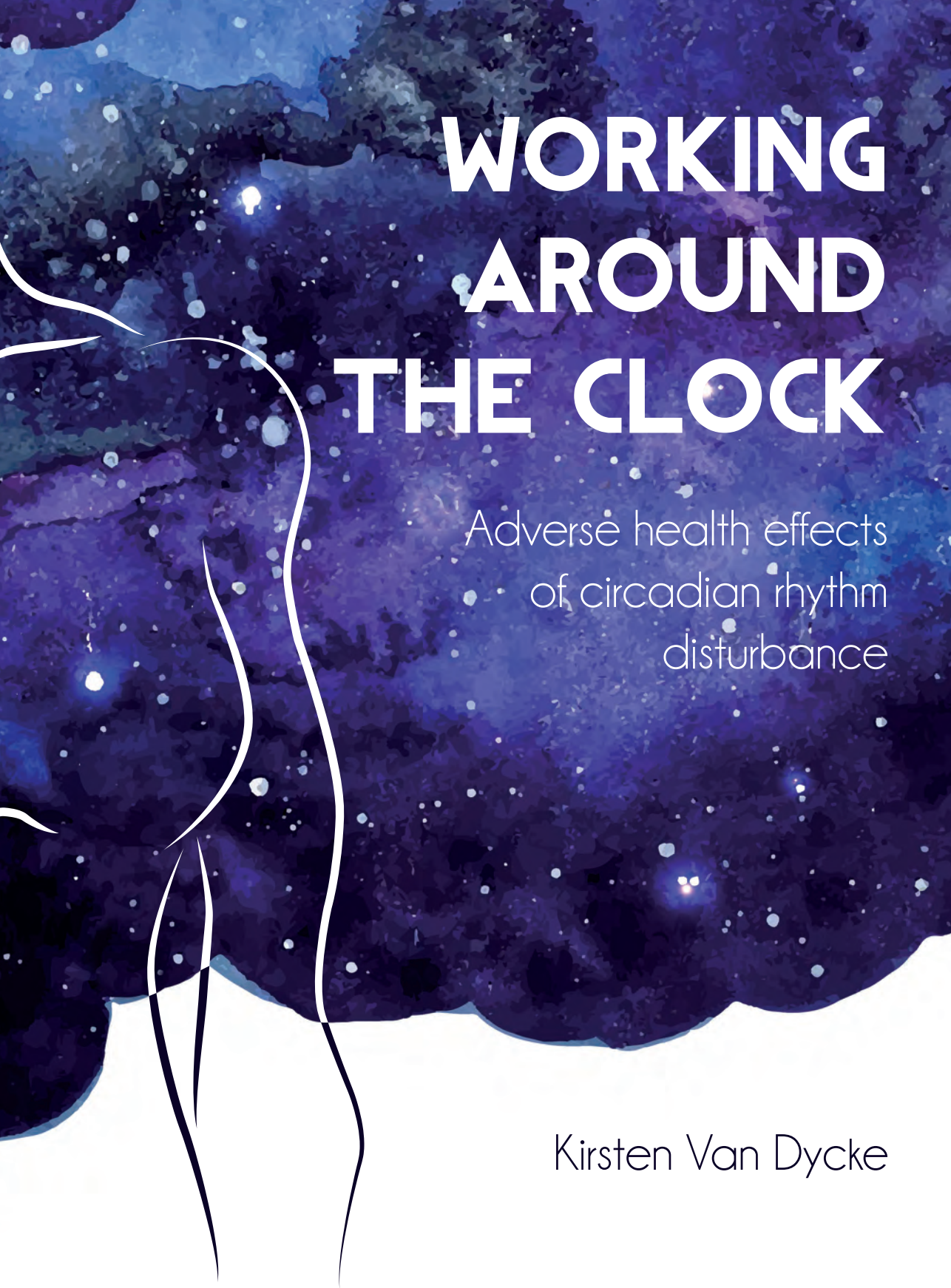

WORKING AROUND THE CLOCK

Adverse health effects of circadian rhythm disturbance

Kirsten Van Dycke

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Werken rond de klok:

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

The circadian clock

To anticipate the recurring environmental changes resulting from the Earth's rotation, most organisms have developed a circadian clock with periodicity of approximately (circa) one day (diem). Circadian rhythms can be found in behavior, physiology and metabolism. Well known examples are the sleep-wake cycle or rhythm in locomotor activity (Hurd et al., 1998, Cahill et al., 1998), daily variations in body temperature (Refinetti and Menaker, 1992) and blood pressure (Pickering, 1990) and melatonin levels that rise at sunset and decrease at sunrise (Hardeland et al., 2006). In mammals, this internal timekeeping system is composed of a master or central clock, the suprachiasmatic nucleus (SCN) located in the brain, and peripheral clock in all other tissues in the body. The SCN consists of two bilateral nuclei in the ventral hypothalamus, just above the optic chiasm and is required to synchronize the peripheral clocks (Moore and Eichler, 1972, Moore, 1983, Van den Pol, 1980).

To keep these approximately 24 hour rhythms synchronized with the environment, circadian rhythms need to be entrained by Zeitgebers. The most important and potent Zeitgeber for the central SCN clock in mammals is light (Bell-Pedersen et al., 2005). The light-input is perceived by specialized retinal ganglion cells in the eye using a photo pigment called melanopsin (Berson et al., 2002). This photo pigment differs from the rhodopsin in the rods and cones, used mainly for vision. The photic signal is transmitted by the retinohypothalamic tract to the hypothalamus, which projects to the SCN. Depending on the timing of the light exposure, the circadian rhythm will delay or advance, resulting in e.g. waking up later or earlier, respectively (Warman et al., 2003, Duffy et al., 1996). Whereas light can affect timing of the clock in the SCN, timed food intake has been shown to entrain circadian rhythms of peripheral clocks, independent of the SCN (Carneiro and Araujo, 2012). Social cues are also sufficient to entrain human circadian rhythms (Aschoff et al., 1971). Furthermore, melatonin is best known as output of the biological clock, but in turn can also entrain or phase shift the clock in several species (Redman, 1997).

These circadian rhythms are endogenous, meaning that the rhythm persist under constant conditions with a period of approximately 24 hours. The circadian rhythm is self-sustaining and regulated on a molecular level by a molecular oscillator composed of clock genes (described in Box 1). The circadian rhythm in the absence of Zeitgebers such as light is called free running rhythm. In humans, the average

free running period has been shown to be slightly longer than 24 hours (Czeisler et al., 1999). In contrast, most mouse strains have an internal rhythm that is slightly shorter than 24 hours, with variation between the strains (Pfeffer et al., 2015, Schwartz and Zimmerman, 1990). As described previously, this internal rhythm is synchronized with the environment by Zeitgebers. The relationship between the timing of the biological clock and the timing of an external time cue is called the phase angle of entrainment and determines chronotype; whether an individual is an early type or a late type (Emens et al., 2009).

Box 1. Molecular clock

At the molecular level, the circadian rhythms are generated by an auto-regulatory transcriptional-translation feedback loop (TTFL) (Lee et al., 2001). In short, the positive limb of the TTFL consists of CLOCK and BMAL1 proteins, driving the transcription of *Period* (*Per1*, *Per2*) and *Cryptochrome* (*Cry1*, *Cry2*) genes. In the negative limb, PER and CRY proteins form a heterodimeric complex that translocates back into the nucleus, repressing the CLOCK/BMAL1 driven transcription, thereby inhibiting their own gene expression (Ko and Takahashi, 2006, Lee et al., 2001, Reppert and Weaver, 2002). This molecular oscillator is coupled to output processes via clock-controlled genes, including transcription factors (Reppert and Weaver, 2002). Micro-array studies have revealed that approximately 10 % of the transcriptome is under circadian control (Hughes et al., 2010, Miller et al., 2007, Panda et al., 2002).

Shift work-associated breast cancer risk

Working around the clock strains and disturbs this tightly regulated biological clock, which eventually might result in adverse health events. Our 24/7 economy demands people to work at irregular times and as a consequence, more and more people are involved in atypical working schedules. Recent surveys in Europe indicate that approximately 19% of the workers in the European Union (EU) work at night and 17% are involved in shift work with permanent or rotating shifts (Eurofound, 2012). In the Netherlands, approximately 1.2 million people work sometimes or regularly during the night (CBS, 2010). Recent epidemiology studies show that long-term shift work increases acute and chronic health risks, like fatigue (Akerstedt and Wright, 2009), gastrointestinal complaints (Knutsson and Boggild, 2010), and developing diabetes (Suwazono et al., 2006, Pan et al., 2011) and cardiovascular disease (Gu et al., 2015, Fujino et al., 2006). Importantly, work in shifts for many years was associated with an increased breast cancer risk

in women (Akerstedt et al., 2015, Schernhammer et al., 2006). Epidemiological studies concerning the relation between shift work or night work and breast cancer risk have been summarized in several meta-analyses with contradicting results (He et al., 2014, Ijaz et al., 2013, Jia et al., 2013, Kamdar et al., 2013, Megdal et al., 2005, Wang et al., 2013). In the first meta-analysis Megdal *et al.* found a pooled relative risk (RR) of 1.51 (95% CI, 1.36–1.68) for the association between shift work and breast cancer, based on six studies (Megdal et al., 2005). Subsequent meta-analyses reported lower RRs of approximately 1.2 (He et al., 2014, Jia et al., 2013, Kamdar et al., 2013, Wang et al., 2013) or limited to no evidence for a shift work breast cancer relationship (Ijaz et al., 2013).

Shift work is a complex combination of exposures, involving multiple aspects that have been suggested to underlie the relationship between shift work and cancer (Fritschi et al., 2011). These aspects include internal desynchronization, suppression of melatonin or vitamin D levels, sleep disruption and lifestyle disturbances, all shown in Figure 1. All suggested hypotheses are extensively discussed by Fritschi *et al*. (Fritschi et al., 2011). In summary, the alternating shifts during shift work may cause rhythms in peripheral function to become out of phase with the central clock or sleep wake cycle, internal desynchronization. Additionally, both timing and the degree of light exposure in shift workers is changed affecting both melatonin and vitamin D levels. Light exposure during the night can result in melatonin suppression (Davis and Mirick, 2006), which is suggested to increase breast cancer development in experimental studies by various mechanisms (e.g. anti-oxidant function) (Blask et al., 2005). Individuals working in night shift are suggested to have less sunlight exposure, resulting in lowered vitamin D levels (Kimlin and Tenkate, 2007), whereas sun exposure has been suggested to be protective of several types of cancer (van der Rhee et al., 2013). Vitamin D has been suggested to be one of the mediating factors in the preventive effect of sunlight on cancer (van der Rhee et al., 2013), for example by inhibition of cell proliferation through various mechanisms (Garland and Garland, 2006). Sleep disruption can cause a decrease in melatonin levels, decreased immune activity and metabolic disturbance, which all have been linked separately with increased cancer risk (Bovbjerg, 2003, Bianchini et al., 2002, Blask et al., 2005). Lastly, lifestyle factors such as smoking, unhealthy eating, age at first birth and duration of breast-feeding are possibly different for shift-workers compared to day-workers and are all factors which increase cancer risk.

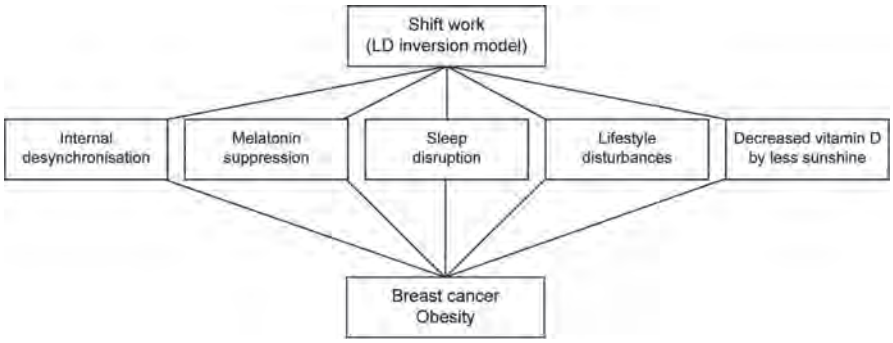

Figure 1. Suggested scenarios underlying the relationship between chronic CRD and breast cancer (adapted from Fritschi *et al*. (Fritschi et al., 2011)).

Due to the observational nature of epidemiological studies, it is unknown which aspect or combination of aspects is responsible for the observed increased health risk in shift workers. Moreover, several factors can be either defined as part of the exposure, intermediate factor or be a result of shift work exposure. For example, significant differences in socio-economic status, smoking behavior, nulliparity, hormone replacement therapy use, and obesity between women that had and had not worked at night have been reported (Wang et al., 2012). An important drawback of the epidemiological studies is that these studies require several years to study effects on chronic diseases prospectively, and the ability to control the aspects related to shift work is limited.

Additionally, exposure assessment of shift work is often lacking or incomplete due to the retrospective nature of many studies and the diversity of shift work schedules used. The first line of epidemiological studies, such as the Nurses' Health Study (Schernhammer et al., 2006), have been able to identify associations between shift work and breast cancer using relatively crude exposure assessment metrics such as 'ever/never conducted shift work' or 'duration of working on a rotating schedule'. Molecular epidemiology studies, as performed by Papantoniou *et al*. assessed the exposure to shift work in more detail, reporting diurnal preference, light exposure, circadian variation in melatonin, and sex hormone production (Papantoniou et al., 2014). The findings of this cross-sectional study among day and night workers show that night shift workers have decreased melatonin levels and differences herein result from diurnal preference and intensity of light-at-night exposure. However,

it will take many years before specific exposures during shift work are proven to be causal for cancer risk in human studies. **To study the causal relationship between circadian disturbance resulting from shift work and increased breast cancer risk, confounding factors should be excluded or taken into account and circadian disturbance exposure should be clearly defined or controlled.**

Animal studies on the shift work-cancer relationship

Animal studies allow detailed analysis of individual aspects of shift work, by allowing the exclusion (melatonin, lifestyle factors) or measurement (internal desynchronization, sleep disruption, vitamin D) of these individual factors. Additionally, such studies can identify underlying changes in biological processes that could provide targets for the development preventive measures. Animal studies have a number of advantages compared to epidemiological studies. Firstly, inter individual variation can be minimized in animal studies. Exposure models can be developed to mimic human shift work allowing controlled uniform exposure, in a genetically homogenous study population. Animal studies are therefore not hampered by the environmental or lifestyle confounding factors, or exposure assessment difficulties and therefore are better suited to study causality. Secondly, in comparison to prospective cohort studies, the effects of shift work on long-term health effect can be determined in a relatively short (1 year) time window. Moreover, in shift work many environmental factors have changed, such as altered light exposure, changed sleep-wake cycle (Grundy et al., 2009) and differently timed food intake (Lennernas et al., 1995). In animal studies, these aspects can be studied separately or in combination, providing insight into which disturbance(s) of the circadian system is (are) (mostly) responsible for the observed health effects.

When using animals to study the relation between circadian rhythm disturbance (CRD) and breast cancer risk, one can manipulate both sides of the equation: circadian rhythm and breast cancer development. Disruption or disturbance of circadian rhythms can be accomplished by alternating or shifting light schedules, constant (dim) light exposure or disruption of the endogenous circadian rhythm. The endogenous circadian rhythms can be disrupted through genetic modification of the molecular clock, by mutating one of the core clock genes. Removing the pineal gland will also result in the loss of circadian rhythmicity. In literature circadian (rhythm) disruption is often used to describe manipulation of the circadian system. In this thesis we differentiate between disruption and disturbance. The models

in which the endogenous circadian system is modified either genetically or anatomically, resulting in a completely or partially non-functional circadian system are referred to as circadian disruption models. Whereas, circadian disturbance refers to strain or stress imposed on the functional circadian system by exogenous exposure, including but not limited to jet lag, altered timing of food intake and day-time sleep.

Most studies make use of circadian disruption models or constant light exposure, of which the majority shows an increase in breast tumor growth. The increased tumorigenesis after disruption of the endogenous circadian rhythm or by the "unnatural" constant light exposure model, which is known to desynchronize mammalian clock neurons, resulting in behavioral and physiological arrhythmicity (Ohta et al., 2005), emphasizes the relation between the circadian clock and tumor development. However, altered light schedules provide a more realistic tool to mimic constant disturbance of the circadian system resulting from shift work. Such an approach has previously been employed by exposure to a nightly light pulse (Travlos et al., 2001) or infusion with blood of women exposed to a nightly light pulse (Blask et al., 2005). Whereas the first study found no effect on chemically induced tumor growth, the latter showed a significant increase in MCF-7 xenograft growth. Additionally, other circadian rhythm disturbance (CRD) models have been described that mimic human shift work, including altered timing of 1) food intake, 2) activity and 3) sleep. A large variety of these models, as well as altered light exposure, have been used to study the metabolic effects of human shift work with a substantial number of indecisive results (Opperhuizen et al., 2015). Previously, altered light schedules have shown to increase chemically induced liver tumor growth (Filipski et al., 2009), but have not yet been employed in combination with breast cancer induction models.

Since breast tumors are relatively rare in animals, studying breast cancer risk in wild type animals requires a large number of animals. Therefore, breast cancer animal models are used to reduce the number of animals needed. Breast cancer models can be divided into chemically induced tumor development and xenografted mammary gland tumors or tumor cell lines. Importantly, carcinogenesis comprises three different stages, namely initiation, promotion and progression (Box 2 and Fig. 2). The majority of the previous studies used chemically induced or xenografted tumors provide evidence that CRD causes enhanced tumor growth and thus

affect promotion and/or progression, but the studies could not give insight into the effect of CRD on tumor initiation.

Box 2. Carcinogenesis

The multistage carcinogenesis theory is generally accepted and describes three different stages between the initial carcinogenic stimulus and the final manifestation of cancer (Berenblum, 1975). Following this theory, carcinogenesis can be divided into *initiation, promotion* and *progression*. During the initiation stage, a carcinogenic stimulus causes irreversible damage to the DNA, resulting in potential for neoplastic development. The mutations causes either the activation of a proto-oncogene or the inactivation of a tumor-suppressor gene. However, without stimulation to proliferate the initiated cell will not develop into a malignant tumor. The promotion stage is characterized by prolonged exposure to promoting stimuli (Upton et al., 1986), causing the selective clonal expansion of the initiated cell, resulting in a pre-neoplastic lesion. These cells become increasingly unresponsive to cellular signals that regulate cell growth and proliferation. Further genetic changes will result in more heterogeneity and aggressive characteristics during the progression stage. The proliferation rate increases and tumor growth becomes invasive, eventually resulting in distant metastasis. In this stage, angiogenesis is required for tumor growth beyond 2 mm size (Folkman, 1985).

