates from permutation analysis under the null hypothesis assuming no relationship between predictors and time categories.

melatonin & cortisol					
Predicted time category	***AUC***	***sens***	***spec***	***ppv***	***npv***
afternoon/evening	0.40 (0.15 - 0.54)	0.69 (0.00 - 0.99)	0.31 (0.00 - 1.00)	0.33 (0.00 - 0.46)	0.41 (0.00 - 0.68)
morning/noon	0.39 (0.10 - 0.55)	0.04 (0.00 - 0.20)	0.96 (0.85 - 1.00)	0.11 (0.00 - 0.44)	0.67 (0.66 - 0.69)
night/early morning	0.38 (0.09 - 0.55)	0.00 (0.00 - 0.02)	1.00 (0.98 - 1.00)	0.02 (0.00 - 0.17)	0.78 (0.77 - 0.78)
HSPA1B, PER1, PER3, TRIB1, THRA1, MKNK2					
Predicted time category	***AUC***	***sens***	***spec***	***ppv***	***npv***
afternoon/evening	0.46 (0.32 - 0.57)	0.80 (0.69 - 0.91)	0.18 (0.01 - 0.35)	0.43 (0.40 - 0.48)	0.48 (0.15 - 0.67)
morning/noon	0.45 (0.29 - 0.57)	0.15 (0.00 - 0.34)	0.83 (0.72 - 0.95)	0.25 (0.00 - 0.44)	0.66 (0.63 - 0.70)
night/early morning	0.45 (0.28 - 0.58)	0.02 (0.00 - 0.13)	0.98 (0.92 - 1.00)	0.07 (0.00 - 0.38)	0.77 (0.77 - 0.79)
MKNK2, HSPA1B, PER3, melatonin & cortisol					
Predicted time category	***AUC***	***sens***	***spec***	***ppv***	***npv***
afternoon/evening	0.45 (0.30 - 0.57)	0.82 (0.72 - 0.94)	0.16 (0.01 - 0.33)	0.43 (0.41 - 0.48)	0.47 (0.10 - 0.67)
morning/noon	0.45 (0.29 - 0.57)	0.13 (0.00 - 0.32)	0.85 (0.74 - 0.97)	0.24 (0.00 - 0.44)	0.66 (0.64 - 0.70)
night/early morning	0.44 (0.26 - 0.58)	0.02 (0.00 - 0.10)	0.98 (0.93 - 1.00)	0.07 (0.00 - 0.38)	0.77 (0.77 - 0.78)

Accuracy estimates were derived based on multinomial logistic regression using leaving-one-out cross validation, except the dependent variable (time categories) was randomly permuted (k=1000). Values in the table are means and 5%-95% quartiles. AUC – area under the receiver operating characteristic (ROC) curves; PPV – positive predictive value; NPV – negative predictive value, spec – specificity; sens – sensitivity.

Table S2. Model parameters (multinomial logistic regression betas) of the final models.

Model	*Parameter & marker*	*Predicted time category*		
		afternoon/evening	*morning/noon*	*night/early morning*
melatonin & cortisol	**Constant**	2.27	-0.82	-1.45
	melatonin	-0.01	0.00	0.09
	cortisol	-0.01	0.01	0.00
HSPA1B, PER1, PER3, TRIB1, THRA1, MKNK2	**Constant**	-1.05	-3.80	4.84
	TRIB1	0.07	0.00	-6.99
	THRA1	0.00	-2.00	6.67
	MKNK2	0.00	3.30	-14.50
	HSPA1B	1.10	0.00	-1.12
	PER1	-2.30	0.99	0.00
	PER3	-1.40	0.00	0.92
MKNK2, HSPA1B, PER3, melatonin & cortisol	**Constant**	1.79	-3.64	1.85
	MKNK2	0.00	0.68	-8.26
	HSPA1B	0.60	0.00	-2.48
	PER3	-2.16	0.00	3.04
	melatonin	-0.01	0.00	0.08
	cortisol	-0.01	0.01	0.00



CHAPTER 5

Ascertaining rhythmic metabolites in blood for estimating trace deposition time

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Manuscript in preparation

CHAPTER 6

Discussion

In recent years, outstanding progress has been made in various research areas in forensic genetics and forensic molecular biology. To name three of the most relevant ones: (1) the application of single nucleotide polymorphisms (SNPs) to determine externally visible characteristics of an individual from DNA ('Forensic DNA Phenotyping') (*Liu et al., 2009, 2015a,b; Branicki et al., 2011; Walsh et al., 2011, 2013*) and to infer bio-geographic origin (genetic ancestry) (*Kidd et al., 2014; Lao et al., 2006, 2008; Mendizabal et al., 2012; Bulbul et al., 2015*), both aiming to help finding unknown perpetrators, who cannot be identified with conventional DNA profiling; (2) the identification of RNA markers to determine the cellular origin of a forensic stain found at the crime scene (*Zubakov et al., 2010, 2015; Visser et al., 2011; Lindenbergh et al., 2012*) aiming to link (or not) the DNA–identified sample donors with the criminal act; and (3) advancement in DNA and RNA analysis technology, such as next generation sequencing (NGS), to obtain more and more forensically relevant information from limited crime scene material (*Yang et al., 2014; Chaitanya et al., 2015; Parson et al., 2015; Ralf et al., 2015; Zubakov et al., 2015*). Despite of all the progress made, one issue still remains a challenge for the forensic community – estimation of time.

Time in the forensic context can be regarded as either the time of death of an individual, or as sample age, i.e. the time that has passed since the biological material has been left at the crime scene. This thesis however, addresses a new aspect of forensic time - trace deposition time, i.e. when during the day/night a human biological trace was deposited at the crime scene, and thus brings chronobiology into the forensic field. This thesis describes the research undertaken to explore the suitability of diverse biomolecules, characterized by 24 hour rhythmicity in their abundance and/or expression, for the purpose of blood trace deposition timing. The main objective was to first identify and then verify rhythmic biomarkers in human blood samples, and subsequently test their suitability for trace deposition timing using newly designed statistical time prediction models. This thesis summarizes the work done on this topic, and further discusses the results obtained in relation to similar findings and potential

future research.

The benefits of using rhythmic biomolecules in forensic research have been first recognized in 1994, when the hormone melatonin, extracted from the pineal gland, was used for time of death estimation (*Mikami et al., 1994*). Melatonin is characterized by diurnal changes in secretion levels and its concentration profile in either blood plasma or saliva is considered the most reliable measure of human circadian timing (*Arendt 2005, 2006*). Melatonin has also been used in a proof-of-principle study by *Ackermann et al. (2010)* for trace deposition time estimation in blood and saliva, together with cortisol – another hormone, with a confirmed circadian pattern (*Orth et al., 1979; Van Cauter et al., 1996*). As the most suitable, given their strong circadian rhythms, hormones had been already tested for the purpose of trace deposition timing, I focussed on exploring additional biomarker sources, such as microRNA (miRNA), messenger RNA (mRNA), and metabolite markers.

In **chapter 2**, I described the investigation of two miRNAs for trace deposition timing. MiRNAs are small molecules (22 – 25 nucleotides), resistant to degradation, characterized by tissue-specific expression; all of which makes them very promising for forensic applications (*Bartel 2004; Wienholds & Plasterk 2005; Zubakov et al., 2010; Wang et al., 2013; Sijen 2014*), especially for forensic tissue identification (*Zubakov et al., 2010; Courts & Madea 2010, 2011*). Lately, miRNAs have also been found to be involved in circadian clock mechanism (*Cheng et al., 2007; Chen et al., 2013; Mehta et al., 2013*) and some were shown to exhibit rhythms in their abundance in certain tissues as well (*Cheng et al., 2007; Mehta et al., 2013*). As I described in **chapter 2**, together with my collaborators, I have established the 24 hour day/night expression profiles of two miRNA markers, miRNA-142-5p and miRNA-541, in human blood, and addressed their suitability for trace deposition time estimation. We were motivated by the findings of *Odriozola et al. (2013)*, who observed diurnal differences in expression levels of miR-142-5p and miR-541 in vitreous humour and found them suitable for time of death determination. Our study demonstrated that

miR-142-5p levels in blood do not exhibit 24 hour rhythms, while miR-541 was present in blood in very small amounts, thus not allowing for its reliable quantification using the sensitive RT-qPCR approach we applied (**chapter 2**). One of the most plausible explanations for the discrepancies between our results from blood and the results of *Odriozola et al. (2013)* in vitreous humour is that the expression patterns and/or expression levels of both miRNA markers are different in the two different tissues used. Since most miRNAs are characterized by tissue-specific expression, it is possible that this particular miRNAs exhibit rhythmic changes in the vitreous humour and not in blood. However, a recent report (*Corradini et al., 2015*) has shown that miRNA-142-5p is expressed differentially in blood of subjects that died either during day time or night time, though the results presented for miR-541 agreed with ours. Nevertheless, due to conflicting findings, we would suggest further testing on miR-142-5p and miR-541 expression in vitreous humour, blood and other human tissues before these markers are applied for time of death estimation and trace deposition timing.

The study I presented in **chapter 2** was the first to test the suitability of miRNA for trace deposition timing. Despite finding the two miRNAs unsuitable for our goal, this research area should not be abandoned. In this study, we focused our efforts on only two particular miRNA markers because of their previously suggested suitability for time of death estimation from very different human material. It would be worth using systematic approaches, such as microarrays or deep-sequencing, to search for rhythmic miRNA markers in blood samples collected around the clock. A more comprehensive approach, including activity and function assessment, could potentially uncover not only involved pathways but also targeted transcripts and their protein products, some of which might display rhythmicity in abundance. As miRNAs in general are involved in gene regulation (*Lee et al., 1993; Wightman et al., 1993;* reviews by *Sevignani et al., 2005; Shivdasani 2006; Wu & Belasco 2008; Bartel 2009*) it is not unexpected that such systematic screening for rhythmic miRNAs would find candidate markers to be further investigated for the purpose of trace

deposition timing.

Motivated by previous positive findings on circadian hormones and not being discouraged by our negative findings on miRNA, we investigated another type of molecular marker – mRNA. Various mRNA markers have previously been applied in forensic research for determining the age of bloodstains, based on differential in vitro degradation of mRNA (*Bauer et al., 2003; Anderson et al., 2005*), and for forensic tissue identification, using tissue-specific mRNA markers (*Zubakov et al., 2008, 2009; Visser et al., 2011; Lindenbergh et al., 2012*). Previous studies showed that in all tissues studied to date, approximately 5 to 10% of the transcriptome displays oscillations in expression, however with little overlap between them (*Panda et al., 2002; Storch et al., 2002; Duffield 2003*; reviewed by *Cermakian & Boivin 2009*). Therefore, the mRNA population is in principle a vast source of potential novel, rhythmic markers that could be useful for trace deposition time estimations (*Kayser & de Knijf 2011*). In 2011, for the first time, rhythmic genes *BMAL1*, *REV-ERBa* and *PER2*, have been proposed for estimating the time of death (*Kimura et al., 2011*). The study described in **chapter 3**, focuses on verifying the expression patterns of selected clock-controlled genes in human blood samples. Prior to this study, the knowledge about rhythmic expression patterns of these genes in human blood was scarce, as most of them were only analysed in rodents or in cell cultures. These genes were later investigated to determine their suitability for blood trace deposition timing, and used for designing time prediction models (**chapter 4**).

In **chapter 3,** together with my collaborators, I analysed the expression patterns of nine clock-controlled genes in human blood samples collected previously (*Ackermann et al., 2012, 2013*) and used in the studies described in **chapters 2-5**. When investigating the presence of 24 hour rhythmicity in concentration and/or expression levels of candidate biomarkers, the sampling strategy is very important and is one of the several reasons as to why we conducted all the experiments in these blood samples. Furthermore, from a forensic perspective, blood is encountered quite commonly at crime scenes, typically with the occurrence of violent crimes. Our

sample collection approach however, was designed in mind with the circadian aspect of the research. Blood sampling around the clock is relatively feasible and minimally invasive. The samples were obtained from individuals who participated in a study conducted in a sleep laboratory, a center designed to monitor and record sleep patterns and sleep disorders. Therefore, the facility is adapted so that the sleep of the subjects is not disrupted upon nightly blood collection, which is virtually impossible in other circumstances.

The design of the study (designated as S/SD study), fully described in *Ackermann et al. (2012, 2013)* and *Davies et al. (2014)*, featured stringent selection criteria of the participants, including health monitoring, chronotype assessment, restriction of sex and age influence (*Dijk et al., 2000; Duffy & Czeisler 2002*). The external conditions that can influence the internal rhythms of the participants (light, meal composition and timing, physical activity) were controlled in the sleep laboratory, yet the protocol still resembled natural everyday settings, which is important when confronted with the real-life, forensic scenarios. Additionally, in the design of the S/SD study, a second period of 24 hours, that incorporated a night of sleep deprivation, was included. The reason for this was to determine, whether any of the candidate markers identified in the samples collected in the first 24 hours of the S/SD study, would be affected by this condition. Sleep deprivation is known to influence the immune system (*Lange et al., 2010*), levels of various hormones, such as cortisol, melatonin and leptin (*Redwine et al., 2000; Mullington et al., 2003*), metabolic processes (*Knutson et al., 2007*), metabolome (*Dallmann et al., 2011; Davies et al., 2014*), and transcriptome (*Möller-Levet et al., 2013*). It was shown that the rhythmic expression of many transcripts becomes dampened, i.e. a decrease in amplitude was observed (*Möller-Levet et al., 2013*), and that prolonged periods of insufficient sleep alter gene expression in human blood cells (*Möller-Levet et al., 2013*). Therefore, for our forensic objective this condition was particularly valuable, as (1) it allowed us to preliminarily characterize what factors can affect the identified candidate markers; and (2) it could be used for screening for specific

markers of sleep and/or sleep deprivation, which not necessarily have to be rhythmic. In future studies, samples collected during the second 24 hour period of the S/SD study could be used to identify transcripts, proteins etc., whose expression/abundance levels in blood change significantly during sleep deprivation. With a sufficient amount of informative markers and validation testing, a valuable tool for alibi testing (awake/asleep claims) could be generated.