The circadian clock is involved in several aspects of carcinogenesis. DNA repair pathways are in place to prevent irreversible damage to the DNA, resulting in tumor initiation. It has been suggested that all aspects of the cellular response to DNA damage are controlled or influenced by the circadian clock (Sancar et al., 2010). Furthermore, it has been shown that the rate of tumor growth is highly rhythmic during the day (You et al., 2005).

Overall, many animal experiments have been performed to elucidate the causal relationship between circadian rhythm disturbance and mammary gland tumor development (for references see Table 2). Table 1 summarizes all breast cancer specific animal experiments published up to June 2015. The majority of the studies used an exposure model resulting in disruption of the endogenous circadian rhythm (n=13) or complete suppression of the circadian rhythm by continuous light (n=11). Breast cancer induction in the majority of the studies involved mouse models with rapidly growing tumors, either induced chemically (n=16) or xenografted (n=5).

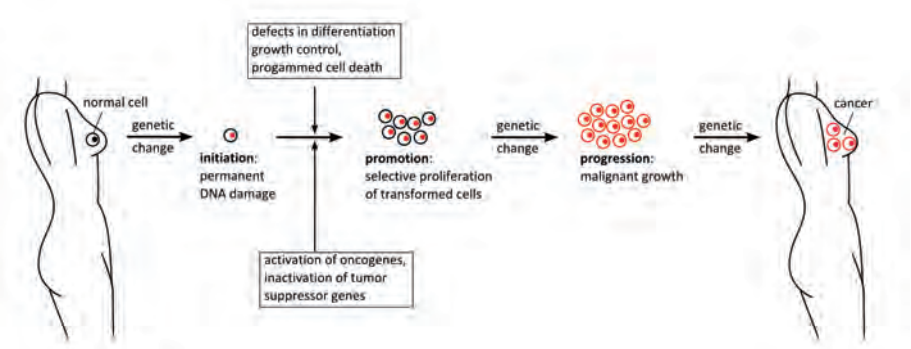

Figure 2. Carcinogenesis (Adapted from Weston & Harris Cancer Medicine 6th edition)**.** Carcinogenesis comprises three stages: *initiation, promotion* and *progression*. In the initiation stage, a mutation occurs in a gene of one of the key regulatory pathways of the cell. Subsequently, the initiated cell selectively proliferates during promotion. Due to the genetic instability, additional mutations occur resulting in malignant cell growth: progression and ultimately to metastasis.

Overall, many animal experiments have been performed to elucidate the causal relationship between circadian rhythm disturbance and mammary gland tumor development (for references see Table 1). Table 1 summarizes all breast cancer specific animal experiments published up to June 2015. The majority of the studies used an exposure model resulting in disruption of the endogenous circadian rhythm (n=13) or complete suppression of the circadian rhythm by continuous light (n=11). Breast cancer induction in the majority of the studies involved mouse models with rapidly growing tumors, either induced chemically (n=16) or xenografted (n=5).

Table 1. Studies investigating the relationship between CRD and breast cancer development, shown as studies with positive outcome/all studies.

Circadian disruption / disturbance model / Breast cancer Induction	Altered light schedules	Continuous light exposure	Endogenous circadian rhythm disruption	Total
Spontaneous cancer development	-	1/3	2/2	3/5
Chemically induced cancer	0/1	4/6	4/9	8/16
Xenografted tumors	1/1	2/2	2/2	5/5
Total	1/2	7/11	8/13	16/26

Although various animal models show increased tumor development or growth upon circadian disruption, none of these studies fully recapitulate the human shift work and carcinogenesis situation. For the circadian disruption models holds that

the lack of a functional clock is different from the shift work situation where a functional circadian clock is continuously strained or disturbed. Obviously, models using xenografted tumors or tumor cell lines can only study tumor growth and chemical induction of tumors is extremely potent, leaving limited space for tumor initiation by CRD. Moreover, the window of exposure in these models is limited to weeks due to the rapid tumor development, which does not allow mimicking long-term exposure scenarios as experienced by shift workers. Interestingly, no studies were performed combining spontaneous breast tumor development with an exposure protocol focused on disturbing rather than disrupting the circadian rhythm. **Consequently, there is a need for studies that combine relevant models for CRD exposure and breast tumor development to provide experimental evidence for the shift work-cancer connection.**

Li-Fraumeni mouse model

A mouse model with a predisposition for human relevant breast cancer provides a unique tool to experimentally study the shift work breast cancer relationship. Mutations in the tumor suppressor gene p53 are associated with increased tumor development (Coles et al., 1992), progression (Borresen-Dale, 2003), recurrence (Norberg et al., 2001) and decreased response to therapy. Additionally, Li-Fraumeni syndrome patients, carrying germ line p53 mutations, are predisposed to developing breast cancer at a relatively early age (Varley et al., 1997). Given the apparent important role of p53 in preventing breast tumor development in humans, a mouse model with a similar defect in p53 is a valuable tool to study breast tumor development. Therefore, a transgenic mouse model was developed with mammary gland specific expression of the p53.R270H mutation, resulting in spontaneous mammary gland tumorigenesis mimicking human breast cancer development (Wijnhoven et al., 2005). Expression of the mutation in mammary tissue was achieved by crossing p53.R270H mutant mice with mammary-specific Cre transgenic mice having Cre recombinase under the control of the hormone-inducible Whey Acidic Protein (WAPCre mice; (Wagner et al., 1997)). This mouse model has been shown to develop human relevant mammary gland tumors, including hormonal receptor status, with a latency time of approximately one year. **This combination provides a human relevant endpoint and sufficient time for exposure to study the relationship between chronic CRD and breast cancer development experimentally.**

Table 2. Detailed information of all studies included in Table 1.

Study/author	Study population	Endpoint	Breast cancer induction	Exposure model	Findings
(Anisimov et al., 2004)	Mice	Tumor spectrum	Spontaneous	Constant (dim) light	Increased tumor incidence
(Khaetski, 1965)	Rats	Mammary gland tumor	DMBA	Constant (dim) light	Increased tumor incidence
(Hamilton, 1969)	Rats	Mammary gland tumor	DMBA	Constant (dim) light	Increased tumor incidence
(Kothari et al., 1984, Mhatre et al., 1984, Shah et al., 1984)	Rats	Mammary gland tumor	DMBA	1) Constant (dim) light 2) Pinealectomy	1) Increased tumor incidence 2) No effect
(Cos et al., 2006)	Rats	Mammary gland tumor growth	DMBA	Constant (dim) light	Increased tumor growth
(Anderson et al., 2000)	Rats	Mammary gland tumor	DMBA	Constant (dim) light	Decreased tumor incidence
(Blask et al., 2003)	Rats	MCF7 tumor graft	Tumor cell line graft	Constant (dim) light	Increased tumor growth
(Blask et al., 2005)	Rats	MCF7 tumor graft	Tumor cell line graft	Infusion with blood of women exposed to a nightly light pulse	Increased tumor growth
(Subramanian and Kothari, 1991)	Rats	Mammary gland tumor	DMBA	Pinealectomy	Increased tumor incidence and growth
(Blask et al., 1991)	Rats	Mammary gland tumor	NMU	Pinealectomy	No effect
(Shah et al., 1984)	Rats	Mammary gland tumor	DMBA	Pinealectomy	No effect
(Lapin, 1978)	Rats	Mammary gland tumor	DMBA	Pinealectomy	Increased tumor incidence
(Aubert et al., 1980)	Rats	Mammary gland tumor	DMBA	Pinealectomy	No effect
(Tamarkin et al., 1981)	Rats	Mammary gland tumor	DMBA	Pinealectomy	Increased tumor incidence
(Travlos et al., 2001)	Rats	Mammary gland tumor	NMU	1) Pinealectomy 2) Nightly light pulse	1) No effect 2) No effect
(Climent et al., 2010)	Mice	Mammary gland tumor	1) DMBA 2) MMTV-Neu 3) Spontaneous	*Per3* knock-out	1), 2), 3) Increased tumor incidence
(Yang et al., 2009b)	Mice	MTCL tumor graft	Tumor cell line graft	*Per2* knock-down	Increased tumor growth
(Yang et al., 2009a)	Mice	MTCL tumor graft	Tumor cell line graft	*Per1* knock-down	Increased tumor growth
(Vinogradova et al., 2009)	Rats	Tumor spectrum	Spontaneous	Constant (dim) light	Increased tumor growth, no effect on mammary gland tumor
(Vinogradova et al., 2010)	Rats	Tumor spectrum	Spontaneous	Constant (dim) light	Increased tumor growth, no effect on mammary gland tumor
(Wu et al., 2011)	Rats	MCF7 tumor graft	Tumor cell line graft	Constant (dim) light	Increased tumor growth
(Blask et al., 2014)	Rats	MCF7 tumor graft	Tumor cell line graft	Constant (dim) light	Increased tumor growth

Monitoring CRD for interventions

Given the wide variety of observed health effects, biomarkers are needed that detect chronic CRD (long) before adverse health effects occur. Biomarkers are a valuable source of information, enabling early or non-invasive detection of disease or disease risk factors. Currently available or gold standard biomarkers for circadian rhythm studies are melatonin, corticosterone and body temperature. These markers are often used in studies to assess the effect of shift work (Niu et al., 2015, Papantoniou et al., 2014, Sack et al., 1992), but have several limitations for the use in large populations. An overall drawback of these classical markers of the circadian rhythm is the need for around the clock measurement. In relation to this circadian rhythmicity, for melatonin and body temperature the peak of the rhythm depends on chronotype (Lack et al., 2009), which in turn alters with age, complicating the use of these markers in longitudinal studies. Additionally, melatonin peak serum levels can be suppressed by light exposure during the night (Davis and Mirick, 2006) making routine assessment more difficult. Moreover, classical markers are acutely altered after impingement on the circadian system and are not related to the (chronic) adverse health outcomes. **Therefore, universal biomarkers for circadian rhythm disturbance are needed to compare CRD models, to evaluate the effect of preventive measures and to measure the personal risk of a shift worker.**

Aim and outline of this thesis

The aim of this thesis is to study the causal relationship between chronic circadian disturbance and adverse health effects. Additionally, identifying underlying mechanisms and potential biomarkers for CRD to aid the development and study of preventive measures are objectives as well. Finally, a new model to study health effects of shift work in animal studies is presented.

In **chapter 2** we used a unique breast cancer-prone mouse model to study the causal relation between chronic CRD and breast cancer development. Besides breast cancer risk, the effects of CRD on body weight gain, sleep probability and classical circadian markers were investigated. As described previously, several scenarios have been suggested to underlie the relationship between shift work and cancer, in this chapter we show the relevance or irrelevance of these mechanisms.

The presence of a causal relationship between shift work and adverse effects, warrants the detection of biomarkers for early detection of disturbance in order to develop preventive measures. Transcriptomic studies provide a valuable tool to obtain a complete overview of gene activity in tissues of interest. In **chapter 3**, a proof of principle is given using a comparative transcriptomics approach to identify potentially blood detectable biomarkers for breast cancer. A similar approach was used to identify biomarkers for CRD, independent of time of day, in **chapter 4**. Besides using biomarkers for CRD, in large cohort studies one could opt for the use of biomarkers related to disease outcome. The measurement of these biomarkers in a molecular epidemiology-like setting requires knowledge of their daily blood level patterns. **Chapter 5** provides a descriptive study of the daily variation of hormonal and lipid biomarkers, often used in large scale studies. Development of evidence-based preventive measures further require insight into underlying biological mechanisms.

In **chapter 6**, hepatic gene expression was analyzed to identify genes and processes affected by chronic CRD. Both the effects on genes that show circadian rhythmicity under normal conditions and on overall gene expression are described. Subsequently, the role of these genes in biological processes relevant for the observed phenotype was investigated.

CHAPTER 2

CHRONICALLY ALTERNATING LIGHT CYCLES INCREASE BREAST CANCER RISK IN MICE

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Current Biology, 2015, 25(14): 1932-7

SUMMARY

Although epidemiological studies in shift workers and flight attendants have associated chronic circadian rhythm disturbance (CRD) with increased breast cancer risk, causal evidence for this association is lacking (Pukkala et al., 1995, Schernhammer et al., 2006). Several scenarios have been proposed to contribute to the shift work-cancer connection: (i) internal desynchronization, (ii) light at night (resulting in melatonin suppression), (iii) sleep disruption, (iv) lifestyle disturbances and (v) decreased vitamin D levels due to lack of sun light (Fritschi et al., 2011). The confounders inherent in human field studies are less problematic in animal studies, which are therefore a good approach to assess the causal relation between circadian disturbance and cancer. However, the experimental conditions of many of these animal studies were far from the reality of human shift workers. For example, some involved xenografts (addressing tumor growth rather than cancer initiation and/or progression) (Blask et al., 2003, Filipski et al., 2004), chemically induced tumor models (Hamilton, 1969, Shah et al., 1984) or continuous bright light exposure, which can lead to suppression of circadian rhythmicity (Anisimov et al., 2004, Wu et al., 2011). Here, we have exposed the breast cancer-prone *p53*$^{R270H\Delta/+}$ *WAPCre* conditional mutant mice (in a FVB genetic background) to chronic CRD by exposing them to a weekly alternating light-dark (LD) cycle throughout their life. Animals exposed to the weekly LD-inversions showed a decrease in tumor suppression. In addition, these animals showed an increase in body weight. Importantly, this study provides the first experimental proof that CRD increases breast cancer development. Finally, our data suggest internal desynchronization and sleep disturbance as mechanisms linking shift work with cancer development and obesity.

EXPERIMENTAL PROCEDURES

Experimental set-up

To study the effect of chronic circadian disturbance (CRD) on the development of breast tumors, breast cancer-prone female *p53*$^{R270H©/+}$*WAPCre* conditional mutant mice in an FVB genetic background were chronically exposed to a LD-inversion protocol. The p53 R270H mutation in human Li-Fraumeni patients occurs in their germline and patients consequently develop tumors in a variety of tissues, including breast cancer. Since we are specifically interested in breast tumor development in the current study, we used the conditional p53$^{R270H©/+}$ model, in which the mutation was specifically activated in mammary gland tissue through WAP-driven cre-recombinase. The generation of these mice has been previously described (Wijnhoven et al., 2005). Cre recombinase expressing mammary gland cells have been shown without pregnancy (Derksen et al., 2011), therefore virgin mice were used in this study. At 8 weeks of age, mice were randomly assigned to remain under a normal 12:12 hour light-dark (LD) cycle or to undergo a weekly alternating 12:12 hour light-dark cycle. After approximately 18 shifts, on day 7 of the last shift, a cross-sectional sample of animals was sacrificed around the clock, with 4 hour intervals (cross-sectional study, n=4 per time point). Blood and tissues were collected for further analysis. The remainder of the animals (longitudinal study, n=25 per group) stayed under LD or weekly LD-inversion (CRD) conditions and were sacrificed after when tumors reached approximately 1 cm^3 or when animals were found moribund. Due to premature animal loss, 21 animals per condition were available for further analysis in the cross-sectional experiment. In the longitudinal study, 20 and 21 animals were available for body weight and tumor-free survival analyses for the control and CRD groups, respectively. In an additional group of mice (n=5 per group), a radio transmitter (Physio Tel, TA11 TA-F10; Data Sciences, St. Paul, MN) was implanted in the peritoneal cavity to record locomotor activity and core body temperature every ten minutes.

The animal handling in this study was performed in compliance with national legislation, including the 1997 Dutch Act on Animal Experimentation, and the experiments were approved by the institute's Animal Experimentation Ethical Committee.

Breast tumor development

Mice were palpated once a week to check for tumor development. The time of first mammary tumor detection was registered and used to determine latency times. To confirm the presence of a tumor and determine tumor type, paraffin-embedded, formalin-fixed tumor sections were stained with H&E for histopathological evaluation.

Body weight and food intake

Mice and food were weighed weekly to determine body weight gain and food intake. Body weight gain was expressed as percentages of body weight at the start of the experiment and was statistically analyzed at 28 weeks of exposure, the last time point without tumor-bearing animals. Animals were group-housed with three to four animals per cage; therefore, food intake was expressed as average weekly food intake. Food intake was measured between 8 and 15 weeks of exposure in the cross-sectional study.

Gene expression, corticosterone and vitamin D levels

Circadian expression levels of clock genes *Bmal1*, *Per1*, *Per2*, *Dbp* and cell cycle control gene *c-Myc* were determined in livers of control and weekly alternated LD cycle exposed cross-sectional animals, using quantitative reverse transcription polymerase chain reaction (RT-PCR). All oligonucleotide primers were obtained from Life Technologies (Bleiswijk, The Netherlands). Total RNA was extracted from RNA*later* (Invitrogen, Grand Island, NY, USA) protected liver tissues using the miRNeasy Mini Kit (Qiagen Benelux, Venlo, The Netherlands). Clock gene expression was given as relative expression compared to a reference pool containing all samples. Corticosterone and vitamin D serum levels were determined using ELISA assays (Yanaihara Institute Inc. Shizuaka, Japan and Immunodiagnostics Systems, Frankfurt am Main, Germany, respectively).

Body temperature and activity

Body temperature and behavioral activity were recorded for 2 weeks at baseline, after 1 shift and after 18 shifts. Cosine curves were fitted using the R statistical software environment (http://www.r-project.org) to determine the acrophase of activity and body temperature rhythms (*i.e.*, peak time). Acrophase was expressed in External Time (ExT) (Daan et al., 2002), with ExT 0 indicating mid-dark. Sleep patterns were determined using mice telemetry data as described previously for

human wrist actimetry data (Juda et al., 2013b). In short, telemetric activity data were collected in 10-min bins, and sleep episodes were automatically detected by the method described earlier. Based on a non-rhythmic trend (calculated by using centered moving 24-h averages), sleep (=1) and wake (=0) t were dichotomized according to being below or above a selected threshold (15% of the trend). Onsets and offsets of sleep episodes were then assessed by a correlation method as earlier described in detail (Juda et al., 2013a, Roenneberg et al., 2015).