As I described in **chapter 3**, the nine genes were additionally tested in blood samples collected during a different study conducted at a sleep laboratory. This second study (designated as CR study) followed a protocol, known in the chronobiology field as constant routine, which is the accepted "gold" standard to demonstrate the endogenous nature of observed rhythmicity (*Duffy & Dijk 2002*). Therefore, the CR study described in **chapter 3** was designed so that the inherent circadian rhythms were exposed in the absence of external cues. After health and chronotype assessment, and meeting other eligibility criteria, the participants were admitted into the sleep laboratory. There, they underwent a 33 hour long protocol, which included total sleep deprivation, hourly isocaloric meals, dim light conditions and maintaining a semirecumbent posture. Those specific conditions allow, as mentioned, to exclude external cues, such as light, feeding, activity and sleep/wake cycle, from influencing the subjects' internal rhythms, thus unmasking the endogenous rhythms, in this particular example, in gene expression, and melatonin and cortisol concentration.

The main objective of the research described in **chapter 3**, was to discriminate between genes, whose rhythms in expression are driven mainly by exogenous factors such as light, feeding etc., from genes with expression rhythms persisting in the absence of such external cues, i.e. circadian genes. The latter would exhibit expression rhythms in the S/SD study samples and also in the CR study samples, due to the endogenous component driving their rhythmic expression. The analyses of rhythmicity, described in **chapter 3**, were performed on two levels – group level, assessed by a non-linear mixed model analysis and on single individual level,

assessed by the single cosinor analysis (*Minors & Waterhouse 1988; Ackermann et al., 2013*). Thus, the results obtained were interpreted with this distinction in mind (**chapter 3**). Out of these nine genes, four (*SREBF1*, *STAT3*, *THRA1* and *TRIB1*) were shown to display statistically significant expression rhythms, in both S/SD and CR studies, on a group level. Because genes ascertained during this project were to be used for subsequent forensic studies, we considered the group level analysis more in accordance with our research concept. The prospective forensic tools need to be of universal use, therefore information about significant averaged expression levels was deemed necessary for further testing, compared to that regarding rhythms in expression within a single individual. This does not, by any means, presume that the results of the single cosinor method are inferior. Detection of significant rhythms in many studied individuals, allows to anticipate that expression rhythmicity will be detectable also on a group level; however we keep in mind that due to the single cosinor method's nature, the statistical power to detect rhythmicity on a single individual level is lower compared to an analysis of several individuals simultaneously.

As previously mentioned, the data presented in **chapter 3** served as the basis for the research I described in **chapter 4**. We selected eight clock-controlled genes together with three core clock genes (*Ackermann et al., 2013*) and used them for trace deposition time estimations with various subsets of biomarkers in prediction models. The prediction models were based on multinomial regression analysis, frequently used in genetic epidemiology to ascertain the genetic risk of a disease (*Jostins & Barrett 2011*) and also proposed for predicting externally visible characteristics from genotype data (*Liu et al., 2009; Branicki et al., 2011; Walsh et al., 2011, 2013*). We built in total three prediction models, one with mRNAs as predictors, a second with melatonin and cortisol as predictors, and a third with both hormones and mRNAs as predictors (**chapter 4**). Out of the three models, the one comprised of mRNAs and hormones, showed the best prediction accuracy with AUC values above 0.88 for all categories. Comparison between the models made it clear that adding the mRNA markers

to the hormones improves the prediction accuracy of predicting day/night categories. However, before this model and its markers can be implemented in forensics, additional testing as well as improvements need to be considered. First, the model combining hormones and mRNAs, uses in total only five biomarkers (two hormones and three genes), selected based on their significant independent input into prediction accuracy. In general using a smaller number of informative markers in forensics is preferred, usually due to limited amount of evidentiary material found at the crime scene, and thus the limited amount of independent tests that can be performed. Secondly, the model we present in this thesis gives a categorical outcome, in this particular case as three established time categories (**chapters 4 & 5**), each spanning 8 hours, as most of the markers we identified peaked in similar time frames. Therefore, an RNA sequencing approach would be recommended to identify other transcripts that would exhibit different expression peak times than the ones presented here, as this could lead to increasing the prediction accuracy of the model by including more time categories.

The research described in **chapters 2, 4** and **5** of this thesis was performed in blood samples collected from males, and consequently the results we present should be considered as a male-specific, until females are tested. A global study published in 2013 (*Gibbons 2013*), found that males accounted for approximately 96% of all homicide perpetrators in the world, and that nearly 79% of homicide victims worldwide were men. In this respect, the development of the trace deposition time prediction model for males is warranted, however the need for a universal, or, at the latest, an additional female-specific model exists, as women are more likely to be victims of domestic (~64%) and sex-related (~82%) homicides (*Gibbons 2013*). Any future studies aimed at developing such a model, should take into account the potential issue of sex-related differences in circadian rhythms, despite the fact that the evidence are conflicting and study results inconsistent (*Burgess & Eastman 2005; Cain et al., 2010; Gunn et al., 2016*). Some researchers describe the existence of differences in the timing of circadian phase markers between men and women (*Van*

Cauter et al., 1996; Larsson et al., 2009), while others demonstrate, that no sex-related differences in circadian phase timing exist (*Burgess & Fogg 2008; Gunn et al., 2016*). Furthermore, additional evidence exists on the influence of oral contraception on female hormone levels, including melatonin and cortisol (*Kostoglou-Athanassiou et al., 1998; Simunkova et al., 2008*). These references emphasize that any future studies, aiming to develop a universal or female-specific model for forensic trace deposition time estimations, should be explored with caution, and with regards to potential sex-related changes in circadian timing that may influence the final marker and model selection. If indeed, sex differences between biomarkers and models for trace deposition timing exist and are strong enough to significantly impact the final time prediction, this would cause no problem in the final application to forensic case work, as sex information is obtained from every commercial DNA profiling test and thus available for every case where forensic DNA profiling is done.

Furthermore, the markers we present here were not yet validated for forensic purposes, i.e. sensitivity and in vitro stability testing of the mRNA markers were not yet performed. The latter is especially vital for forensic applications, as crime scene sample collection typically occurs days, weeks, or even months after sample deposition. Hence, to avoid erroneous time estimations, it needs to be experimentally established, that the candidate biomarkers do not change in their abundance during sample storage time due to in vitro degradation. *Ackermann et al. (2010)* demonstrated that in blood samples stored for up to 28 days no changes in melatonin concentration were seen, however the concentration of cortisol was slightly reduced, possibly due to degradation. Although in general, mRNA is considered to be less stable then DNA, and prone to rapid in vitro degradation (*Alberts et al., 2007*), it has also been shown that many mRNA markers show strong time-wise stability (*Setzer et al., 2008; Zubakov et al., 2008, 2009*). For biological trace time estimations, in vitro stability of rhythmic mRNA markers, introduced in **chapter 4**, is a prerequisite for future forensic applications, and will need to be addressed in prospective studies. Furthermore, although

blood represents a common biological trace encountered at crime scenes, other biological trace materials, such as saliva, semen, vaginal secretion, and skin cells, are also found at the crime scenes. Thus, ascertaining the expression of the candidate mRNA markers in these forensically relevant tissues would be the next validation step and a prerequisite for a prospective forensic tool development. As gene expression is often tissue-specific, only careful investigation of the mRNA markers in other human tissues will determine if these biomarkers can be applied to traces other than blood stains. For now, it needs to be emphasized that the proposed mRNA markers are only suitable for estimating the deposition time of blood. Additionally, as both melatonin and mRNA were previously applied for time of death estimations (*Minami et al., 1994; Kimura et al., 2011*), it may be expected that the particular mRNA markers, presented in **chapter 4** of this thesis, could be suitable for time of death estimations as well. Up to date, however, **chapter 4** represents the first study assessing the suitability of mRNA markers for blood trace deposition timing and is the first to propose a statistical model for estimating blood deposition time using molecular biomarkers.

In the studies I described in **chapters 2 – 4**, the markers we ascertained were all from the RNA family. However, circadian rhythms are present in many aspects of human behaviour and physiology, such as metabolism; thus it can be expected that other types of biomarkers would also be suitable for trace deposition timing. Metabolites, as well as mRNAs have previously been implied in internal body time (BT) estimations for prospective diagnostic and chronotherapy purposes (*Ueda et al., 2004; Minami et al., 2009; Kasukawa et al., 2012*). In 2004, *Ueda et al.,* have proposed a method of internal body time estimation based on whole genome expression profiles, termed the molecular timetable method. Few years later, follow-up papers on "metabolite timetable" were published (*Minami et al., 2009; Kasukawa et al., 2012*). The authors of those papers proposed quantification of hundreds of clock-controlled metabolites using LC-MS (liquid chromatography mass spectrometry) to construct a molecular timetable of blood metabolites. With this timetable,

they were able to measure internal body time (BT) under various conditions in mice and in humans; however, using two antiphase samples. Estimating internal body time is an akin, however not an equivalent concept to estimation of trace deposition time. This is mainly because the samples in the study by *Kasukawa et al. (2012)* were collected during a constant routine and semi-constant routine studies. Due to the design of a constant routine protocol, the collected samples are not fully suitable for marker identification for forensic applications, as they do not reflect natural conditions encountered every day. They are, however, useful to determine the nature of the rhythmicity, which could be beneficial in characterizing the candidate markers in order to prove their suitability for forensic purposes before validation and tool development. Furthermore, in the metabolite timetable method to determine the internal BT, at least two antiphase samples are required, which is a prerequisite that cannot be applied in a typical forensic case scenario.

In **chapter 5,** I investigated a selection of oscillating metabolites for the purpose of blood trace deposition timing. Metabolites, belonging to either acylcarnitines, amino acids, biogenic amines, hexose, glycerophospholipids or sphingolipids, and two hormones, melatonin and cortisol, were measured in the blood samples from the S/SD study, as described in detail elsewhere (*Davies et al., 2014*). After rhythmicity assessment on group and single individual levels, 58 candidate metabolite and hormone markers were preselected for model building. Three time prediction models were built (**chapter 5**) and compared together. We found that the model using mRNA, hormone and metabolite markers as predictors was better in terms of prediction accuracy, compared to other models presented in this study.

However, we noted that most of the metabolites (50 out of 56) had the highest concentration in the afternoon, which did not allow us to increase the number of the day/night time categories to be predicted. Therefore, oscillating metabolites with peak times during different times of the day would be necessary in order to increase the resolution of time predictions. In terms of marker identification for

forensic trace deposition timing (similar to mRNA), an untargeted approach to identify novel rhythmic metabolites, followed by an approach targeting the newly identified candidate markers, would be preferable for designing a forensic tool. With the noticeable progress in NGS (next generation sequencing) technology development and implementation, also in forensics, the opportunity for introduction of LC-MS-based forensic equipment and assays seems to be just a matter of time. In the future, research aiming at combining the molecular timetable method and the statistical models presented in this thesis could result in a valuable approach to determine not only trace deposition time for forensic purposes, but also internal body time, necessary for treatment optimization and personalization, as well as for chronotherapy strategies.

The metabolite data described in **chapter 5** were obtained by analysing human plasma samples. Most published LC-MS studies rely on plasma samples for metabolite profiling, however in a typical forensic setting, the evidentiary material is often a dried blood stain, which makes plasma separation impossible. From an analytical point of view, whole blood is a complex sample and is not typically used for LC-MS measurements. It is not stable during long storage, unless frozen; however freezing might cause lysis of the cellular components, as well as other changes in sample composition and metabolism. In recent years, however, whole dried blood samples have been utilized on the LC-MS platforms in toxicology or pharmacokinetic studies (*Deglon et al., 2009*; reviewed by *Li & Tse 2010; Zukunft et al., 2013*). This proves the potential and feasibility of using these samples, keeping in mind their much more complex nature, when analysing and interpreting the results. This issue will need to be explored in future studies.