Statistics

All data are expressed as means ± standard error of the mean (SEM) and were visualized and statistically analyzed using GraphPad Prism software version 6.04 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Differences in body weight gain at 28 weeks, tumor latency time and vitamin D serum levels were statistically tested using Kolmogorov-Smirnov test. Differences between LD-inversion and LD animals in food-intake, gene expression and corticosterone serum levels were analyzed using a two-way analysis of variance (ANOVA) followed by Sidak's posttest. Linear regression was performed to analyze changes in total sleep, sleep in light and sleep in dark over time. Subsequently, differences between the slopes for the LD and CRD group were analyzed. CircWave Batch v5.0 software (Roelof Hut, www.euclock.org) was used to analyze circadian rhythmicity of gene expression and serum levels. The ChronoSapiens software was used to analyze behavioral data (Roenneberg et al., 2015).

RESULTS

Classical circadian rhythms

To investigate the potential causal links between chronic CRD and enhanced cancer risk in more detail, we placed breast cancer prone *p53*$^{R270H©/+}$ *WAPCre* conditional mutant mice (further referred to as p53$^{R270H©/+}$ *WAPCre* mice (Wijnhoven et al., 2005) in a 12 hour light, 12 hour dark cycle (LD 12:12). At the end of every week, the light or dark phase was extended to 24 hours to invert the LD cycle. Mice were sacrificed after tumor development (longitudinal study) or around the clock after approximately 18 LD-inversions (cross-sectional study). To monitor the extent to which this protocol affected the circadian steady state, we recorded locomotor activity and core body temperature (CBT) in an additional group of animals. Under

baseline LD schedules, all *p53*$^{R270H/+}$ *WAPCre* animals showed regular daily activity and CBT rhythms (Fig. 1a for CBT; activity data not shown). The temperature maximum is reached approximately at External Time (ExT) 20. After the LD-inversions, the CBT rhythm re-established a stable phase of entrainment after 3-4 days of transients, which appeared to be more gradual after the first inversion (Fig. 1b) compared to inversion-week 18 (Fig. 1c). On day 7 after the 18th LD-inversion, representing steady state, CBT showed a circadian rhythm, but its peak was significantly delayed by 2 hours compared with age-matched control animals ($p = 0.010$).

Long-term health effects

In the longitudinal experiment, as shown in Figure 2a, mice exposed to weekly LD-inversions showed a larger increase in relative body weight compared to the animals kept in a stable LD cycle (RM-ANOVA, group: $p = 0.0319$; time: $p < 0.0001$; interaction: $p < 0.0001$). Although already apparent at week 6, group differences only became significant after week 24 (Sidak's posttest $p < 0.05$). The difference in body weight gain between the groups did not reach significance in the cross-sectional experiment (RM-ANOVA, group: $p = 0.1410$; time: $p < 0.0001$, interaction, $p = 0.4049$), probably due to a shorter experiment time and fewer LD-inversions (Fig. S1a). Differences in the amount of food-intake cannot explain the general weight-gain in the LD-inversion groups, since we even found a small, significant decrease in food intake in these animals compared to the stable LD controls (Fig. S1b).

The latency to mammary gland tumor development was reduced by 17% in the CRD-exposed mice compared to the LD control mice (Fig. 2b, median latency time: 42.6 versus 50.3 weeks, respectively; Kolmogorov-Smirnov $p = 0.0127$). Chronic LD-inversion affected neither the number of tumor-bearing mice nor tumor type (mammary gland tumors or other tumors). In both groups, approximately 80% of the animals developed mammary tumors, including carcinomas and carcinosarcomas (Table S1).

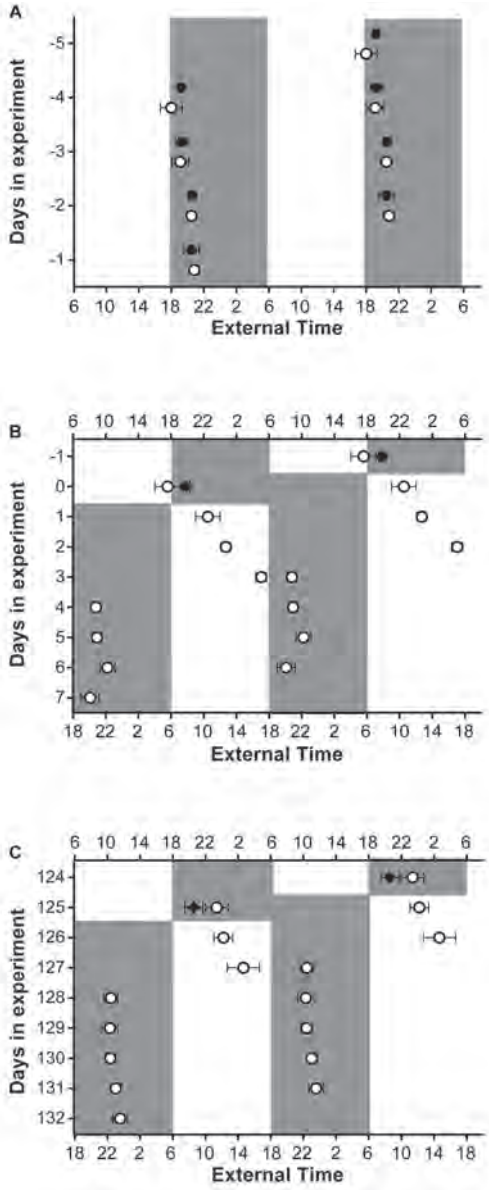

Figure 1. Peak temperature phases under LD and CRD conditions. (A-C) of *p53*$^{R270H/+}$*WAPCre* animals maintained under stable LD 12:12 conditions (closed symbols) or weekly alternating light cycles (open symbols) (n=5 animals per group) (a) before start of the light inversions, (b) at the first LD-inversion and (c) after 18 LD-inversions. Per graph, subsequent days are plotted from top to bottom. Time of day on the x-axis is expressed as External Time (ExT), with ExT 0 corresponding with mid-dark. The upper axis indicates the ExT before the LD-inversion. Values represent the mean ± SEM. Diamonds indicate the average temperature peak times of animals maintained under normal LD conditions. Data are presented as double plots to help visualizing phase shifts (day 0+1, day 1+2, day 2+3 etc. on consecutive lines). Grey areas indicate darkness.

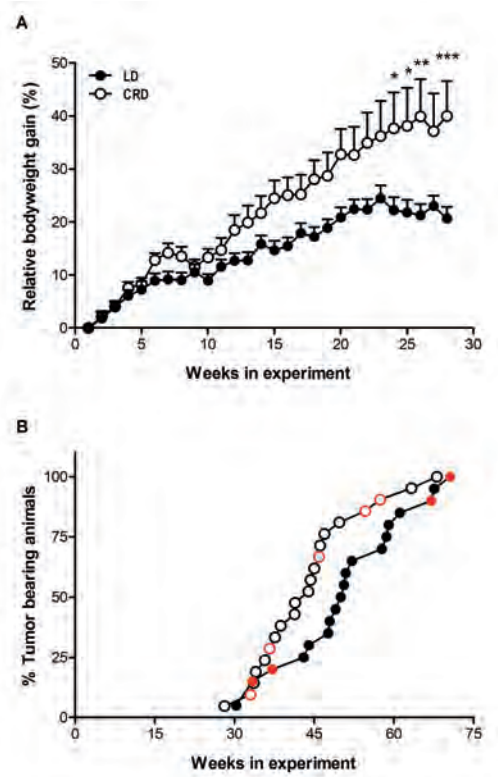

Figure 2. Long-term health effects resulting from CRD exposure. (a) Relative body weight gain of *p53*$^{R270H/+}$*WAPCre* animals exposed to a regular LD cycle (closed symbols, n=20) or weekly alternating LD cycles (open symbols, n=21) in the longitudinal study. Note the significantly stronger weight gain of animals exposed to chronically alternating light cycles compared with animals maintained under a regular LD cycle in the longitudinal study (RM-ANOVA, group: $F(1, 39) = 4.950$, $p=0.0319$; time: $F(27, 1053) = 42.48$, $p<0.0001$; interaction: $F(27, 1053) = 3.738$, $p<0.0001$). Values represent the mean ± SEM. (b) Percentage of mice with palpable tumor in normal LD cycles (n=20; closed symbols) or chronic CRD conditions (n=21; open symbols). Black color indicates mammary gland tumor, whereas red color indicates other tumor types. See Table S1 for pathology data.

Investigating proposed mechanisms of shift work-related carcinogenesis

To gain more insight into these increased health risks resulting from chronically alternating light cycles, we focused on the proposed mechanisms linking shift work to cancer (Fritschi et al., 2011). We analyzed clock (*Per1, Per2, Bmal1)* and clock-controlled (*Dbp, c-Myc*) gene expression in liver and corticosterone serum concentrations (Fig. 3) to identify alterations and desynchronization among organ-specific clocks and/or between central and peripheral clocks. In line with behavior and CBT, *Per1, Per2 and Dbp* hepatic gene expression re-entrained within 7 days in the new LD regime (tested after 18 LD-inversions; CircWave all p-values smaller than

0.05; Two-way ANOVA all p-values larger than 0.05). Only *Bmal1* showed significant interaction between group and time, clearly resulting from increased expression in the CRD group at ExT10. In contrast to the unimodal 24-hr corticosterone rhythm in control animals, we found a significant bimodal (12-hr) rhythm in the CRD mice with a major peak at ExT 14 and a minor peak at ExT 2. We also analyzed the cell cycle control gene *c-Myc*, which was previously suggested to be clock-controlled and to play a role in accelerated tumor growth after chronic jet-lag (Filipski et al., 2005, Fu et al., 2002). Although a slight phase advance appears to be induced by CRD exposure, no significant differences in expression kinetics were observed between the two groups.

We did not find an effect of CRD on 25-hydroxy-vitamin D levels (data not shown). This might be well explained by the fact that laboratory animals are not exposed to sun light, and accordingly are not stimulated to synthesize vitamin D.

To study whether chronically alternating light cycles caused sleep disruption, we used the activity recordings to determine the total amount of predicted sleep. As we estimated sleep based on periods of inactivity, in our study, sleep refers to sleep probability rather than actual sleep. As shown in Figure 4 (left panels), the total amount of sleep showed a significant increase both in CRD mice ($p < 0.0001$; slope = 0.01543) and controls ($p < 0.05$; slope 0.003814) with increasing time in the experiment. However, the increase over time was significantly different ($p < 0.001$) between the two groups. While the controls increased their total sleep over the course of the experiment by 10%, CRD mice slept 50% more. Under LD conditions, the slight increase in sleep results mostly from sleep consolidation during the light phase (upper middle panel). In contrast, CRD-exposed animals increased their sleep in both light and darkness (lower left panels).

As to be expected from the frequent need to re-entrain after LD-inversions (see also Fig. 1), sleep in light was reduced to half of baseline levels after the inversion (day 0; Fig. S2, lower panels) and consequently increased to 150% in the dark phase. However, while sleep in dark returned to approximately baseline after 4 days in the new regime, sleep in light almost increased to 175% of baseline. This imbalance shows that the increase of total sleep shown in Figure 4 is mainly due to overcompensation during the normal sleep times of this nocturnal rodent. LD controls showed normal variations in sleep timing across the week (for separate analyses of the recorded weeks, see Fig. S3a and Fig. S3b).

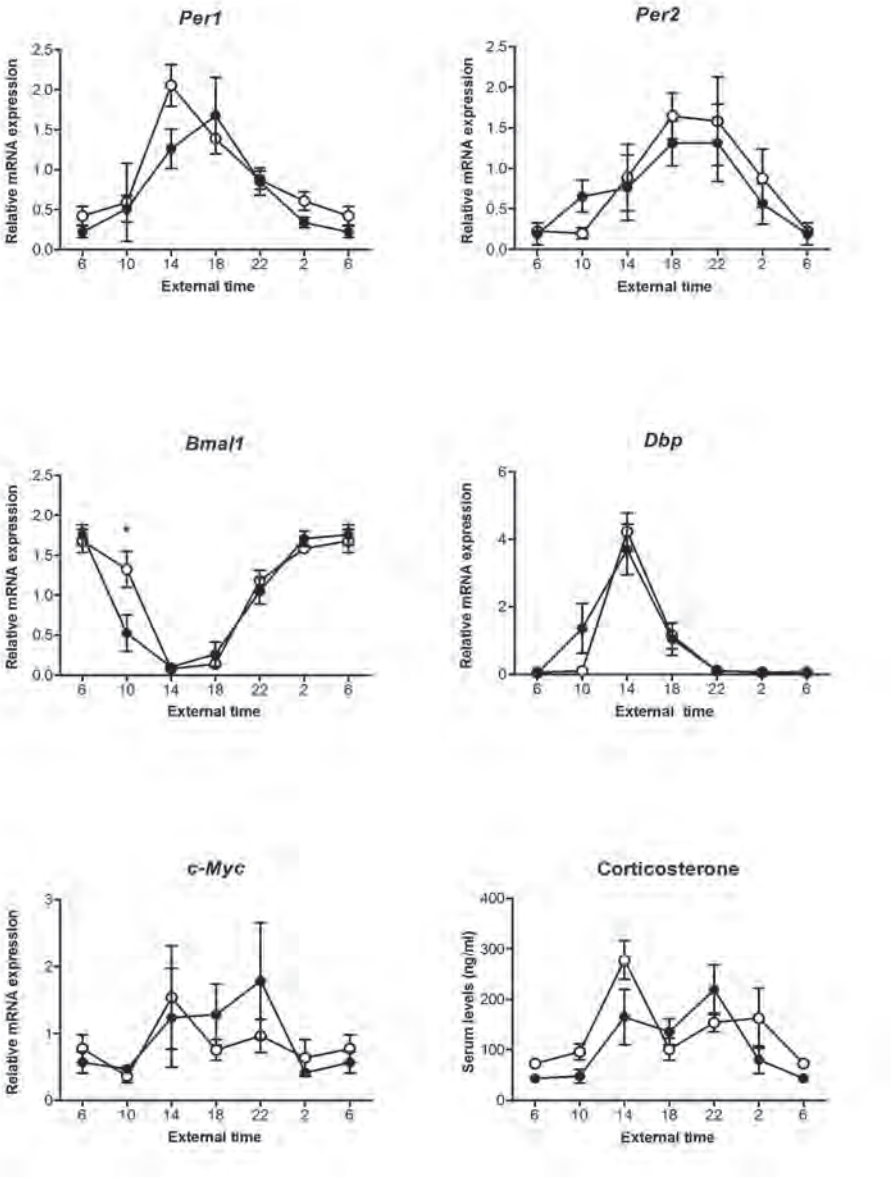

Figure 3. Circadian expression of clock genes and c-Myc and corticosterone serum levels. Closed symbols = LD, open symbols = chronic LD-inversions. Circadian expression of clock genes *Bmal1*, *Per1*, *Per2*, *Dbp* and cell-cycle control gene *c-Myc* in liver and serum corticosterone levels (n= 3 or 4 mice per time point). Chronic LD-inversions did not significantly affect circadian expression of clock genes. Only minor differences were found at an individual time point for *Bmal1* (*Sidak's posttest $p<0.05$). The expression of *c-Myc* appears to show a phase advance in CRD-exposed animals, however no statistical differences were found. Corticosterone levels, however, were affected by the alternating light cycles. In contrast to the circadian rhythmicity in LD animals, corticosterone exhibits a significant 12-hr rhythm after 18 LD-inversions

with a major peak at ExT 14 and a minor peak around ExT 2 ($p<0.05$). Values at "lights on" were double plotted to help visualize circadian patterns. Values represent the mean ± SEM. Time of day on the x-axis is expressed as External Time (ExT), with ExT 0 corresponding with mid-dark.

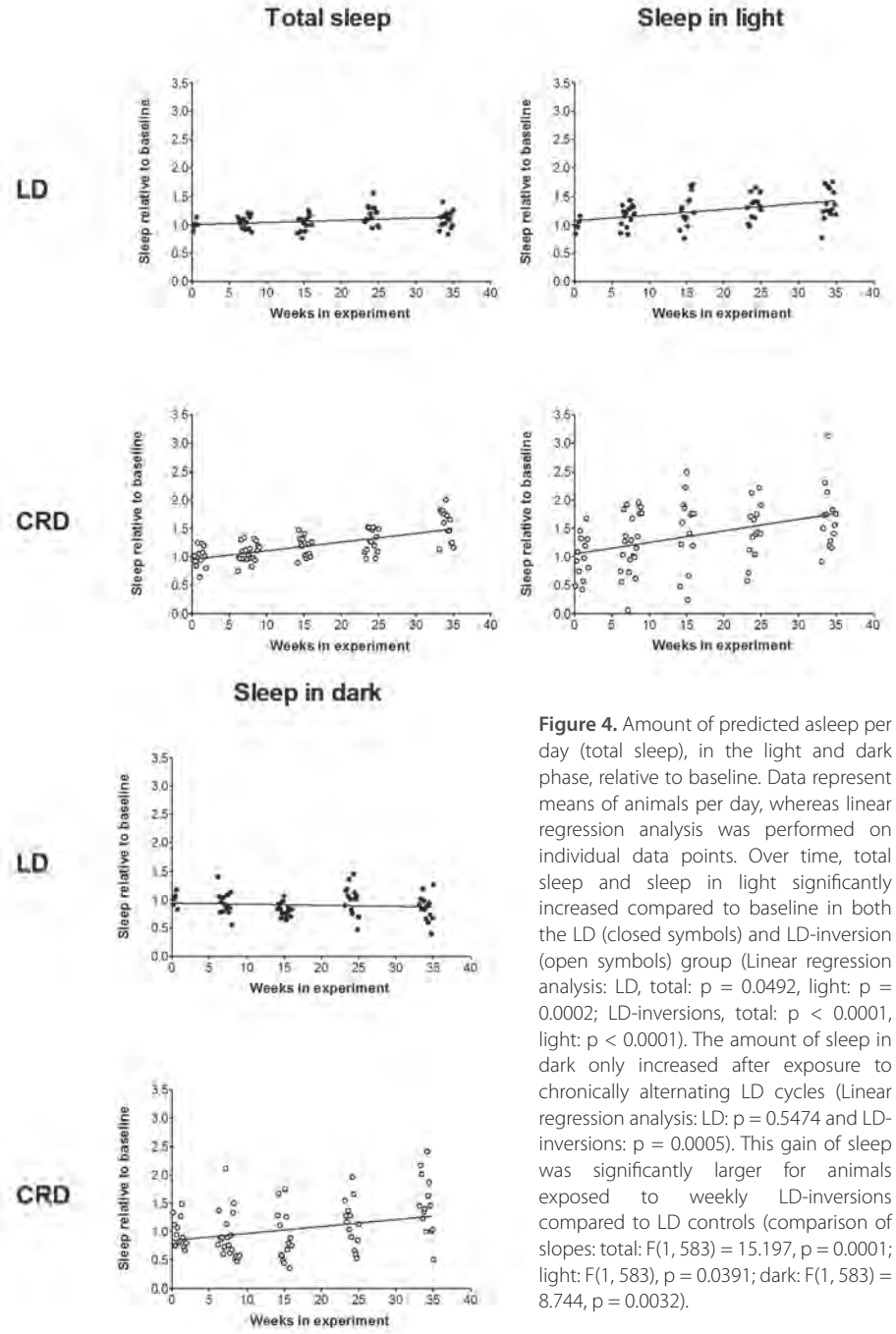

Figure 4. Amount of predicted asleep per day (total sleep), in the light and dark phase, relative to baseline. Data represent means of animals per day, whereas linear regression analysis was performed on individual data points. Over time, total sleep and sleep in light significantly increased compared to baseline in both the LD (closed symbols) and LD-inversion (open symbols) group (Linear regression analysis: LD, total: $p = 0.0492$, light: $p = 0.0002$; LD-inversions, total: $p < 0.0001$, light: $p < 0.0001$). The amount of sleep in dark only increased after exposure to chronically alternating LD cycles (Linear regression analysis: LD: $p = 0.5474$ and LD-inversions: $p = 0.0005$). This gain of sleep was significantly larger for animals exposed to weekly LD-inversions compared to LD controls (comparison of slopes: total: $F(1, 583) = 15.197$, $p = 0.0001$; light: $F(1, 583)$, $p = 0.0391$; dark: $F(1, 583) = 8.744$, $p = 0.0032$).

DISCUSSION

Human field studies only offer limited insights into potential causalities between shift work and cancer due to the complex network of influencing variables, such as different shift work schedules, genetic heterogeneity, or individual shift work history (including the healthy workers phenomenon) – to name only a few (IARC, 2010). The *p53*$^{R270H©/+}$ *WAPCre* mouse model for spontaneous breast cancer development allowed us to study the effects of chronically alternating light cycles on breast cancer initiation and/or progression; thus, this model differs from previous xenograft and chemically induced tumor models that do not reliably recapitulate the human situation (Blask et al., 2003, Cos et al., 2006). To our knowledge, this is the first study that unequivocally shows a link between chronic LD-inversions and breast cancer development. It thereby provides experimental evidence for previous epidemiological data associating shift work and jet-lag with increased breast cancer risk. Epidemiological studies have also indicated an association between social jet-lag, shift work and body weight, although conflicting data exist (Kubo et al., 2011, Nabe-Nielsen et al., 2011, Roenneberg et al., 2012, Parsons et al., 2014). Various mouse studies have shown that circadian disturbance induced by continuous light, forced activity or a disrupted LD cycle causes an increase in body weight (Coomans et al., 2013, Oishi, 2009, Salgado-Delgado et al., 2008). Our study, enforcing circadian strain by using changes in the environmental LD cycle supports the relationship between CRD and weight-gain. The finding that CRD-exposed animals sleep more than controls, logically correlates with less activity and could thus contribute to a stronger weight gain. But to what extent are the results presented here for a causal link between CRD and increased breast cancer risk in a nocturnal rodent also relevant to human shift work?

Shift work involves many aspects that could be involved in the causal mechanisms that lead to increased health risks: internal desynchronization, melatonin suppression due to light at night, sleep disruption, lifestyle disturbances (such as smoking, [lack of] breastfeeding, unhealthy diet, altered timing of food, etc.), and decreased vitamin D levels due to lack of sunlight exposure (Fritschi et al., 2011). The current study provides further insight into the relevance of these separate aspects (for an overview: see Fig. S4). Melatonin suppression, decreased vitamin D levels nor lifestyle disturbances (absent in mice) cannot account for the enhanced cancer risk in animals kept under LD-inversion conditions: (i) p53$^{R270H©/+}$ *WAPCre* mice (FVB background) are melatonin-deficient; (ii) neither CRD mice nor controls

were exposed to sunlight, and we found no difference in 25-hydroxy-vitamin D between groups. This is consistent with a study in night shift workers which found no difference in vitamin D levels between fixed daytime workers, rotating shift workers without night shift and rotating shift workers with night shift (Itoh et al., 2011). (iii) Lifestyle factors did not differ (amount of food intake) in the LD-inversion mice and the controls or were absent (e.g. breast feeding, smoking). Given the combination of decreased breast tumor latency time and increased body weight after chronic LD-inversions, it is tempting to speculate on the role of timing of food intake. Potentially, changed timing of food intake disrupts metabolic processes, resulting in adverse health effects. Previous studies have shown that timed feeding can indeed (partially) rescue tumor and obese phenotypes (Filipski et al., 2005, Fonken et al., 2010).

Desynchrony between the sleep-wake cycle and endogenous circadian rhythmicity was recently shown to disrupt the circadian regulation of the human transcriptome (Archer et al., 2014a) and suggested to be an important factor underlying shift work-mediated breast cancer risk (Fritschi et al., 2013). In the present study, 7 days after the last LD-inversion, we found a disturbance of the circadian rhythmicity of corticosterone levels. It should be noted that blood samples were taken under ketamine/xylazine anesthesia, known to slightly increase corticosterone levels (Arnold and Langhans, 2010). However, as mice in the control and LD-inversion group were treated in similar manner, anesthesia is unlikely to explain the disturbed corticosterone rhythm in LD-inversed mice. In contrast to the disturbed corticosterone rhythm, on day 7 after the last inversion, we did not observe alterations in the daily patterns of hepatic clock gene expression, indicating that the liver clock reentrained within one week. However, disturbance of circadian rhythms at earlier time points is inherent of the re-entrainment to the new LD cycle. Minor differences in *c-myc* expression could be indicative of larger differences at earlier time points. This would be in line with other studies in which phase advances induce *c-myc* expression directly and three days after exposure (Filipski et al., 2005, Iwamoto et al., 2014). Previous studies have shown effects on both corticosterone rhythm and liver clock gene expression by other circadian disturbance protocols (Filipski et al., 2004, Barclay et al., 2012). Corticosterone has been shown to be involved in the entrainment and resynchronization of locomotor activity and peripheral clocks (Kiessling et al., 2010, Sage et al., 2004, Sujino et al., 2012). Liver clock gene expression is more sensitive to input of feeding

for entrainment (Sujino et al., 2012), which might explain the discrepancy and thus desynchrony between the disrupted corticosterone rhythm and reentrained clock gene expression in liver.

Sleep duration, sleep timing and sleep quality are central elements of human shift work studies, both as outcome variables (in the form of descriptives of shift work-related strain (Akerstedt, 2003) or post-intervention measures, (Neil et al., 2014, Vetter et al., 2015)) and as a proposed countermeasure (e.g., schedule-specific sleep strategies (Petrov et al., 2014)). As long-term sleep analysis in mice is challenging, sleep was estimated based on inactivity, and accordingly refers to sleep probability rather than actual sleep. However, there is a good consensus in the literature that extended bouts of inactivity highly correlate with actual electroencephalogram (EEG) and/or polysomnogram (PSG) detected sleep. In the *Drosophila* literature, 5-minute bouts of inactivity are considered sleep (Shaw et al., 2000) and experiments in mice have shown that inactivity periods of 40 sec highly correlate with EEG-scored sleep (Pack et al., 2007). Similarly high correlations are reported for video-recorded immobility and EEG-scored sleep (Fisher et al., 2012). Since the sleep analysis conducted here is based on relative immobility within 10-min bins, we likely underestimate rather than overestimate the duration and frequency of sleep episodes. Despite the potential risk of mistaking immobility in a wake mouse for sleep, we consider our sleep-assessments as a good correlate for actual sleep duration.

In human shift workers, sleep timing constantly changes, which results in shorter sleep (and reduced sleep quality). In contrast, we observed that mice exposed to chronic LD-inversions – not accompanied by forced sleep deprivation due to work – sleep more. The finding that CRD-mice appear to sleep more than control animals seems to contradict findings in shift-workers, who usually sleep less than day-workers (Juda et al., 2013a). However, unlike real shift workers, the CRD protocol in our animal study only involved changes in the LD cycle without the additional sleep restrictions enforced by work-shifts impinging on usual rest times. Assuming that the correlation between inactivity and sleep in mice is not influenced by the exposure to chronically alternating light cycles, we propose that the increase in amount of sleep observed upon CRD represents compensation for the constant perturbations of circadian timing and sleep. Future studies, addressing sleep and wakefulness by EEG rather than sleep probability, should provide a definite answer

to what extend sleep time and quality are affected upon chronically alternating light cycles.

The search for the underlying mechanisms that link shift work and health detriments (including increased cancer prevalence) is difficult in human shift work studies due to the network of potentially mediating factors. Controlled laboratory experiments, though performed in a nocturnal mouse model, allow the identification, isolation and quantification of individual contributors. The present study provides additional evidence for the role of internal desynchronization and sleep disruption in the etiology of CRD-associated health risks and pathologies (e.g., obesity and cancer; see Fig. S4). Female $p53^{R270H©/+}$ *WAPCre* conditional mutant mice are ideal for investigating this etiology for breast cancer risk in depth. Furthermore, the highly controlled experimental conditions allow us to identify molecular biomarkers for CRD or other (shift work-related) exposures. Despite being based on a nocturnal mouse model, our results strongly suggest that individuals with hereditary (breast) cancer predispositions should not be exposed to frequently changing Zeitgebers as they exist for example in shift work or trans-meridian aviation. Due to the growing 24/7 economy, shift work will become increasingly part of our society and will, therefore, increasingly affect public health outcomes. Our experimental setup provides a unique tool for exploring underlying mechanism as well as devising countermeasures.

CHAPTER 3

BIOMARKER DISCOVERY USING A COMPARATIVE OMICS APPROACH IN A MOUSE MODEL DEVELOPING HETEROGENEOUS MAMMARY CANCER SUBTYPES

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ABSTRACT

Identification of biomarkers for early breast cancer detection in blood is a challenging task, since breast cancer is a heterogeneous disease with a wide range of tumor subtypes. This is envisioned to result in differences in serum protein levels. The p53R270H/+WAPCre mouse model is unique in that these mice spontaneously develop both ER– and ER+ tumors, in proportions comparable to humans. Therefore, these mice provide a well-suited model system to identify human relevant biomarkers for early breast cancer detection that are additionally specific for different tumor subtypes. Mammary gland tumors were obtained from p53R270H/+WAPCre mice and cellular origin, ER, and HER2 status were characterized. We compared gene expression profiles for tumors with different characteristics versus control tissue, and determined genes differentially expressed across tumor subtypes. By using literature data (Gene Ontology, UniProt, and Human Plasma Proteome), we further identified protein candidate biomarkers for blood-based detection of breast cancer. Functional overrepresentation analysis (using Gene Ontology, MSigDB, BioGPS, Cancer GeneSigDB and proteomics literature data) showed enrichment for several processes relevant for human breast cancer. Finally, Human Protein Atlas data were used to obtain a prioritized list of 16 potential biomarkers that should facilitate further studies on blood-based breast cancer detection in humans.

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer and leading cause of cancer death among women worldwide (Ferlay et al., 2010). The most efficient way to reduce cancer mortality and morbidity is detection at an early stage, allowing effective therapeutic intervention. One way to improve cancer detection could be by means of a non-invasive test based on blood biomarkers. Preferably, these serum markers detect breast cancer across multiple tumor types regarding histopathology or diagnostic marker status, in an early stage of the disease. Currently, CA15-3, CA27-29 and CEA are clinically used breast cancer serum biomarkers for postoperative surveillance in patients with no evidence of disease and monitoring therapy in patients with advanced breast cancer rather than early detection (Sturgeon et al., 2008, Ludwig and Weinstein, 2005). Additionally, the use of HER2/neu/ERBB2 (in shed form) in serum for disease monitoring in patients without elevation of other tumor markers and for monitoring of Trastuzumab treatment is under evaluation (Sturgeon et al., 2008), and CA125 has also been suggested as tumor marker for advanced breast cancer (Norum et al., 2001). However, these clinical serum biomarkers are only used for disease monitoring and prognosis, and there are currently no FDA approved biomarkers for *early* detection of breast cancer. Therefore, much international effort is put into identification and validation of novel serum biomarkers for early breast cancer diagnosis (EDRN, Kretschmer et al., 2011, Böhm et al., 2011, Opstal-van Winden et al., 2011).

Breast cancer is a heterogeneous disease, with a large variety of tumor subtypes (Sorlie et al., 2001). Due to this heterogeneity, the identification and use of a single biomarker for breast cancer detection is challenging. Here, we focus on the identification of a panel of biomarkers which allows for detection with high specificity and sensitivity across various kinds of breast cancer tumor types to maximize screening applicability. In addition to a biomarker panel for all tumor types, it would be beneficial to be able to distinguish between tumor subtypes, as the prognosis and therapeutic approach is different for each subtype. Such additional information on prognosis and therapy could lead to better follow-up and referral of newly diagnosed patients. Relevant information in this respect would be e.g. estrogen receptor alpha (ER) and HER2/neu status as well as histopathological classification.

A growing body of evidence demonstrate that mouse models for cancer (summarized in (Kelly-Spratt et al., 2008)) show common molecular, biological and clinical features compared to human cancers and as such these models are considered a promising tool for identification of cancer biomarkers (Kelly-Spratt et al., 2008). The human relevance of this approach is shown in a mouse model for breast cancer where the overlap of the plasma proteome is compared to proteomics studies of human breast cancer cell lines (Pitteri et al., 2008). However, mouse models used thus far for breast cancer biomarker identification (Whiteaker et al., 2007, Pitteri et al., 2008, Schoenherr et al., 2011, Whiteaker et al., 2011) exclusively develop ER negative mammary gland tumors, while estrogen is thought to play an important role in breast tumors development (Nandi et al., 1995). Moreover, the majority of human breast tumors are ER positive. In our study, we use an innovative mouse model developed previously in our laboratory (Wijnhoven et al., 2005) which spontaneously develops ER positive and negative tumors, resembling the human situation. This $p53^{R270H/+}$WAPCre mouse model develops tumors of two different histopathological types: carcinomas and carcinasarcomas. Carcinoma is the most common tumor type found in women and therefore most relevant in this study. Although carcinosarcomas are rare in humans, this subclass shows a poor prognosis and early detection is highly necessary, since 5-year overall survival reaches zero if detected at later stages (Gutman et al., 1995). In addition to the estrogen receptor status, and the two histological subtypes, we were also able to determine different HER2/neu status based on gene expression data. HER2/neu amplification in human breast cancers is associated with lower 5-year survival (Sotiriou and Pusztai, 2009). However, a positive HER2/neu status makes women eligible for trastuzumab treatment which improves survival significantly (Gianni et al., 2011). As such, HER2/neu based differentiation is highly important.

In this study, we use a genomics approach on this mouse model mimicking heterogeneous breast tumor development in humans, to identify candidate protein biomarkers relevant for early blood-based detection of human breast cancer. First, we compare different types of tumor and control tissue from the $p53^{R270H/+}$WAPCre mouse model to determine genes which are informative *across* multiple tumor subtypes versus control, or which are able to distinguish *between* tumor subtypes. In the next step, we determine which of these mouse genes have a human equivalent that encodes for a protein potentially detectable in human serum. Finally, we compare the obtained lists with relevant information in other

databases to functionally characterize and prioritize the proteins for follow-up studies.

MATERIALS AND METHODS

Animal experiment

This study was approved by the Animal Experimentation Ethical Committee of our institute. Animal handling in this study was carried out in accordance with relevant Dutch national legislation, including the 1997 Dutch Act on Animal Experimentation.

Generation of p53R270H/+WAPCre mice and the phenotypic characteristics have been described previously (Wijnhoven et al., 2005). Heterozygous p53R270H and WAPCre mice, originally in a hybrid background (Wijnhoven et al., 2005), were crossed to FVB mice to generation F6. Female p53R270H/+WAPCre mice were generated by crossing female heterozygous p53R270H mice with male heterozygous WAPCre mice. All mice were weighed weekly and checked for the development of tumors by palpation. Mice were sacrificed immediately after detection of a palpable tumor (62±12 weeks of age), since our analysis was aimed at the identification of biomarkers for early detection of breast cancer. All tumors were dissected and weighed, the tumor size ranged from 52 to 988 mg with a median tumor weight of 328 mg. Control mammary glands were isolated from the same animals at the opposite site of the tumor. Tumors were processed for histopathology following standard procedures. For comparison with control tissue from a wild type strain, female FVB/N mice were sacrificed at 55 weeks of age and mammary tissue was collected.

Histology and immunohistochemistry

Paraffin-embedded, formalin fixed tumor sections were stained with H&E for histopathological evaluation. ER expression was analyzed using the rabbit polyclonal antibody MC-20 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA). In this study we found that 74 percent of the mammary tumors showed ER positivity, which is comparable to earlier studies with this mouse model and the human situation (Wijnhoven et al., 2005).

Erbb2/HER2/neu status

In clinical diagnosis, mRNA expression levels have been shown to be a reliable method for the determination of HER2/neu status of breast tumors (Capizzi et al., 2008). Therefore, we used our microarray expression data for Erbb2 (the murine form of HER2) to determine the HER2/neu status of the mammary gland tumors in our study. We used a cutoff value of mean log transformed expression of normal mammary gland tissue plus 3 standard deviations (Capizzi et al., 2008) to define which tumors were HER2/neu positive. In our data, this corresponded to a three-fold up-regulation of Erbb2 compared to control tissue. Using this cutoff, we found that 21 percent of the mammary tumors were HER2/neu positive, which is close to the average reported value of 26% in human breast cancer (Revillion et al., 1998). Please note that we will use the phrase HER2 positive/negative throughput the paper to relate murine Erbb2 gene expression to the terms used in human clinical diagnostics.

Microarray analysis

RNA was extracted from control mammary glands and mammary tumors using the miRNeasy Mini Kit (Qiagen Benelux, Venlo, The Netherlands). RNA concentrations were measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The integrity of the RNA samples was determined with the BioAnalyzer (Agilent Technologies, Amstelveen, The Netherlands) using the RNA nano 6000 kit (Agilent Technologies). RNA was further processed for hybridization to Affymetrix HT Mouse Genome 430 PM Array Plates at the Microarray Department of the University of Amsterdam, the Netherlands. RNA amplification, labeling and genechip hybridization, washing and scanning were carried out according to Affymetrix protocols.