In this thesis, I have introduced a novel concept of forensic time estimations using molecular biomarkers, which I suggest to be beneficial for forensic practise after careful validation of the analysis methods and biomarkers proposed here. Trace deposition time estimation, when assessed appropriately, could provide important investigation leads and information relevant for verifying the

alibis/testimonies of suspects and/or witnesses. With this thesis I present candidate biomarkers of various types together with statistical prediction models for estimating blood deposition time. Starting with the first publication by our group (*Ackermann et al., 2010*), where melatonin and cortisol were introduced and evaluated, we have been steadily testing other rhythmic biomarkers in order to determine those that can be used for this intent. Melatonin and cortisol by themselves have shown great potential in distinguishing between night and day (*Ackermann et al., 2010*) and later on, between three day/night time categories (**chapter 4**), yet with each new type of marker added – mRNAs and metabolites, the accuracy of the prediction models has improved (**chapters 4 – 5**). Future research should focus on identifying additional biomarkers, preferentially for all types of forensically relevant tissue types, so that trace deposition timing can be performed in traces other than blood. Furthermore, the peak times in oscillations of these biomarkers should "cover" the whole 24 hour period, to allow time estimations in greater detail by using more than the three predicted time categories that were established in this thesis. The biomarkers identified here (and in the future) must then be carefully validated for forensic purposes, particularly regarding in vitro stability, as well as potential sex differences, before they can be applied in forensic casework. Overall, the research presented here has the potential to improve not only blood deposition time estimations, but with additional tests it could be applied for time of death determination, as well as providing a knowledge base in other relevant research areas, such as chronotherapy and personalized medicine.

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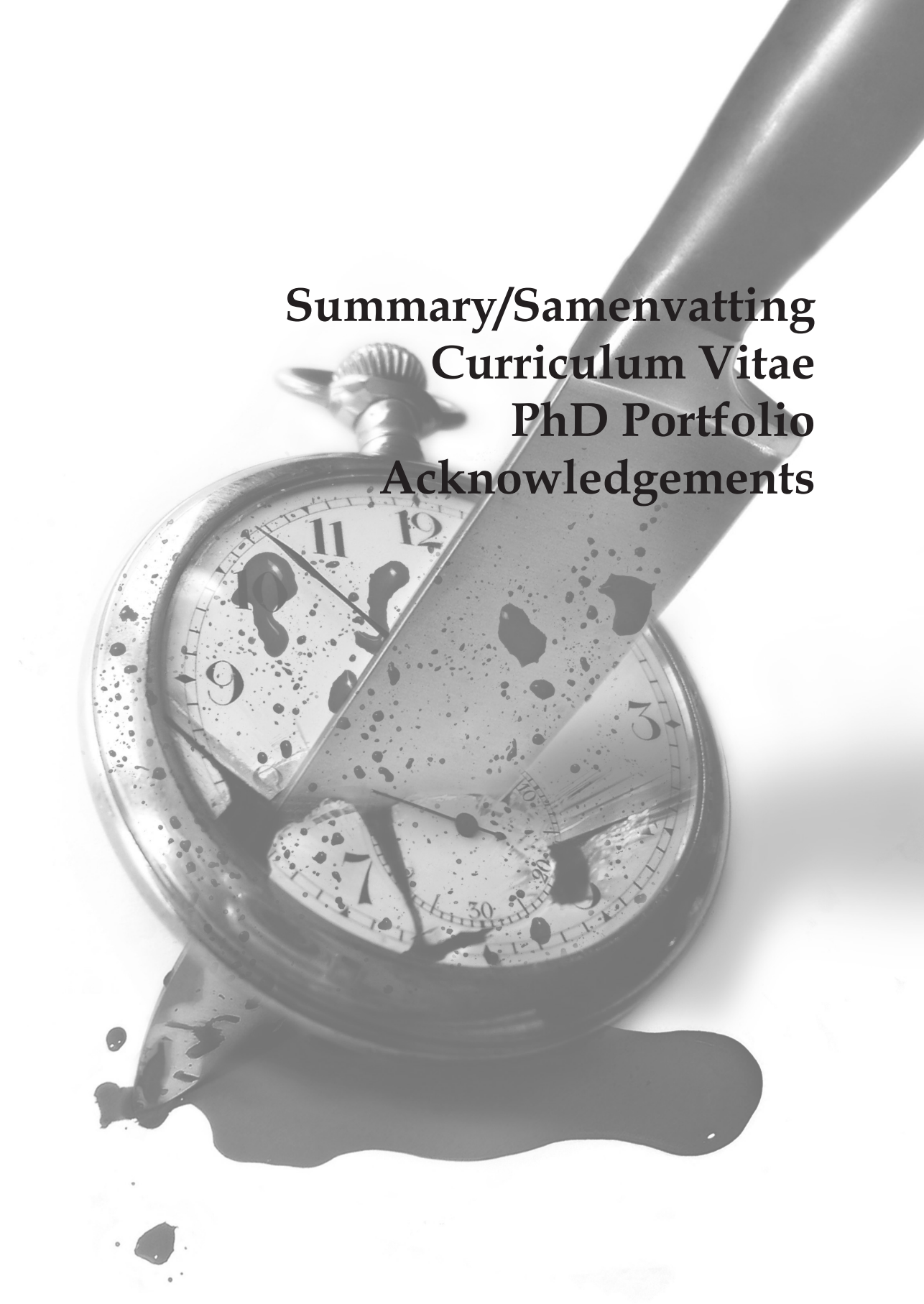

Summary/Samenvatting
Curriculum Vitae
PhD Portfolio
Acknowledgements

Summary

In recent decade, the field of forensic molecular biology and genetics has witnessed a drastic development in many of its research areas, some of which have already led towards practical casework applications. Introduction of SNP-based phenotype determination (*Liu et al., 2009, 2015a; Walsh et al., 2011, 2013*), identification of the cell or tissue origin of a trace using RNA biomarkers, or more recently, rapid development and implementation of NGS technology (*Chaitanya et al., 2015; Ralf et al., 2015; Zubakov et al., 2015*), with properly validated tools all contribute to the improvement of criminal investigations. However, one of forensic fields, namely forensic time estimations, lagged behind despite the importance of time information in many forensic cases. The various research outcomes presented in this thesis have started to overcome this lack of knowledge by unravelling one of the aspects of forensic time estimations, i.e. when during the day or night a blood sample was left behind. This scientific progress was made possible with the joint application of circadian and forensic biology.

In Chapter 1 of this thesis a comprehensive description of circadian rhythmicity is presented. It provides the information on the nature of circadian rhythms, their generation (on a molecular level) and organization, together with the processes that display such oscillations, due to their association with the circadian system. Furthermore, an introduction on the application of the circadian biology in forensics is provided.

In **Chapter 2** the utility of miRNA markers miR-142-5p and miR-541, previously proposed for forensic time of death determination (*Odriozola et al., 2013*), for blood stain deposition timing was investigated. These candidate miRNAs were analysed in venous blood samples collected from 12 healthy individuals every 4 hours during the 24 hour day/night period under controlled sleep-laboratory conditions. Differences in miRNA-142-5p abundance in samples collected during daytime and night time were

not statistically significant (one-way ANOVA p=0.81), and it also did not display statistically significant rhythmicity during the 24 hour day/night period (cosine fit for all individuals p>0.05, averaged data p=0.932). The second miRNA (miRNA-541) was present in the blood samples in very small amounts, which did not allow for reliable quantification and data generation. Overall, our data suggest that these two miRNA markers are not suitable for estimating the deposition time of forensic bloodstains.