Altogether, 30 samples were used for further analysis: 6 control FVB, 5 control $p53^{R270H/+}$WAPCre (Control TG), 9 carcinomas (Car) ER+, 2 carcinomas ER–, 5 carcinosarcomas (Csc) ER+, and 3 carcinosarcomas ER–. Of the 9 ER+ carcinoma samples, 4 were HER2/Neu positive; all other samples were HER2/neu negative.

Data analysis

Affymetrix CEL files were normalized using the Robust Multichip Average (RMA) algorithm and the MBNI custom CDF (http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/) for this chip. RMA output consisted of 16492 probe sets corresponding to unique Entrez GeneIDs. Quality control

consisted of visual inspection of residual plots and other RMA QC parameters. Further data analysis such as ANOVA and Principal Component Analysis (PCA) was carried out using R software unless indicated otherwise. Gene expression data between (two or more) experimental groups were compared using ANOVA, where p values < 0.001 and False Discovery Rate (FDR) < 5% were considered significant. Additionally, for tumor vs. control comparisons, only genes up-regulated in the tumor group(s) were considered in further analyses, as the aim of this study was to detect tumor-originating markers for early cancer detection in serum-based diagnostics. By making further Boolean combinations of regulated gene lists, we obtained genes informative *across* or *between* tumor subtypes.

To determine which genes code for proteins potentially detectable in human serum, we determined which proteins have a human equivalent that is annotated in Gene Ontology as extracellular, in UniProt as secreted, or has been experimentally detected in plasma or serum as part of the Human Plasma Proteome project (Anderson et al., 2004).

Functional annotation and overrepresentation analysis was carried out using DAVID (http://david.abcc.ncifcrf.gov) (Huang et al., 2009) for generally known databases such as Gene Ontology and UniProt. Further gene set overrepresentation analyses were performed using an in-house developed tool (based on the DAVID methodology) for other custom functional gene set libraries based on BioGPS tissue expression data (www.biogps.org, (Wu et al., 2009)), MSigDB-C2 gene sets curated from genomics literature (www.broadinstitute.org/gsea/msigdb/, (Liberzon et al., 2011)), MSigDB-C4 cancer gene expression modules (Segal et al., 2004) the cancer signatures GeneSigDB (http://compbio.dfci.harvard.edu/genesigdb/, (Culhane et al., 2010)), and an in-house made collection of protein lists from mouse blood-based proteomics literature (Pitteri et al., 2008, Whiteaker et al., 2011, Whiteaker et al., 2007, Schoenherr et al., 2011).

Protein expression in human breast cancer and normal mammary tissue was compared using the Human Protein Atlas (www.proteinatlas.org) (Ponten et al., 2008). This site contains semi-quantitative (absent – weak – moderate – strong) data. We considered a protein up-regulated in human tissue if at least 50% of the tumor samples in the database displayed an expression level at least one level higher than that of normal mammary gland.

A visual summary of the workflow used in the data analysis is given in Figure 1.

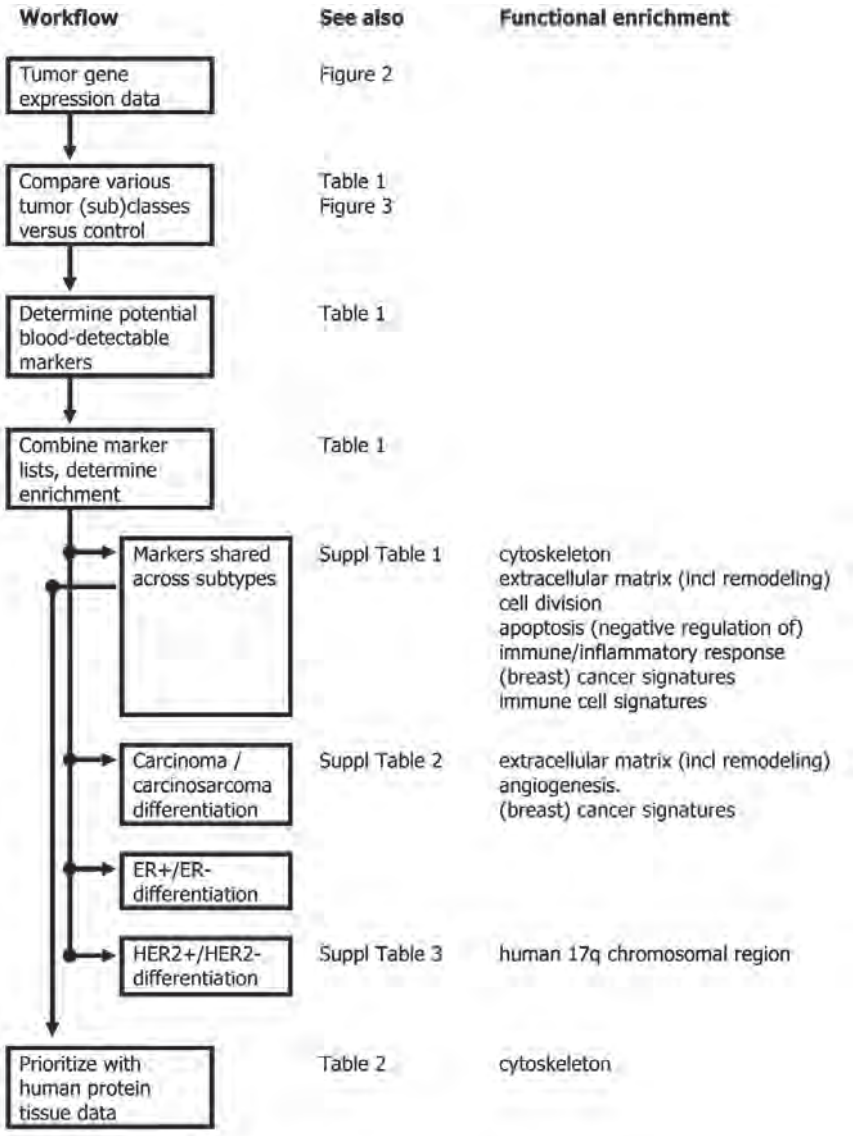

Figure 1. Summary of the data analysis workflow, with cross-references to corresponding table and figures, and a summary of the functional enrichment analysis results.

RESULTS

Principal Component Analysis

Prior to statistical analysis, whole genome transcription profiles were visualized in a PCA (Figure 2). This revealed that, on the whole, tumor samples are clearly distinct from control samples. Additionally, the PCA indicated that several tumor subclasses with regards to histology and receptor status could already be distinguished (e.g. carcinoma HER+, carcinoma ER–, carcinosarcoma ER+ versus ER–), whereas the two control groups appeared highly similar. This latter finding was confirmed by statistical analysis: there are no genes with significant expression changes between the two mammary tissue controls groups (Table 1) and therefore these groups were combined in further analyses.

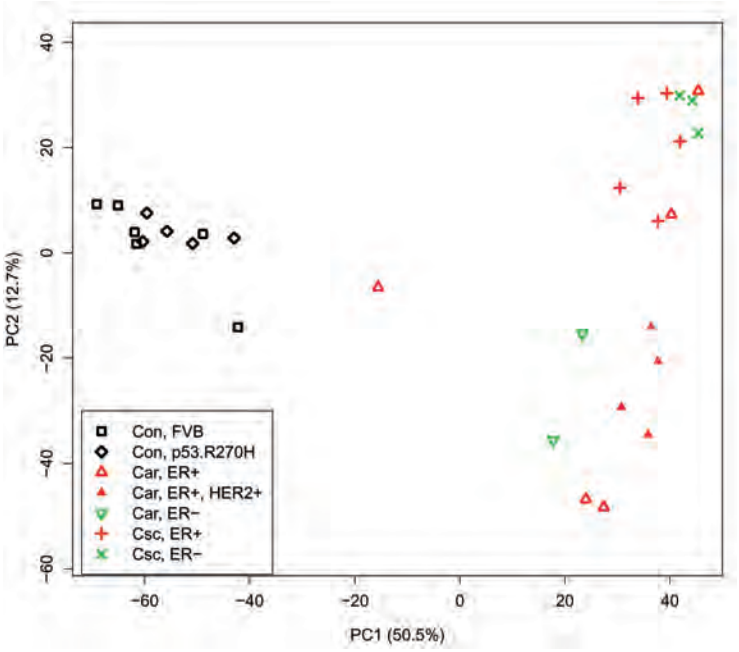

Figure 2. Principal Component Analysis on tumor and control tissue gene expression data.

Similarities in genes expression changes across tumor subtypes

We compared gene expression changes between controls and tumor samples for either all tumors or by subset based on histopathology, ER, or HER2 status (Table 1, Figure 3). Since this study was set up to specifically identify tumor-originating markers for cancer detection by means of a blood-based test, we not

only considered statistical significance ($p < 0.001$ and FDR 5%), but also set the demand that genes are up-regulated in tumor vs. control. After calculating the number of significantly up-regulated genes in tumor groups, we also determined how many of these have a human equivalent that is potentially blood-detectable, again with eventual screening implementation in mind. Numbers of genes meeting these criteria are given in Table 1. For tumor (subtype) versus control comparisons, we found between 2185 and 3471 up-regulated genes, of which 238 to 458 (on average 11%) were potentially detectable in human blood. The fold ratio for these up-regulated genes ranged from 1.21 to 39.71 (median 1.85), for the potentially blood-detectable markers the fold up-regulation ranged from 1.28 to 39.71 (median 2.21).

Table 1. Statistical comparisons[a] and further combinations

Query	Comparison	Regulated genes	Of which human blood detectable
A	Control FVB <> Control TG	0	0
B	all tumor > all control	2850	313
C	carcinoma > all control	2330	238
D	carcinosarcoma > all control	3471	458
E	ER+ > all control	2768	309
F	ER– > all control	2185	247
G	HER2+ > all control	2768	307
H	HER2– > all control	2749	313
J	carcinoma <> carcinosarcoma	350	76
K	ER+ <> ER–	0	0
L	HER2+ <> HER2–	178	24

Combination	Description	Regulated genes	Of which human blood detectable
C AND D	carcinoma and carcinosarcoma vs control	1942	200
(C OR D) AND J	carcinoma/carcinosarcoma differentiation	197	51[b]
C AND J	specific for carcinoma	53	11
D AND J	specific for carcinosarcoma	171	48
(C AND D) AND J	applicable for both	27	8
E AND F	ER+ and ER– vs control	1815	202
(E OR F) AND K	ER+/ER– differentiation	0	0
G AND H	HER2+ and HER2– vs control	1887	203
(G OR H) AND L	HER2+/HER2– differentiation	117	15[c]
G AND L	specific for HER2+	109	14
H AND L	specific for HER2–	25	2
(G AND H) AND L	applicable for both	17	1
ALL (B,C,D,E,F,G,H)	Shared across all subtypes	1395	149[d]

[a] based on $p < 0.001$ and FDR < 5%
[b] Supplementary Table 2
[c] Supplementary Table 3
[d] Supplementary Table 1

Next, we determined the overlap between the various gene lists. We noticed that for each of these lists, roughly half (37% - 74%) was present in an overlap with other sets (Table 1, Figure 3). This indicated that there is a common set of genes that discriminates the various tumor groups from control tissue, but not the sub-tumor types by themselves. The most comprehensive overlap across all of the comparisons resulted in 1395 regulated genes of which 149 met the criteria for protein blood detectability. These 149 genes are listed in Supplementary Table 1. Fold up-regulations ranged from 1.30 to 16.80 (median 2.13) for the set of 1395 genes, and from 1.39 to 16.34 (median 2.36) for the 149 potentially blood-detectable genes. As for the single tumor (subtype) versus control comparisons, there is a trend in that the potentially blood-detectable subset shows larger fold up-regulations.

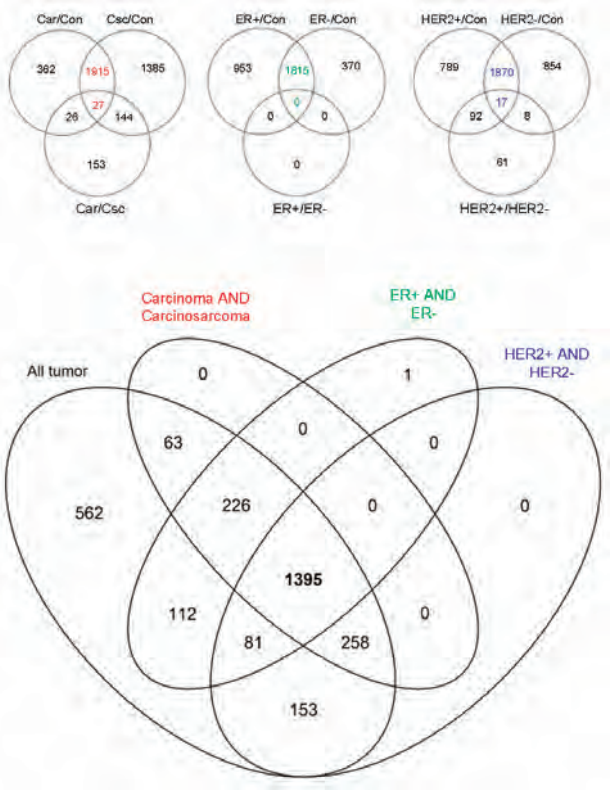

Figure 3. Venn diagram for the various comparisons made. Overlap of discriminative genes based on histological classification (red), ER status (green), HER2 status (blue), and across all tumor subtypes (bold).

GO functional annotation analysis on this list revealed enrichment for terms related to the cytoskeleton, extracellular matrix (including remodeling), cell division, (negative regulation of) apoptosis, and immune/inflammatory response (Figure 1).

Enrichment analysis on the MSigDB-C2 database showed mainly significance (FDR 5%) for gene sets related to cancer, including several breast cancer sets (Poola et al., 2005, Schuetz et al., 2006, Sotiriou et al., 2006), with other significant gene sets being mainly involved in stress and immune responses. Enriched MSigDB-C4 cancer modules were related to cytoskeleton, extracellular matrix, cell division, and inflammatory response. The BioGPS-derived tissue-associated gene set library did not reveal a match between normal mammary tissue and the shared 149 marker set, but instead showed enrichment for predominantly immune set-related signatures, perhaps reflecting the endogenous response to the tumor (Figure 1). Comparison to literature mouse plasma breast cancer proteomics sets revealed one significant enrichment, namely to Table S6g of Whiteaker et al (Whiteaker et al., 2011) which describes their verification of plasma biomarkers on individual mice. This overlap comprised six genes (*Hsp90b1, Hspa5, P4hb, Pfn1, Tnc, Ywhaz*). Finally, the cancer GeneSigDB was used to determine if there was significant overlap to cancer literature gene sets. The analysis showed significant enrichment for 171 of the 2142 cancer signatures. These signatures were derived from several tissues, although the fraction of significant breast cancer signatures (63 enriched out of 476 breast cancer sets) was relatively highest, being 1.7 times that for the overall (171 / 2142) significant fraction. The most significant breast cancer gene set was that by Lauss et al (Lauss et al., 2008) containing consensus genes from literature studies to predict breast cancer recurrence. Next, we determined which genes are most characteristic for the 63 enriched breast cancer signatures versus the rest of the database. We found that this enrichment could mainly be ascribed to genes involved in cell division. Overall, the high match to literature gene sets suggests our marker set has considerable resemblance to marker sets found in (predominantly) human breast cancer.

Differences between tumor subclasses

Besides biomarkers common to all tumor subclasses, we also set out to find biomarkers that might help to discriminate between various tumor subclasses. Indeed, the PCA in Figure 2 indicated this would be possible. For the carcinoma vs. carcinosarcoma comparison we found 350 differentially expressed genes, 76 of which are potentially human blood-detectable (Table 1, Figure 3). Since latter implementation would only be practical if a biomarker was increased in either of the two subclasses when compared to a control, we determined the overlap of the carcinoma vs. carcinosarcoma genes with the sets of genes up-regulated in

carcinoma and/or carcinosarcoma to control. This led to 51 genes, which are listed in Supplementary Table 2. As can be seen from this table, 48 of these genes show higher expression in carcinosarcoma than in carcinoma. Additionally, GO functional enrichment analysis showed enrichment for extracellular matrix, extracellular matrix remodeling, and angiogenesis. These findings are in line with the more aggressive clinical phenotype of this subtype. Of the 51 genes in Supplementary Table 2, 8 overlap with the set of 149 shared genes (Supplementary Table 1), namely *Actn1*, *Inhba*, *Msr1*, *Ncam1*, *Plau*, *Pls3*, *Tpm4*, and *Wisp1*. Enrichment analysis on C2, C4 and BioGPS datasets found significance for (breast) cancer, extracellular matrix (remodeling), angiogenesis, and immune response gene sets (Figure 1). No significant overlap with literature mouse plasma cancer proteomics sets was found. In the GeneSigDB sets there was enrichment for 79 sets, 26 of which were breast cancer related. Here the breast cancer-specific enrichment was mainly due to extracellular matrix (remodeling) associated genes.

When the analogous comparison was made for ER+ and ER– tumors vs. each other and the control, no differentially expressed genes were found. Apparently, following the genomics strategy described here, no potential biomarkers are identified to discriminate between ER+ and ER– breast cancers in human blood.

For the HER2 status, 178 genes were differentially expressed between HER2+ and HER2– tumors, 117 of which were up-regulated in either subtype to control (Table 1, Figure 3). Of these, 15 were potentially blood-detectable (Supplementary Table 3). Of these, 14 were higher expressed in HER2+ tumors, including, as could be expected, the Erbb2 gene itself. Mutual comparisons showed no overlap between this list and the list of 149 markers shared across tumor subtypes versus control, nor the 51 genes discriminating carcinoma from carcinosarcoma. No GO enrichment was found for these 15 genes. Additional enrichment analyses only showed enrichment for one gene set across all five databases, namely the C2 entry for an amplification hot spot in the human chromosome 17q11.1-q21; 17q25 region described by Myllykangas et al (Myllykangas et al., 2006), which indeed contains the (human homolog of the) *Erbb2* gene (Figure 1).

Further prioritization of potential biomarkers

To translate our results obtained using a mouse model to the human situation and prioritize the markers for further human follow-up studies, we used the data

available on the Human Protein Atlas to compare protein expression in human breast cancer tissue and normal mammary gland for the 149 shared genes in Supplementary Table 1. For the 116 proteins where such human information was available, 16 showed an increased expression in human cancer tissue compared to normal mammary gland. These 16 proteins are given in Table 2. Their gene expression fold up-regulations ranged from 1.81 to 7.28 (median 2.13) with standard deviation values (see also Supplementary Table 1) being approximately a third of the fold up-regulation (median relative SD 36%). A relatively high standard deviation (5.62, 77% relative SD) was found for VCAN. Functionally, the majority of these 16 proteins are associated with the cytoskeleton.