Chapter 3 presents the characterization of several clock-controlled genes (CCGs), which previously were mostly studied in rodents only, in human peripheral blood samples collected during sleep/sleep deprivation (S/SD) and constant routine (CR) studies from 12 participants of the S/SD study, and across 33 h in 12 participants of the CR study. We found that out of nine candidate genes tested, four, i.e. *STAT3*, *SREBF1*, *TRIB1*, and *THRA1* displayed statistically significant expression rhythms during both the S/SD and the CR studies, which implies a role of the endogenous factor in rhythms generation. In addition, we confirmed the circadian rhythms of the clock genes *PER1*, *PER3*, and *REV-ERBa* during constant routine. Overall, our results show that the expression of clock and selected clock-controlled genes in human blood cells, is partially driven by exogenous factors (sleep and fasting state) and in part by the endogenous oscillator. This study provides rhythmic candidate mRNA markers for follow-up studies regarding future medical and forensic applications (for the latter, see **chapter 4**).

In **chapter 4,** mRNA transcripts characterized in the study described in **chapter 3**, were applied for blood deposition time estimations, together with melatonin and cortisol, previously proposed for this purpose (*Ackermann et al., 2010*). Eleven significantly rhythmic mRNA candidate markers were used to establish statistical models for day/night time category prediction. Overall, mRNA-based time prediction was less accurate compared to hormone-based time estimation. However, the combined use of three mRNA markers *HSPA1B*, *MKNK2* and *PER3* together with melatonin and cortisol improved the time prediction accuracy relative to the

models based on mRNA and the hormones alone, with AUC values of 0.88, 0.88, and 0.95, for night/early morning, morning/noon, and afternoon/evening categories, respectively. This study was the first to apply mRNAs for blood deposition timing and demonstrate their advantage for time category prediction, and the first to present a statistical model for estimating day/night time categories based on molecular biomarkers.

In **Chapter 5** oscillating metabolites were identified and used for estimating blood deposition time with a statistical model. From 171 metabolites measured in human blood plasma collected around the clock at 2 hourly intervals for 36 hours under controlled, yet real-life resembling conditions, from 12 individuals, 56 oscillating metabolites were identified. After additional tests, 11 metabolites together with melatonin, cortisol, and mRNA biomarkers, were used as day/night time categories predictors in statistical models. The model with five metabolites (C16, C18:1, C4, Ile, SMC24:1), 1 hormone (melatonin), and 1 mRNA marker (*MKNK2*), delivered the most accurate time prediction with AUC values of 0.96, 0.85, and 0.89, for night/early morning, morning/noon, and afternoon/evening categories, respectively, compared to other models tested. Overall, this study exemplifies the suitability of metabolite biomarkers for trace deposition timing, and demonstrates that combining them together with rhythmic mRNAs and circadian hormones improves the accuracy of estimating day/night time categories.

The final chapter **(chapter 6)** provides a general discussion on the results described in **chapters 2-5** of this thesis. The research presented here, introduces a new concept in forensic molecular biology: trace deposition timing using molecular biomarkers of different types (RNA, metabolites, hormones). We propose newly identified candidate molecular markers, characterized by robust rhythms/oscillations in expression and/or abundance and provide statistical models in order to facilitate future development of a forensic tool for trace deposition time estimations. Forthcoming studies should find more rhythmic biomarkers with peaks in concentration and/or expression at times other than those introduced

in this thesis, to improve the precision of trace deposition timing. Finally, the biomarkers introduced in this thesis, as well as those that will be identified in the future, will need to undergo stringent forensic validation, particularly regarding their time-wise stability and resistance against in vitro degradation. The proposed statistical prediction models will be revalidated using a larger group of individuals, before the markers and the final models can be applied to trace deposition timing in actual forensic cases. Furthermore, prospective research should investigate whether the biomarkers and models, established in this thesis for trace deposition timing, may also become useful for time of death estimation, as may be expected by the link between both aspects of time in forensics.

Samenvatting

In het afgelopen decennium heeft het veld van forensische moleculaire biologie en genetica drastische ontwikkelingen ondergaan in veel van zijn onderzoeksgebieden, waarvan sommige al hebben geleid tot praktische toepassingen in de behandeling van forensische zaken. Introductie van op SNPs gebaseerde fenotype bepalingen (*Liu et al., 2009, 2015a; Walsh et al., 2011, 2013*), identificatie van de afkomst van het cel of weefseltype van een biologisch spoor door gebruik te maken van RNA biomarkers, of meer recentelijk, de relatief snelle ontwikkeling en implementatie van de NGS technologie (*Chaitanya et al., 2015; Ralf et al., 2015; Zubakov et al., 2015*), waardoor gevalideerde tools gezamenlijk hebben bijgedragen aan de verbetering van criminele onderzoeken. Eén van de forensische werkvelden, namelijk forensische tijdsbepalingen, is daarentegen achter gebleven in de ontwikkeling, ondanks dat het beschikken over informatie betreffende tijd essentieel is in veel forensische zaken. De wetenschappelijke uitkomsten die gepresenteerd worden in dit proefschrift leggen de basis om dit gebrek aan kennis te overkomen, door één van de aspecten van forensische tijdsbepalingen te ontrafelen; op welk tijdstip van de dag of nacht een bloedspoor is achtergelaten. Deze wetenschappelijke vooruitgang werd mogelijk gemaakt door de gezamenlijke toepassing van circadiane (dag-nacht cyclus) en forensische biologie.

In **hoofdstuk 1** van dit proefschrift wordt een uitgebreide beschrijving gepresenteerd van circadiane ritmiek. Het verstrekt informatie over de natuur van circadiane ritmes, hoe ze tot stand komen (op moleculair niveau), hoe ze georganiseerd zijn, en over de processen die door hun associatie met het circadiane systeem dergelijke oscillaties laten zien. Verder wordt een introductie gegeven van de toepassing van circadiane biologie in forensisch onderzoek.

In **hoofdstuk 2** wordt de bruikbaarheid onderzocht van miRNA markers miR-142-5p en miR-541 voor het bepalen van het moment van de dag waarop een bloedspoor is achtergelaten.

Deze miRNAs waren eerder voorgesteld als biomarkers voor het bepalen van het tijdstip van overlijden (*Odriozola et al., 2013*). Deze kandidaat miRNAs werden geanalyseerd in veneuze bloedsamples die gedurende het 24 uurs dag/nacht ritme elke 4 uur verzameld zijn van 12 gezonde individuen, onder controle van condities die door het slaap-laboratorium zijn vastgesteld. Verschillen in hoeveelheden van aanwezigheid van miRNA-142-5p in bloedsamples die verzameld zijn tijdens de daguren ten opzichte van die hoeveelheden tijdens de nachturen waren statistisch gezien niet significant (eenweg ANOVA $p=0.81$), en er werd ook geen statistisch relevant ritme gedetecteerd tijdens de 24 uurs dag/nacht periode (cosinus fit voor alle individuen $p>0.5$, gemiddelde data $p=0.932$). De tweede miRNA (miRNA-541) was in erg lage hoeveelheden aanwezig in de bloedsamples, waardoor het niet mogelijk was om betrouwbare berekeningen te doen en/of om data te genereren. Onze data in zijn geheel suggereert dat deze twee miRNA markers niet geschikt zijn voor het bepalen van het moment van de dag waarop een bloedspoor is achtergelaten.