Table 2. Prioritized shared serum markers, based on human tissue expression data

Human GeneID	Human symbol	Description	Average ratio Tumor/Control[a]	Human protein staining: median breast cancer versus normal breast tissue[b]
60	ACTB	actin, beta	2.00	moderate vs weak
79879	CCDC134	coiled-coil domain containing 134	1.81	moderate vs weak
1072	CFL1	cofilin 1 (non-muscle)	2.45	moderate vs weak
2146	EZH2	enhancer of zeste homolog 2 (Drosophila)	2.92	moderate vs negative
2316	FLNA	filamin A, alpha	2.10	weak vs negative
10905	MAN1A2	mannosidase, alpha, class 1A, member 2	2.33	strong vs moderate
4627	MYH9	myosin, heavy chain 9, non-muscle	2.09	moderate vs weak
4830	NME1	non-metastatic cells 1, protein (NM23A) expressed in	2.27	strong vs moderate
11333	PDAP1	PDGFA associated protein 1	2.72	moderate vs negative
29108	PYCARD	PYD and CARD domain containing	2.17	strong vs moderate
26064	RAI14	retinoic acid induced 14	3.77	weak vs negative
54910	SEMA4C	semaphorin 4C	2.40	weak vs negative
7168	TPM1	tropomyosin 1 (alpha)	2.59	weak vs negative
51592	TRIM33	tripartite motif containing 33	2.12	moderate vs weak
1462	VCAN	versican	7.28	weak vs negative
7511	XPNPEP1	X-prolyl aminopeptidase (aminopeptidase P) 1, soluble	2.01	weak vs negative

[a] additional values for tumor subclass ratio averages and standard deviations can be found in Supplementary Table 1
[b] retrieved from Human Protein Atlas (www.proteinatlas.org)

DISCUSSION

Breast cancer is the leading cause of cancer death worldwide (Ferlay et al., 2010). Early diagnosis of cancer is necessary for improvement of survival and reduction of morbidity. Therefore, there is a need for sensitive testing strategies specifically for early detection. For improvement of current early detection rates, blood-based diagnostics could be a valuable addition to (or next to) existing screening programs, because of their low costs and flexible logistics. The first step towards implementing such blood-based detection is the identification of biomarkers that detect across multiple tumor types, to increase overall sensitivity. A secondary step would be the identification of biomarkers that are able to differentiate between tumor subtypes to improve follow-up and referral of newly diagnosed patients, i.e. a more personalized approach. In the study described here, we use a genomics approach on tumor tissue compared to normal mammary gland to identify protein candidate biomarkers relevant for blood-based early detection of human breast cancer. Interestingly, we hereby differentiated the mammary tumors with regards to histological subtype and receptor status, to reflect human variation in these tumor characteristics. Moreover, we used an experimental set up specifically aimed at the early detection of cancer, rather than a set up with more progressed cancers.

Mouse models are frequently applied in biomarker discovery research (Kelly-Spratt et al., 2008). Earlier studies have shown that although both human and mouse breast tumors have a heterogeneous profile, similarities can be found and therefore, mouse models are useful in human breast cancer biomarker discovery studies (Klein et al., 2007, Pitteri et al., 2008). In recent years, some studies have focused on early detection, such as a plasma proteomics study between PyMT transgenic tumor-bearing mice and matched controls by Pitteri et al. (Pitteri et al., 2008), and several between tumor and normal mammary tissue from a conditional HER2-driven mouse (Schoenherr et al., 2011, Whiteaker et al., 2011, Whiteaker et al., 2007). However, these literature studies are limited in number, and none have taken into account the differentiation between tumor subtypes within a single mouse model. Here, we first report the use of a humanized mouse model for breast cancer that represents the heterogeneity of breast cancer, within an identical genetic background. Two different histological subtypes, and ER as well as HER2 positive and negative tumors are represented in this model. We did not determine

progesterone receptor (PR) status, as clinically PR expression has little prognostic value and is merely used as an additional factor next to ER for the response to hormonal therapy (Sotiriou and Pusztai, 2009).

In this study we identified 149 potentially blood-detectable biomarkers (Supplementary Table 1). The identified genes were significantly up regulated in all tumor types compared to normal mammary gland tissue and potentially blood-detectable according to GeneOntology, Uniprot or the Human Plasma Proteome Project. Moreover, we compared protein expression in human breast tumors and normal breast tissue using the Human Protein Atlas. With these steps we translated mouse mammary gland tumor gene expression to 16 prioritized candidate blood-detectable human breast cancer markers (Table 2).

Our biomarker panel is enriched for several biological functions, such as cytoskeleton, extracellular matrix (including remodeling), cell division, (negative regulation of) apoptosis, and immune/inflammatory response (Figure 1). These terms are related to cancer processes in general, although not specifically to breast cancer. This is corroborated by the enrichment for MSigDB-C2 and GeneSigDB signatures for breast- but also other types of cancers. If the intended use of the biomarker panel would be specifically breast cancer, biomarkers specific for breast cancer (or other tumors) should also be included. Additionally, it can be noted that several immune/inflammatory response markers can also be triggered by other diseases and such markers would need to be combined with non-immunological markers to provide sufficient specificity for (breast) cancer.

We did not detect any significant differential expression for the clinically used markers CA15-3, CA125, CEA, and CA27-29. This can probably be attributed to the fact that – in line with our aim of finding early detection biomarkers – we dissected mice as soon as a palpable tumor was detected. These clinical markers are more often found at more advanced tumor stages and are considered more useful for tumor monitoring and prognosis rather than early detection (Ludwig and Weinstein, 2005, Zeiwar et al., 2007, Molina et al., 2010, Tampellini et al., 2006).

We identified more potential biomarkers across tumor types than we did for discrimination between tumor types. As mentioned before, breast cancer is a heterogeneous disease and in this study several tumor types were represented. It

seems that despite this heterogeneity there are still substantial similarities across tumors types, which involve known cancer deregulated processes such as cell division and tissue invasion. This would be advantageous to finding biomarkers to improve current breast cancer screening. In the future, additional differentiation between tumor subtypes based on tumor type specific biomarkers or genetic make-up may be beneficial.

Although our mouse model represents the heterogeneity found among human breast tumors and the regulated genes found in this study are involved in mechanisms relevant for human breast cancer, the use of any mouse model for human breast cancer has inherent limitations and application of our biomarker panel in a large-scale screening setting would require several validations steps. Therefore, naturally, our potential biomarker panel needs to be tested in human blood samples, to determine the usefulness, and subsequently sensitivity and specificity of this panel for early detection of breast cancer. The main potential limitation in human samples is that the variation between individuals is much larger and statistical power for early tumor detection would decrease considerably. In fact, studies have shown that it is even difficult to find reproducible markers within a clinically homogenous group of patients due to the individual variations and heterogeneities (Ein-Dor et al., 2005). Further studies in small human cohorts or humanized mouse models, using multi-marker assay methods such as Luminex or antibody arrays are therefore needed to determine which markers in our panel are sufficiently robust for eventual testing on a larger human cohort.


3

CHAPTER 4

BIOMARKERS FOR CIRCADIAN RHYTHM DISTURBANCE INDEPENDENT OF TIME OF DAY

Adapted from:

Biomarkers for circadian rhythm disruption independent of time of day

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ABSTRACT

Frequent shift work causes disturbance of the circadian rhythm and might on the long-term result in increased health risk. Current biomarkers evaluating the presence of circadian rhythm disturbance (CRD), including melatonin, cortisol and body temperature, require 24-hr ("around the clock") measurements, which is tedious. Therefore, these markers are not eligible to be used in large-scale (human) studies. The aim of the present study was to identify universal biomarkers for CRD independent of time of day using a transcriptomics approach. Female FVB mice were exposed to six shifts in a clockwise (CW) and counterclockwise (CCW) CRD protocol and sacrificed at baseline and after 1 shift, 6 shifts, 5 days recovery and 14 days recovery, respectively. At six time-points during the day, livers were collected for mRNA microarray analysis. Using a classification approach, we identified a set of biomarkers able to classify samples into either CRD or non-disrupted based on the hepatic gene expression. Furthermore, we identified differentially expressed genes 14 days after the last shift compared to baseline for both CRD protocols. Non-circadian genes differentially expressed upon both CW and CCW protocol were considered useful, universal markers for CRD. One candidate marker *i.e.* CD36 was evaluated in serum samples of the CRD animals versus controls. These biomarkers might be useful to measure CRD and can be used later on for monitoring the effectiveness of intervention strategies aiming to prevent or minimize chronic adverse health effects.

INTRODUCTION

Human behavior, physiology and metabolism are subject to daily rhythms, which are controlled by the circadian clock. This endogenous time keeping system provides a temporal organization of our body functions in relation to environmental time and allows us to anticipate to daily recurring events (Mohawk et al., 2012). Chronic circadian rhythm disturbance (CRD), as encountered by frequent night shift work or multi time zone travelling might result in an increased risk for long-term health effects. Indeed, epidemiological studies among shift workers and flight personnel have associated frequent shift work and jet lag with an increased incidence of breast cancer, obesity and metabolic syndrome (Reynolds et al., 2002, Schernhammer et al., 2006, Kubo et al., 2011). These adverse health effects occur after many years of shift work, and at present it is unclear what mechanism is causing adverse health effects and how these effects of shift work can be minimized. The ability to measure chronic CRD associated with shift work would allow measuring effects of interventions on chronic CRD and monitoring adversity in shift workers and ultimately will help to design intervention strategies.

Studies on the beneficial effects of interventions to prevent shift work-driven adverse health outcomes assess effects on CRD using classical circadian markers, including melatonin, cortisol and body temperature (Mirick and Davis, 2008). These markers allow monitoring circadian rhythm and acute CRD using multiple measurements around the clock before health effects occur. In addition to classical circadian markers, recent research on circadian clock controlled output genes has shown that up to 10% of the transcribed genes is under circadian control, providing additional rhythmic markers to estimate body time in blood and tissues (Panda et al., 2002, Ueda et al., 2004). However, both the classical circadian markers and cycling clock and clock-controlled gene markers are non-eligible as CRD markers in large-scale human cohort studies due to two important pitfalls. Firstly, circadian markers require around the clock measurements, resulting in higher costs and larger impact on participating subjects compared to single measurements. Secondly, classical biomarkers are useful for demonstrating acute CRD, but provide no or only limited information on long-term CRD and accumulation of adversity over time. To acquire information on biological adversity of CRD and to explore the effectiveness of CRD preventive measures, new biomarkers are needed to evaluate the presence of chronic CRD in a time of day independent manner.

Shift work involves a multitude of aspects, including phase desynchronization, light at night, sleep disruption and lifestyle disturbances, all of which potentially play a role in causing CRD and associated adverse health effects (Fritschi et al., 2011). Many different shift work schedules are in use, varying in rotation speed and direction, including forward (counterclockwise) or backward (clockwise) rotating shift schedules. Experimental studies in which mice were subjected to (chronic) shifts in the light-dark cycle (as such resembling jet lag), have shown that both counterclockwise (CCW) and clockwise (CW) schedules cause CRD (McGowan and Coogan, 2013). Additionally, several human studies have shown disturbed circadian rhythms by both CCW and CW work schedules, without major differences between the schedules (Barton et al., 1994, Boquet et al., 2004, Tucker et al., 2000). However, in aged mice CCW shifts appeared more disruptive than CW shifts, as evident from the increased mortality (Davidson et al., 2006).

The aim of the present study was to identify universal biomarkers for CRD independent of rotation direction and time of day. Two different rotations of chronic jet lag were used to induce CRD. Since blood biomarker discovery is technically challenging, we selected the liver to identify biomarkers, as the target tissue of metabolic effects of CRD and as previously used for circadian transcriptomics studies (Ueda et al., 2004). By comparing the liver transcriptome of animals under normal, CW rotating and CCW rotating light schedules, we identified a set of hepatic gene expression markers that report on the presence of CRD. Additionally, we identified non-circadian, age-independent genes differentially expressed after CRD compared to baseline that are potentially blood detectable. One candidate biomarker *i.e.* CD36 was validated in blood, allowing future use in large-scale human studies.

METHODS

Study design

Animal studies were performed in compliance with national legislation, including the 1997 Dutch Act on Animal Experimentation, and experiments were approved by the Animal Experimentation Ethical Committee of the National Institute for Public Health and the Environment in Bilthoven. All surgery was performed under isoflurane anesthesia and appropriate analgesics were used to minimize suffering.

Female FVB mice, 8 to 12 weeks of age, were kept under a normal 12:12 hour light-dark (LD) cycle for approximately three weeks, with Zeitgeber Time 0 (ZT0) corresponding to lights on. Animals were group-housed and food and water were provided *ad libitum*. Prior to the first shift, animals (n=24) were sacrificed around the clock at four hour intervals (n=4 at each time point). Subsequently, the remaining group of animals underwent six shifts in either a clockwise (CW) or counterclockwise (CCW) rotating light schedule. Specifically, mice were exposed to a phase delay or phase advance of eight hours every five days, respectively (S1 Figure). Hereafter, mice were again kept under LD, referred to as recovery. After one shift, six shifts, five days recovery and fourteen days recovery n=30 animals per group were sacrificed around the clock with four hour intervals (n=5 per time point) by orbital bleeding under Ketamine/Xylazine anesthesia. Liver and blood were collected for transcriptomics and serum analyses, respectively. A detailed overview of the experimental design is depicted in S1 Figure.

Four additional mice per group received a radio transmitter (Physio Tel, TA11 TA-F10; Data Sciences, St. Paul, MN) in the peritoneal cavity to record core body temperature every ten minutes. Body temperature was recorded throughout the experiment. Cosine curves were fitted using the R statistical software environment (www.r-project.org) to determine the phase (i.e., peak time) of activity and body temperature rhythms.

Microarray analysis

RNA was extracted from livers (n=4 per time point at baseline and n= 2 per time point for the CCW and CW groups) using the miRNeasy Mini Kit (Qiagen Benelux, Venlo, The Netherlands). RNA concentrations were measured using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, the Netherlands).

RNA was processed for gene expression analysis at the Microarray Department of the University of Amsterdam, the Netherlands, using methods described in Pennings *et al* (Pennings et al., 2011). Experimental samples (each corresponding to RNA from one individual mouse) were labelled with Cy3 and the common reference sample (made by pooling equimolar amounts of RNA from experimental samples) was labelled with Cy5. Samples were hybridized to Nimblegen Mus

musculus 12 x 135 k microarrays (Roche NimbleGen, Germany). This type of microarray contains 44,170 gene probes with three spots per probe. Slides were scanned with an Agilent G2565CA DNA microarray scanner. Feature extraction was performed with NimbleScan v2.5 (Roche NimbleGen), resulting in a table containing individual probe signal intensities for both dyes.

The raw data were subjected to a set of quality control checks to ensure comparable signal average and distribution. Raw microarray data for gene-coding probes were normalized in R (www.r-project.org) using a four step approach (Pennings et al., 2011): (1) natural log-transformation, (2) quantile normalization of all scans, (3) correcting the sample spot signal for the corresponding reference spot signal and (4) averaging data from replicate probe spots. The normalized data of 44,170 probes were further analyzed in R and Excel (Microsoft Corporation, USA). Probe to gene annotation data was downloaded from NCBI. Classification and statistical analysis was performed on the probe level. Significantly predictive or regulated probe sets were annotated to the corresponding genes for further biological interpretation. To this end, when multiple probes corresponding to the same gene were significant, they were counted as one gene in further analysis; probes that did not correspond to genes according to the current NCBI database were excluded from further analysis.

Complete raw and normalized microarray data and their MIAME compliant metadata have been deposited at GEO (www.ncbi.nlm.nih.gov/geo) under accession number GSE65346.

Classification approach

After normalization, a classification approach was applied to identify a set of genes able to classify samples into either circadian rhythm disrupted (CRD) or non-disrupted (ND) 14 days after the last shift. Three different algorithms were used: Random forests (RF) (Breiman, 2001), Support vector machine (SVM) (Rifkin et al., 2003) and Prediction analysis for Microarrays in R (PAM-R) (Tibshirani et al., 2002) (R statistical software). To ensure time-of-day independence of the classifier, for each classification algorithm, a 'leave-one-time point-out' cross-validation approach was used for the classification. Here, time point refers to ZT time point. To this end, the data were repeatedly split into a training set and a test set, in which the training set comprised the data for all-but-one time point, and the test set the data for the

remaining time point. The prediction model obtained for the training set was used to predict the test set data. This approach keeps replicate (time point) samples together and therefore gives a more reliable estimation of the prediction accuracy than cross-validation with random sample selection. The prediction accuracy was calculated as the average over all the test set predictions. As each classifier builds a different prediction model (potentially using different genes) for each training set, genes included in the majority of the models for each classifier were taken as consensus gene set for each type of classifier.

As we aim to find biomarkers which applicability does not depend on a specific choice of algorithm, only genes present in all three classifiers consensus sets were considered potentially valuable biomarkers to classify CRD versus non-disrupted (van der Veen et al., 2013, Vandebriel et al., 2010). To validate prediction accuracy of the consensus gene set after one shift, six shifts and after 5 days recovery, the 14 days recovery dataset was used as the training set to build the prediction model.

Sequential approach

To identify differentially expressed genes (as compared to baseline) after 14 days recovery for the CCW and CW protocol separately, we performed a one-way ANOVA with Qlucore Omics Explorer (Qlucore AB, Lund, Sweden) in which $p<0.001$ was considered statistically significant. CircWave Batch v5.0 software (Hut, R., www.euclock.org/results/item/circ-wave-batch.html) was used to analyze circadian rhythmicity of gene expression. P-Values were false discovery rate (FDR) corrected (Benjamini and Hochberg, 1995); genes with an FDR <0.05 were considered rhythmically expressed. Genes that were rhythmically expressed at any time point during the experiment (baseline, 1 shift, 6 shifts 5 or 14 days recovery) were excluded as potential biomarker. The GenAge Database of ageing-related genes (www.genomics.senescence.info) was used to identify (human) age-dependent genes, which were excluded also.

To determine which candidate biomarker genes are potentially detectable in human serum or plasma, we determined which genes code for proteins that are annotated in Gene Ontology as extracellular or in UniProt as secreted. Additionally, we determined which genes have human equivalents that have been experimentally detected with high confidence in plasma or serum (Farrah et al., 2011) or as part of the Human Plasma Proteome Project (Nanjappa et al., 2014).

Biomarker serum levels

Corticosterone serum levels were determined using ELISA assays (Yanaihara Institute Inc. Shizuaka, Japan) and subsequently visualized and analyzed using GraphPad Prism software version 6.04 for Windows (GraphPad Software, San Diego California USA). Five outliers (out of 251 samples) were excluded based on Grubbs analysis (alpha 0.1). Phase and circadian rhythm were analyzed using CircWave Batch v5.0 software. CD36 was determined in serum with a dedicated ELISA assay (Abcam, Cambridge, United Kingdom). Differences between CRD exposed groups and baseline were statistically tested using a two-sided student's t test. P-values <0.05 were considered statistically significant.

RESULTS

Classical circadian markers

First, we evaluated whether the two different schedules, counterclockwise (CCW) and clockwise (CW), affected circadian rhythm by analyzing classical circadian markers: core body temperature and corticosterone rhythms. At baseline, animals showed regular daily body temperature rhythms, with peaks at approximately ZT12. Core body temperature rhythms re-entrain to the new light-dark cycle after the first shift and following shifts, for both CCW and CW groups (Fig. 1, panels A & B, respectively).

As expected, corticosterone serum levels at baseline showed a major peak at ZT12. Additionally, a minor peak at ZT0 was also observed, corresponding with a small increase in activity at this time point specific for FVB mice (CircWave tau = 12, $p = 0.0003$). Differential effects between the two schedules could be observed after peak phase analysis of corticosterone rhythms (Fig. 2). After the first shift, the corticosterone rhythm shows peak levels at ZT16 for the CW group, representing an incomplete shift ($p = 0.005$). In the CCW group peak time was at ZT20, indicative of a lack of phase shift at this time point ($p = 0.052$). Although there is a tendency toward circadian rhythm, no significant circadian corticosterone rhythm could be detected in either group upon six shifts (CCW: $p = 0.369$, CW: $p = 0.700$). At 5 days and 14 days after the last shift, rhythms were detected in both groups. After 14 days recovery these rhythms were less robust for CCW and CW ($p = 0.093$ and $p = 0.105$, respectively) compared to 5 days after the last shift (CCW: $p = 0.007$, CW: $p =$

0.003). Overall, these results show that circadian rhythms of corticosterone levels heavily disturbed upon prolonged exposure to CRD, but reappear when animals are back under normal LD conditions.

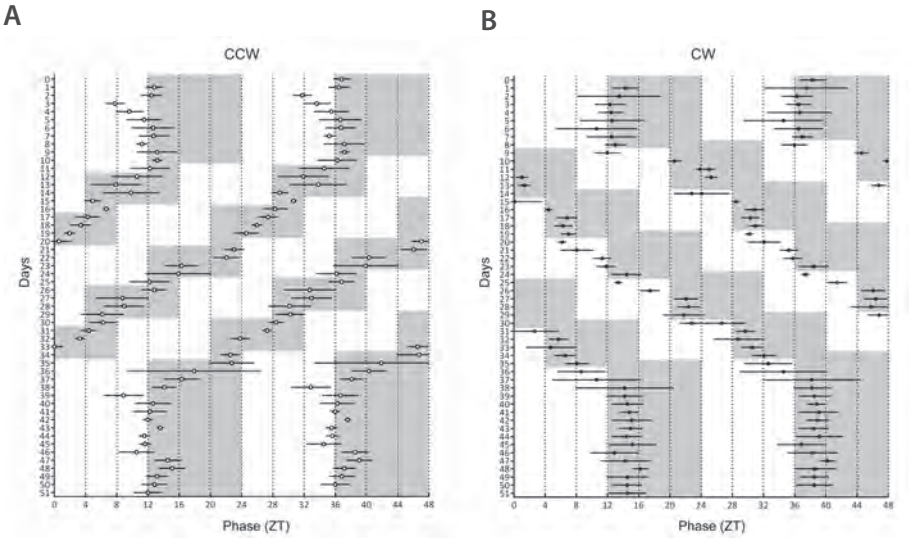

Figure 1. Double plots of peak phase of core body temperature rhythms. A. the counterclockwise schedule and **B.** the clockwise schedule. A cosine function was fitted to determine peak phase (n=4 mice per group). Data are presented as mean peak phase ± sd. Grey areas indicate periods of darkness.

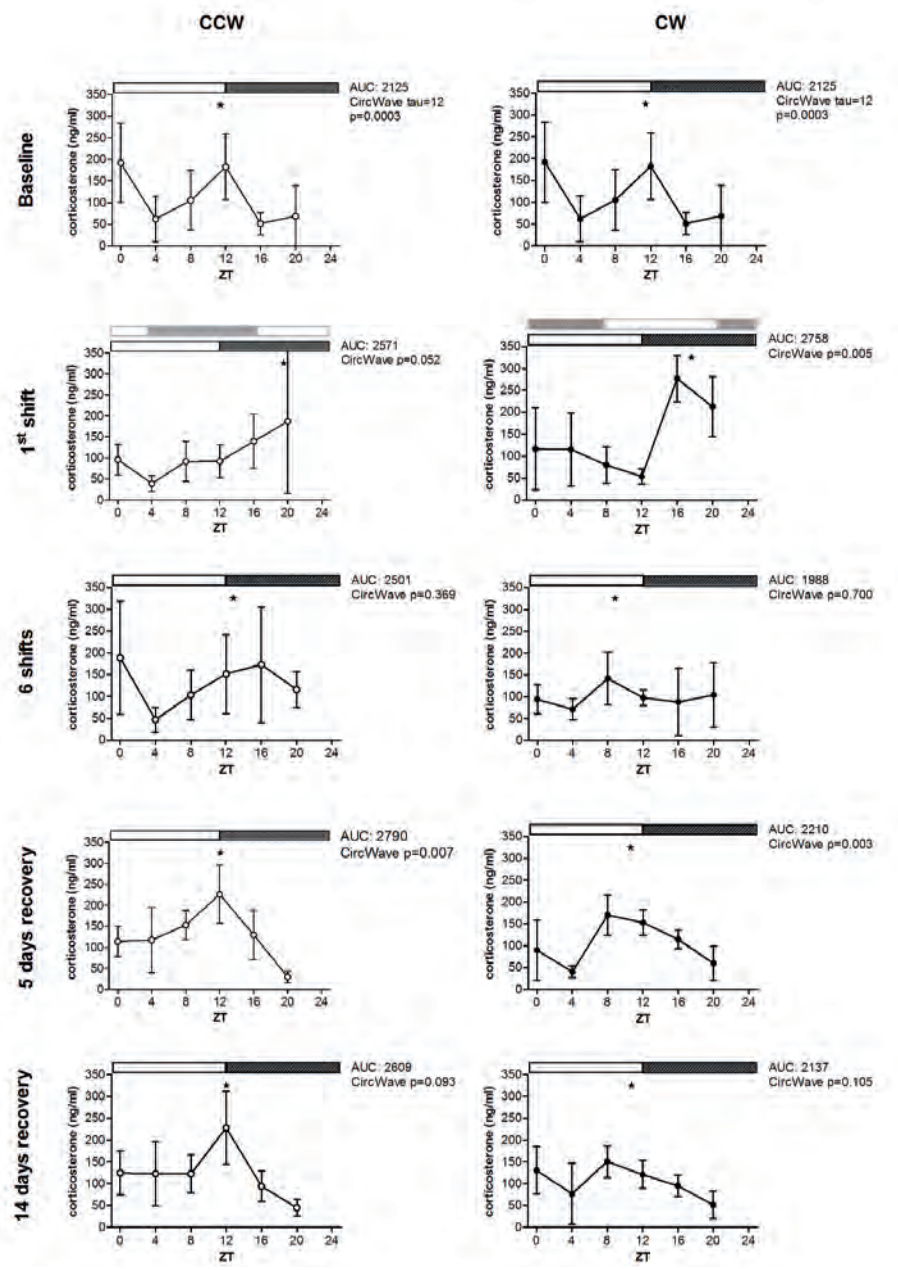

Figure 2. Corticosterone serum levels for the counterclockwise (CCW) and clockwise (CW) jet lag protocols. Serum levels were determined at baseline, after 1 shift, 6 shifts, 5 days recovery and 14 days recovery. Data are presented as mean ± SD. For the first shift, the light dark schedule before the shift is depicted in grey. At baseline a significant 12-hr rhythm was detected, due to the major peak at ZT12 and another peak at ZT0. CircWave peak phases were indicated with (★). Note: as circadian rhythmicity of serum corticosterone levels was lost after 6 shifts, peak phases at the 6th shift are just indicative of the best cosine fit.

In summary, both experimental jet lag schedules, CCW and CW rotation affected the classical circadian markers, indicating CRD. Minor differences in classical circadian markers were found between the two schedules directly after a shift. However, for both schedules the effects were transient, largely recovering within 14 days after the last shift.

Predictive set op hepatic transcriptome markers

Since liver is the target tissue of metabolic effects and can be used for future studies, we performed an around the clock analysis of the liver transcriptome at baseline and after 14 days recovery. To identify a predictive set of hepatic transcriptome markers for chronic CRD with time of day-independent expression levels, we applied a classification approach. Three different classification algorithms were used: RF, SVM and PAM-R (complete overview in Fig. 3a). Using a 'leave-one-time point-out' cross-validation approach, we identified one consensus set of genes per algorithm optimally classifying samples as CRD versus non-disrupted, after 14 days recovery independent of time of day. The SVM approach resulted in a consensus set of 226 probes, RF in 42 and PAM-R in 46. Only the 18 probes, corresponding to 15 individual genes, present in all three classifiers were considered potentially robust biomarkers to classify CRD versus non-disrupted (van der Veen et al., 2013, Vandebriel et al., 2010) (Table 1, S2 Figure). Based on this consensus gene set prediction accuracy was achieved ranging from 90 % to 98% (S1 Table) showing that the set of 15 genes could distinguish CRD-exposed animals from non-disrupted controls with high accuracy independent of sampling time.

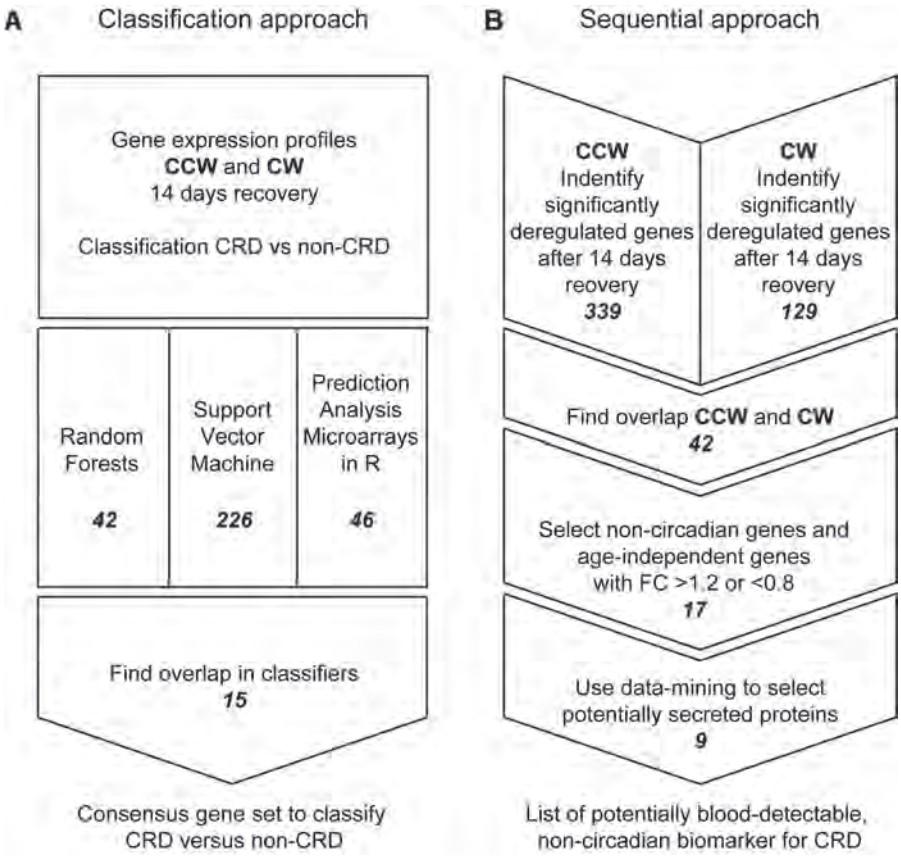

Figure 3. Schematic overview of the microarray analyses. A. Overview of the classification approach, resulting in a set of transcriptomics biomarkers including 15 individual genes. B. Outline of the sequential approach of the data-analyses from hepatic gene expression profiles to 9 potential blood-detectable protein biomarkers.

Subsequently, the ability of the consensus gene set to detect CRD after one shift, 6 shifts and 5 days recovery was determined. Detection of acute CRD was limited, as after the first shift only 29% to 33% of the samples of phase shifted animals were correctly classified as CRD samples, depending on classification method. Accumulation of CRD was detected in the samples taken after six shifts, here, 92% to 88% of the samples were correctly classified as CRD exposed. Samples taken five days after the last shift were also well classified by the gene set, 75% to 88% depending on the algorithm (S2 Table). Overall, this hepatic transcriptome marker set is well able to detect chronic CRD independent of the time point the sample is collected within 14 days recovery.

Table 1. Potential biomarkers for CRD as identified by classification and sequential approach

Gene symbol	Function	Consensus gene set (classification approach)	Non-invasive blood detectable marker (sequential approach)
Cyp2c29	Cytochrome P450 epoxygenase	x	x
Cyp2b10	Cytochrome P450		x
Rbp1	Vitamin A transport	x	x
Sult2a1	Sulfotransferase	x	
Cd36	Scavenger receptor	x	x
Ntrk2	Kinase signaling	x	x
Tusc3	Tumor suppression	x	
Armcx3	Tumor suppression	x	
Gspt2	Cell cycle progression	x	
Snrpn	Transcription	x	x
Tceal8	Transcription	x	
Fkbp11	Protein folding	x	
Orm2	Acute phase plasma protein	x	
Gm3787	Unknown	x	
Gm9299	Unknown	x	
D630033O11Rik	Unknown	x	
Igh-VJ558	Immune		x
Srgap3	GTPase activity		x
Tram1	Translocation proteins		x

Non-invasive biomarkers for CRD

Although predictive of CRD, use of hepatic gene expression markers is still invasive, where non-invasive methods are preferable. However, direct biomarker discovery in blood is technically challenging. To find non-invasive biomarkers eligible to monitor CRD, we aimed to identify potential blood markers for CRD from the gene expression dataset. Therefore, we selected genes of which expression (i) increased or decreased with accumulating CRD exposure and (ii) remained deregulated after 14 days recovery. Furthermore, we excluded genes with circadian expression levels as these have drawbacks earlier described. Compared to control animals, 339 genes were differentially expressed in mice exposed to the CCW schedule. For mice in the CW rotation schedule (p <0.001) 129 were differentially expressed, of which 42 genes were significantly expressed in both groups. Of these 42 genes, only genes with a fold change (FC) larger than 1.2 or smaller than 0.8 were considered relevant to increase the probability of detectable differences in blood protein levels. This resulted in 17 genes all showing an approximate pattern of up or down regulation with accumulating shifts and remaining differentially expressed after 14 days recovery compared to baseline.

Of these 17 genes, 9 genes were found to encode potentially blood-detectable biomarkers according to (i) annotation as "secreted" or "extracellular", and/or (ii) previous proteome-based experimental detection in human plasma or serum: *Cd36*, *Ntrk2*, *Igh-VJ558*, *Srgap3*, *Tram1*, *Snrpn*, *Rbp1*, *Cyp2b10* and *Cyp2c29* (see Fig. 3b and S3 Table for the sequential flow of gene selection). A substantial overlap with the classification consensus gene set was found, 5 genes were identified by both approaches (Table 1). Four out of the 9 genes showed increasing up regulation with number of shifts and remained up-regulated during recovery and five genes showed a similar pattern in the opposite direction (Fig. 4).

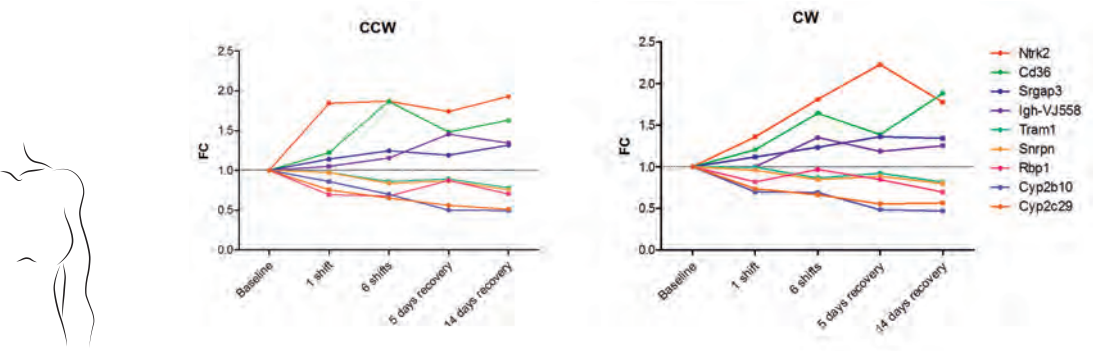

Figure 4. Potentially blood-detectable marker for CRD. Fold ratio of selected differentially expressed genes encoding potentially blood-detectable protein biomarkers for CRD. Expression of these genes is up-regulated or down-regulated by both the CCW and the CW schedule and remains up or down regulated during recovery.

Based on expression patterns and the availability of a serum ELISA assay, CD36 was selected for validation in blood. At 14 days after the last shift, CD36 serum levels showed a significant increase of 18% in animals exposed to CCW shifted light schedules independent of time of day, compared to animals at baseline (Fig. 5). For the animals exposed to the CW light schedule, an 11% increase was found. For both shifting schedules, the increase in blood levels was less pronounced than the increase observed in the gene expression data.