Hoofdstuk 3 presenteert de karakterisatie van verschillende klok-gecontroleerde genen, welke eerder voornamelijk in knaagdieren bestudeerd zijn, in humaan perifere bloedsamples die verzameld zijn tijdens slaap/slaap-deprivatie (S/SD) en constante routine (CR) studies bij 12 deelnemers van de S/SD studie, en gedurende 33 uur bij 12 deelnemers van de CR studie. We vonden dat van de negen kandidaat genen die getest zijn, vier genen, oftewel *STAT3*, *SREBF1*, *TRIB1* en *THRA1*, gen expressie ritmes lieten zien die statitisch gezien significant waren in zowel de S/SD als de CR studie, en dit impliceert een rol van endogene, circadiane factoren in de regulatie van de ritmische gen-expressie van deze 4 genen. Daarbovenop hebben we de circadiane ritmes bevestigd van de 'klok' genen *PER1*, *PER3* en *REV-ERBa* tijdens constante routine. In zijn algeheel tonen onze resultaten aan dat de expressie van de klok genen en de geselecteerde klok-gecontroleerde genen in humane bloedcellen gedeeltelijke aangedreven worden door exogene factoren (slaap en honger status) en gedeeltelijk door de endogene oscillator. Deze studie presenteert ritmische kandidaat mRNA markers welke

geschikt zijn voor vervolgstudies betreffende medische en forensische toepassingen (voor laatstgenoemd, zie **hoofdstuk 4**).

In **hoofdstuk 4** worden de mRNA markers, waarvan de karakterisatie in hoofdstuk 3 is beschreven, toegepast voor het bepalen van het tijdstip waarop een bloedsample is achterlaten, samen met melatonine en cortisol, welke eerder zijn voorgesteld voor deze toepassing (*Ackermann et al., 2010*). Elf significante ritmische mRNA kandidaat markers zin gerbuikt om statistische modellen op te zetten voor het voorspellen van dag/nacht tijdscategorien. In zijn algeheel bleek dat de voorspelling minder accuraat was met op mRNA-gebaseerde markers dan met op hormonen-gebaseerde markers. Desalniettemin bleek dat door een combinatie van 3 mRNA markers, *HSPA1B*, *MKNK2*, *PER3*, en de hormonen melatonine en cortisol de nauwkeurigheid van de tijdsvoorspelling verbeterd kon worden ten opzichte van het gebruik van alleen de mRNA markers of alleen de hormonen, met AUC waardes van 0.88, 0.88 en 0.95, voor respectievelijk nacht/vroege ochtend, ochtend/middag, en namiddag/avond tijdscatergorien. Deze studie toont voor het eerst aan dat mRNAs gebruikt kunnen worden voor het bepalen van het tijdstip waarop een bloedspoor werd achtergelaten, en voordelig zijn voor tijdscategorie voorspelling, ook wordt in deze studie voor het eerst een statistisch model gepresenteerd dat is gebaseerd op moleculaire biomarkers, met als doel het bepalen van dag/nacht categorien.

In **hoofdstuk 5** worden oscillerende metabolieten geidentificeerd, welke vervolgens worden gebruikt om door middel van een statistisch model het tijdstip waarop bloed is achtergelaten te bepalen. Van 171 metabolieten gemeten in humaan bloedplasma samples welke verzameld zijn in 2-uurs intervallen gedurende 36 uur onder gecontroleerde, doch 'real-life' gelijkende condities, van 12 individuen, werden 56 oscillerende metabolieten geidentificeerd. Na aanvullende testen, werden 11 metabolieten samen met melatonine, cortisol, en mRNA biomarkers gebruikt als dag/nacht tijdscategorie voorspellers in statitische modellen. Het model met vijf metabolieten (C16, C18:1, C4, Ile, SMC24:1), 1 hormoon (melatonine) en 1 mRNA

marker (*MKNK2*) leverde in vergelijking met andere geteste modellen, de meest accurate tijdsvoorspelling met AUC waardes van 0.96, 0.85 en 0.89 voor respectievelijk nacht/vroege ochtend, ochtend/middag en namiddag/avond tijdscategorien. In zijn algeheel laat deze studie zien dat metaboliete biomarkers bruikbaar zijn voor het bepalen van het tijdstip waarop een biologisch spoor is achtergelaten, en demonstreert dat het combineren van metabolieten met ritmische mRNAs en circadiane hormonen leidt tot verbeterde nauwkeurigheid van het bepalen van dag/nacht tijdscategorien.

Het laatste hoofdstuk (**hoofdstuk 6**) geeft een algemene discussie van de resultaten beschreven in **hoofdstuk 2-5** van dit proefschrift. Het onderzoek dat hier gepresenteerd wordt, introduceert een nieuw concept in forensische moleculaire biologie: bepaling van het tijdstip waarop een biologisch spoor is achtergelaten gebruikmakend van diverse moleculaire biomarkers (RNA, metabolieten, hormonen). We presenteren nieuw geidentificeerde moleculaire markers, deze worden gekarakteriseerd door robuuste ritmes/oscillaties in expressie en/of aanwezigheid, en leveren statistische modellen die gebruikt kunnen worden voor toekomstige ontwikkeling van een forensische toepassing voor het bepalen van het tijdstip waarop een biologisch spoor is achtergelaten. In toekomstige studies zullen meer ritmische biomarkers gevonden kunnen worden, welke pieken hebben in concentratie en/of expressie op andere tijdstippen dan die gepresenteerd zijn in dit proefschrift, om zodoende de nauwkeurigheid van het bepalen van het tijdstip waarop een biologisch spoor is achtergelaten, te verbeteren. Uiteindelijk zullen de biomarkers die geintroduceerd zijn in dit proefschrift, alsook de biomarkers die in de toekomst geidentificeerd worden, strenge forensische validatie moeten ondergaan, met name gericht op de tijdsgebonden stabiliteit en weerstand tegen in vitro degradatie. De voorgestelde statistische voorspellingsmodellen zullen opnieuw dienen te worden gevalideerd gebruikmakend van een grotere groep individuen, voordat de markers en de uiteindelijke modellen toegepast kunnen worden voor het bepalen van het tijdstip waarop een biologisch spoor is achtergelaten in werkelijke

forensische zaken. Daarbovenop zullen toekomstige studies moeten onderzoeken of de biomarkers en de modellen, bevonden in dit proefschrift voor het bepalen van het tijdstip waarop een biologisch spoor is achtergelaten, ook bruikbaar kunnen zijn voor het bepalen van het tijdstip van overlijden, zoals verwacht kan worden door de relatie tussen beide tijdstips-aspecten in forensisch onderzoek.

Curriculum Vitae

Personal details

Name	Karolina Lech
Date/Place of birth	14-05-1986, in Lubin, Poland
Nationality	Polish
Address	
Email	
Mobile	

Education

2012 – 2016	**PhD Studies** Department of Genetic Identification (formerly: Forensic Molecular Biology), Erasmus MC University Medical Center Rotterdam, Rotterdam, The Netherlands PhD thesis: *Circadian Forensics: estimating blood trace deposition time using rhythmic biomarkers.* Promotor: Prof. Dr. Manfred Kayser & co-promotor: Dr. Katrin Ackermann
2008– 2010	**Master of Science in Molecular Biology, track Biotechnology** University of Wrocław, Wrocław, Poland Master Diploma Thesis: *Characterization of phb2/phb6 plants and construction of revertants. Role of type II prohibitins in response to applied stress conditions.* Promotor: Dr. Janusz Piechota
2005 – 2008	B**achelor of Science in Biotechnology** University of Wrocław, Wrocław, Poland Bachelor Diploma Thesis: *Application of mitochondrial DNA in forensic science and archaeology to identify human remains.* Promotor: Dr. Janusz Piechota

Work experience

2012 – 2016	**PhD research** Dissertation title: *Circadian Forensics: estimating blood trace deposition time using rhythmic biomarkers.* Department of Genetic Identification (formerly: Forensic Molecular Biology), Erasmus MC University Medical Center Rotterdam, Rotterdam, The Netherlands
2010 (6 months)	**Erasmus Exchange Program** Research project title: *Role of polyamine catabolism in determining response to chemotherapeutic agents in pancreatic cancer cells.* Department of Biomedical Research, faculty of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark
2008 – 2010	**MSc research** Master thesis title: *Characterization of phb2/phb6 plants and construction of revertants. Role of type II prohibitins in response to applied stress conditions.* Department of Molecular Biology, University of Wrocław, Wrocław, Poland

Publications

***Lech K**, Liu F, Ackermann K, Revel VL, Skene DJ, Kayser M.* Evaluation of mRNA markers for estimating blood deposition time: towards alibi testing from human forensic stains with rhythmic biomarkers. Forensic Sci Int Genet. 2016; 21:119-125

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Russcher M, Chaves I, ***Lech K**, Koch BC, Nagtegaal JE, Dorsman KF, Jong A, Kayser M, van Faassen HM, Kema IP, van der Horst GT, Gaillard CA.* An observational study on disturbed peripheral circadian rhythms in hemodialysis patients. Chronobiol Int. 2015; 32(6):848-57.

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Kreutzer JN, Olsen BB, ***Lech K**, Issinger OG, Guerra B.* Role of polyamines in determining the cellular response to chemotherapeutic agents: modulation of protein kinase CK2 expression and activity. Mol Cell Biochem. 2011; 356(1-2):149-58.

PhD Portfolio

Name PhD Student	Karolina Lech
Erasmus MC Department	Genetic Identification (formerly: Forensic Molecular Biology)
PhD period	April 2012 – March 2016
Promoter	Prof.Dr. Manfred Kayser
Co-promoter	Dr. Katrin Ackermann

General Courses	**Year**	**Workload**
Cell and Development Biology (CDB) course	2013	5 days
Genetics (G) course	2012	5 days
Biochemistry and Biophysics (BB) course	2012	5 days
Biomedical English Writing Course	2015	5 days
Biostatistical Methods I: Basic Principles A (CC02a) course	2014	4 days
Safely Working in the Laboratory	2012	1 day
Specific courses		
Epigenetic regulation in health and disease	2012	1 day
Basic Introductory Course in SPSS Course	2012	3 days
R Course	2012	4 days
Partek Genomics Training Course	2012	2 days
Adobe Photoshop & Illustrator	2014	1 day
Adobe InDesign	2014	0.5 day
Janus Training Course	2013	1 day
PhD Management Course	2013	5 days
Microsoft Access Basic Course	2014	1 day
Microsoft Excel Advanced Course	2014	1 day
Forensic Summer School	2012	3 days
Seminars and workshops		
Medical Genetics Centre (MGC) PhD Workshop Düsseldorf	2012	4 days
Forensic Genomics Consortium Netherlands (FGNC) Yearly Seminars	2013	1 day
MGC PhD Workshop Luxembourg ***Poster***	2013	5 days
Nederlands Forensisch Instituut (NFI) Symposium	2012	1 day
MGC PhD Symposia	2012-2013	2 days
CLHC - Forensic PhD Symposium	2015	1 day
Conferences		
European Biological Rhythms Society (EBRS) Meeting Manchester ***Presentation***	2015	5 days
International Society for Forensic Genetics (ISFG) Conference Kraków ***Presentation***	2015	5 days

Acknowledgements/Podziękowania

"I'm made up of the memories of my parents and my grandparents, all my ancestors. They're in the way I look, in the colour of my hair. And I'm made up of everyone I've ever met who's changed the way I think."

Terry Pratchett

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Dear Debra and Vikki, thank you for the opportunity of collaboration on many projects, and for discussing the outcomes of my research. Thank you and the whole Surrey team for the support in sample collection, data analysis and paper reviewing.

When I started my PhD at Erasmus MC, I did not have sufficient knowledge of the circadian systems; I want to express my gratitude to Prof. Bert van der Horst and the former and current members of the clock group, whom I had the pleasure to know: Annelieke, Aida, Gosia, Ines, Jacqueline, Ed, Filippo, Kira, Roel, Yvonne. Thank you

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During those four years I met many people who were a part of the FMB together with me. I want to thank my colleagues and interns: Arwin, Dmitry, Fan, Iris, Joanna, Kaiyin, Magda, Mannis, Marcus, Marta, Oscar, Susan; and in particular Lakshmi and Mijke. Lakshmi, thank you for being a good friend and colleague, for your support and levelheadedness when I would stress and worry, especially during the last year of the PhD. It has been great to discuss my work with you, but I also really enjoyed our chats about baking and decorating cakes :) Thank you for being there for me during the defence as my Paranymph. Mijke, thank you for all the support and helpful advice I got from you; I appreciate your drive, perseverance and work organization.

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Last but not least, to everyone else that I have met during this four years, thank you for being a part of this wonderful experience!

"This time it had been magic. And it didn't stop being magic just because you found out how it was done."

Terry Pratchett