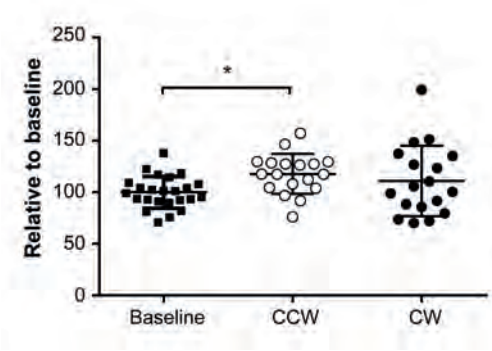

Figure 5. Serum levels of the CD36 protein. Serum levels were determined at baseline and 14 days after the last shift for animals subjected to CCW and CW shifted light schedules. Error bars indicate mean ± sd.

DISCUSSION

Frequent shift work results in a disturbance of the circadian rhythm (CRD) and might on the long-term result in increased health risk. Epidemiologic studies among shift workers and flight personnel have shown increased risk of breast cancer, obesity and metabolic syndrome (Kubo et al., 2011, Reynolds et al., 2002, Schernhammer et al., 2006). The growing 24/7 economy will only lead to increase in shift work and consequently will adversely affect health. Ideally, the level of chronic CRD should be detected before the negative health effects occur. To limit potential health effects, preventive measures to minimize CRD are an attractive option. For these purposes, measuring the presence of chronic CRD is of importance, however, tools to measure chronic CRD are currently lacking. The present study aimed to identify universal biomarkers for chronic CRD using a transcriptomics approach.

To date, CRD and the beneficial effects of preventive measures on CRD are mainly determined using classical circadian markers such as melatonin, cortisol or body temperature (Neil et al., 2014). There is a need of markers that show the cumulative effects of CRD, since the effects on available markers are transient showing only acute effects of CRD. Corticosterone rhythms were heavily disturbed after six subsequent shifts of the light dark schedule and reappear after 5 days recovery, whereas body temperature remains rhythmic and was found to re-entrain to the new light dark schedule within 4-5 days, even after multiple shifts. The present

study identified chronic, non-transient biomarkers for chronic CRD 14 days after the last shift, based on hepatic gene expression. The potentially blood detectable biomarkers report on the presence of chronic CRD, showing an increase or decrease after multiple shifts and non-reversible deregulation upon recovery. The classification markers (consensus gene set) could be used directly after multiple shifts and 5 or 14 days after the last shift, which makes good candidates to determine acute shift work related CRD.

Ideally, for large-scale molecular epidemiology and experimental studies biomarkers of CRD are time-independent. The current available circadian markers require 24-hr measurements, which is challenging in both experimental and field studies. Recent attempts have been made to identify transcriptomics- and metabolomics-based time of day independent biomarkers. In these studies, jet lag and clock mutant mice were used (Minami et al., 2009, Ueda et al., 2004), allowing detection of acute and transient effects of jet lag but not chronic effects. It is challenging to model human shift work since it involves a multitude of aspects, including phase desynchronization, changed social patterns, activity, sleep, nutrition light exposure and sun exposure. Using a chronic jet lag model in mice, and as such mainly mimicking phase desynchronization, we were able to identify biomarkers detecting chronic CRD that can be studied in a single sample, collection of which is independent of time of day. Further studies should point out whether other aspects of shift work (e.g. changes in activity, light-at-night, altered nutritional timing) will affect the same genes.

The set of liver-transcriptome based biomarkers includes genes with a variety of functions. The current study suggests that CD36 could potentially be a blood-detectable biomarker for CRD. CD36 is a scavenger receptor present on many mammalian cell types with a broad range of cellular functions (Febbraio et al., 2001). It has been suggested that CD36 in plasma might represent a marker of the metabolic syndrome (Handberg et al., 2006), a condition that was found associated with frequent shift work (Wang et al., 2014). Furthermore, CD36 was shown to play an important role in breast tumorigenesis (DeFilippis et al., 2012, Uray et al., 2004) potentially associated with the observed increase in breast cancer risk found in shift workers (Wang et al., 2013). Although CD36 is interesting as CRD biomarker, other potentially blood-detectable biomarkers should not be neglected. Especially, the genes that were found upregulated upon CRD are of interest for

further investigation. For example, *Ntrk2* shows a similar induction pattern as CD36 and has also been shown to play a role in breast cancer cell survival and obesity (Xu et al., 2003, Vanhecke et al., 2011). In depth analysis of biological relevant data in this study is limited due to our aim to select non-circadian biomarkers, for which circadian genes were excluded. Full analysis of biological processes affected by CRD requires inclusion of these circadian genes and is subject for further studies.

Our study represents two approaches to identify the most valuable CRD markers based on hepatic gene expression; firstly, an optimal CRD classification set and secondly, a selection of potentially blood detectable biomarkers. An important step that needs to be taken before the identified biomarkers can be applied in experimental and large-scale cohort studies is validation for CRD in humans. For blood-detectable markers, the challenge of the transcriptomics approach is the translation of hepatic gene expression to protein levels in blood. We found that in our homogenous mouse model, CD36 serum levels were also increased in animals exposed to CCW and CW shifted light schedules; however, the increase was smaller compared to gene expression. Potentially, serum CD36 originates from other sources than liver alone, since CD36 is present in many mammalian cell types (Febbraio et al., 2001). Another part of the validation process is the exclusion of post-translational rhythmicity, which is not precluded by the lack of a transcriptional rhythm. In human samples, heterogeneity and variation between samples is much larger and small increases in biomarker blood levels may remain undetected in cross-sectional studies. Preferably, to obtain less inter-individual variation one would opt for longitudinal measurements including baseline measurements per individual before commencing shift work rotations. Furthermore, anchoring with phenotypic endpoint is required to use the selected biomarkers for intervention studies without the need for long-term end-point studies. For this, it should be noted that previous studies have shown that comparable chronic CRD protocols increased negative health effects in mice (Davidson et al., 2006, Filipski et al., 2004).

In conclusion, our study identified a chronic CRD gene-set, comprising 15 genes, potentially useful to study CRD induction by different aspects of shift work and reduction by interventions. Furthermore, we identified 9 candidate genes for blood-detectable biomarkers of CRD, including CD36. Upon validation, these biomarkers provide valuable tools for evaluating CRD in both experimental animal and human studies set up to identify preventive measures for adverse health effects.

CHAPTER 5

DIURNAL VARIATION OF HORMONAL AND LIPID BIOMARKERS IN A MOLECULAR EPIDEMIOLOGY-LIKE SETTING

Adapted from:
Diurnal variation of hormonal and lipid biomarkers in a molecular epidemiology-like setting

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ABSTRACT

Introduction

Many molecular epidemiology studies focusing on high prevalent diseases, such as metabolic disorders and cancer, investigate metabolic and hormonal markers. In general, sampling for these markers can occur at any time-point during the day or after an overnight fast. However, environmental factors, such as light exposure and food intake might affect the levels of these markers, since they provide input for the internal time-keeping system. When diurnal variation is larger than the inter-individual variation, time of day should be taken into account. Importantly, heterogeneity in diurnal variation and disturbance of circadian rhythms among a study population might increasingly occur as a result of our increasing 24/7 economy and related variation in exposure to environmental factors (such as light and food).

Aim

The aim of the present study was to determine whether a set of often used biomarkers shows diurnal variation in a setting resembling large molecular epidemiology studies, i.e. non-fasted and limited control possibilities for other environmental influences.

Results

We show that markers for which diurnal variation is not an issue are adrenocorticotropic hormone, follicle stimulating hormone, estradiol and high-density lipoprotein. For all other tested markers diurnal variation was observed in at least one gender (cholesterol, cortisol, dehydroepiandrosterone sulfate, free fatty acids, low-density lipoprotein, luteinizing hormone, prolactin, progesterone, testosterone, triglycerides, total triiodothyronine and thyroid-stimulating hormone) or could not reliably be detected (human growth hormone).

Discussion: Thus, studies investigating these markers should take diurnal variation into account, for which we provide some options. Furthermore, our study indicates the need for investigating diurnal variation (in literature or experimentally) before setting up studies measuring markers in routine and controlled settings, especially since time – of- day likely matters for many more markers than the ones investigated in the present study.

INTRODUCTION

Many molecular epidemiology studies focusing on high prevalent diseases, such as metabolic disorders and cancer (Danaei et al., 2011, WHO, 2013), make use of metabolic and hormonal markers (for examples see references (Hadji et al., 2014, Jiang et al., 2014). Metabolic markers might serve as important (early) indicators for metabolic disorders, including cardiovascular diseases and type 2 diabetes, and hormonal disbalance is often studied in large epidemiological settings since these are associated with high incidence cancers, such as breast cancer (Mester et al., 2010, Stevens, 2005, Ferlay et al., 2015). In general, for these markers sampling can occur at any time-point during the day or after an overnight fast. However, environmental factors, such as light exposure and food intake might affect the levels of these markers, since they provide input for the internal time-keeping system (Carneiro and Araujo, 2012, Dibner et al., 2010, Hastings et al., 1998, Mohawk et al., 2012). Several markers are well known for their diurnal variation, for example cortisol and melatonin (Gamble et al., 2014). Importantly, heterogeneity in diurnal variation and disturbance of circadian rhythms among a study population might increasingly occur as a result of our increasing 24/7 economy and related variation in exposure to environmental factors (such as light and food). For example, previous studies have indicated that there are substantial changes in the blood transcriptome after experiencing insufficient or mistimed sleep (Archer et al., 2014b, Moller-Levet et al., 2013). This might render accurate determination of markers in molecular epidemiology studies challenging. When the diurnal variation is larger than the inter-individual variation, time of day should be taken into account in molecular epidemiology studies. In this study, we determine whether a set of biomarkers relevant for metabolic disorders and hormone-associated cancers, consisting of endocrine and sex hormones and lipids, shows diurnal variation.

The diurnal variation of various blood markers has previously been studied in different settings, ranging from a relatively uncontrolled routine setting to very tightly controlled laboratory settings. Most information on diurnal variation is derived from these controlled laboratory studies in which factors influencing diurnal variation (such as food intake and/or sleep/wake cycle) are controlled (for example see references (Klingman et al., 2011, Li et al., 2013, Jung et al., 2010, James et al., 2007, Davies et al., 2014, Ang et al., 2012, Dallmann et al., 2012)). Due to the large-scale set up of some molecular epidemiology studies, the possibility

for standardization of sample collection to time of day and food consumption is limited. Therefore, most molecular epidemiology studies collect blood samples to study biomarkers for adverse health outcomes without standardization for time-point, food intake or sleep.

The aim of the present study was to investigate diurnal variation of a set of markers (relevant for metabolic disorders and hormone-associated cancers) in a routine setting in males and females, resembling molecular epidemiology studies: namely, non-fasted and limited control for other environmental influences. In addition, classical markers to study circadian rhythms in chronobiology research are measured: cortisol and components of the molecular biological clock i.e. 'clock genes'. We determined cortisol levels and clock gene expression levels in blood throughout the day to study diurnal variation of these classical markers in a routine setting.

METHODS

Study design and ethics statement

Approval for all procedures was obtained from the ethical committee of the Goce Delchev University in Stip, Republic of Macedonia. All participants signed an informed written consent. This was provided in Macedonian and the translation would be as follows: "I confirm that I am informed about the goals of the Circadian study and voluntary participate in it. I also confirm that all information that I provided with regards to my general health condition and lifestyle is correct". Samples of 17 healthy volunteers, 10 women and 7 men were analyzed in this study. Inclusion criteria were: -age over 21 years old, - apparently healthy without any acute or chronic disease (general medical examination without laboratory analyses), - not taking any drugs, multivitamins and/or supplements, -non-smoking, - not more than 1 (for women) or 2 (for men) alcoholic beverages per day, - no shiftwork and have a normal circadian rhythm (sleeping at nighttime).

The mean age of the males was 24.9 ± 7.2 years and 21.7 ± 0.5 years for the female volunteers. The mean weight was 84.1 ±13.9 kg for the males and 58.5 ±13.6 kg for the females. For more participants characteristics and individual data, see S1 Table. The samples were collected around the clock at four-hour intervals. The first

sample was collected at 08:00 AM after overnight fasting. Participants were asked when they slept between the first and last sampling times (8:00 - 4:00 AM). Total sleep duration between these time-points was 3.4 ± 0.9 hr for males and 3.2 ±1.9 for females. For individual sleeping duration and timing of sleep episodes, see S1 Table. Volunteers were asked to consume their meals one hour after sample collection and otherwise continue their normal daily routines. Meal timing and composition was not further restricted or standardized and meals were consumed at home. EDTA plasma and buffy coat samples (2.7 mL) and serum samples (7.5 mL) were collected in a routine clinical chemistry laboratory by venipuncture of the antecubital vein, using the commercial blood sampling method Sarstedt (Sarstedt AG & Co., Nümbrecht, Germany). Individuals were allowed to leave the laboratory after sample collection and thus were required to wake up and come to the laboratory for sample collection at night.

Plasma and serum analyses

Two methods were used to determine hormonal and lipid biomarkers, an overview can be found in S2 Table. Free fatty acids (FFA), high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides (TG) and cholesterol (CHOL) were determined with an auto-analyzer (Unicel DxC 800, Beckman-Coulter, Woerden, the Netherlands), using kits from Beckman-Coulter. Cortisol (CORT), dehydroepiandrosterone sulfate (DHEAS), estradiol (E2), human growth hormone (hGH), prolactin (PRL), progesterone (PRG), testosterone (TEST), total triiodothyronine (TotT3), thyroid-stimulating hormone (TSH) were determined with an immune-analyzer (Access-2, Beckman-Coulter) using dedicated kits from Beckman-Coulter. Levels of adrenocorticotropic hormone (ACTH), follicle stimulating hormone (FSH), and luteinizing hormone (LH) were determined using commercially available Milliplex Kits (Millipore Corporation, Billerica, MA, USA). All measurements were performed on the same day. The intra-assay variation was determined with three quality control samples (8 tests) for markers measured on the auto-analyzer and with two quality control samples (5 tests) for markers measured on the immune-analyzer. The CVs of markers measured with the Luminex technique were obtained from the manufacturer. See S2 Table for all CVs.

Blood RNA analysis

Expression levels of clock genes *BMAL1* and *PER1* were determined in buffy coats of a subset of volunteers (n=6, female), using quantitative reverse transcription

polymerase chain reaction (RT-PCR). All oligonucleotide primers were obtained from Life Technologies (Bleiswijk, The Netherlands). Total RNA was extracted from buffy coats stabilized with Qiazol, using the miRNeasy Mini Kit (both Qiagen Benelux, Venlo, The Netherlands). cDNA was made using the high capacity cDNA reverse transcription kit (Applied Biosystems by Life Technologies, Bleiswijk, The Netherlands). qPCR was performed on the 7500 Fast Real-Time PCR System (Applied Biosystems by Life Technologies, Bleiswijk, The Netherlands) using Taqman Fast Universal PCR Master Mix and Taqman Gene Expression assays for *BMAL1* (Hs00154147_m1) and *PER1* (Hs01092603_m1) all according to protocol of manufacturer (Applied Biosystems by Life Technologies, Bleiswijk, The Netherlands).

Statistics

To detect all types of diurnal variation, parameters were tested using two types of analyses: cosine fitting analysis and Repeated Measures ANOVA (RM-ANOVA). Depending on the shape of the day curves, these analyses can be overlapping and/or complementary. The RM-ANOVA detects effects of time, i.e. are the levels of the marker different among time-points. The cosine analysis enables detection of rhythms that follow a cosine curve, in manner that is occasionally more sensitive than the RM-ANOVA. For the cosine analysis, CircWave Batch v5.0 software (Roelof Hut, www.euclock.org) was used. A closer fit to cosine gives lower p-value, $p < 0.05$ is assumed a significant cosine curve fit. . Repeated Measures ANOVA (RM-ANOVA) was performed using GraphPad Prism software version 6.04 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). The Greenhouse-Geisser epsilon indicated that sphericity could not be assumed in most analyses, therefore, Greenhouse- Geisser correction was applied. For the RM-ANOVA, $p < 0.05$ was considered statistically significant. If CircWave analysis, RM-ANOVA, or both indicated that signification variation was present, we identified the markers as having diurnal variation in our experiment. Gene expression of *PER1* and *BMAL1* was given as relative expression compared to the mean.

Considering the known difference in levels of several makers between genders, all statistical analyses were performed separately for males and females. The aim of this study was to identify markers with diurnal variation, therefore, differences between males and females were not statistically tested. For all raw data, see S1 File.

RESULTS

We investigated diurnal variation of a set of markers (relevant for metabolic disorders and hormonal cancers) in a routine setting in males and females. To determine the diurnal variation we performed two analyses: 1) Cosine fitting analysis: using CircWave software we determined whether the level of the marker follows a cosine rhythm; 2) RM-ANOVA analysis: determine effects of time of day on parameter levels. If analysis 1, analysis 2 or both indicated that significant variation was present we identified the markers as having diurnal variation in our experiment.

Hormones

Cosine analysis showed that for two of the eleven investigated hormonal markers, a significant cosine curve fit was present in both genders: thyroid-stimulating hormone (TSH) and total triiodothyronine (totT3) (Fig.1). The levels of TSH are highest during the middle of the night, where in males (m) the peak is observed slightly earlier (Clock Time (CT) = 01:54, p = 0.001, amplitude as percentage of the median = 75.89%) compared to females (f) (CT = 03:32, p = 0.006, amplitude = 74.26%). Levels of totT3 are highest at the beginning of the day (m: CT = 06:18, p 0.025, amplitude = 10.14%; f: CT = 07:04, p = 0.041, amplitude = 8.08%). Progesterone (PRG) had a significant cosine curve fit in males, but not in females (m: CT = 07:52, p = 0.003, amplitude = 117.78%; f: p = 0.870) (Fig. 1). For testosterone (TEST), a trend towards a cosine curve fit was present in males, but not females (m: CT = 07:33, p = 0.055, amplitude = 38.38%; f: p = 0.282) (Fig. 1). For a complete overview of CircWave analyses for all 11 hormonal markers, see Fig 1-3 and S3 Table.

For the two markers with a significant cosine fit in both genders, TSH and TotT3, an effect of time was observed with RM-ANOVA as well (for statistics see S3 Table), although for TotT3 in females only a trend is observed (p = 0.078). In addition, for four markers where a cosine curve could not significantly be fitted, a significant effect of time was observed, for TEST, DHEAS, PRG and prolactin (PRL) (Fig. 1 + 2). RM-ANOVA shows that TEST and DHEAS are present at different levels during the 6 time-points in both genders (TEST: m: p 0.021, f: p = 0.023; DHEAS: m: p = 0.045, f: p = 0.002). Furthermore, PRL and PRG are significantly different over the time-points in one gender: PRL (m: p = 0.014, f: p = 0.118) and PRG (m: p = 0.020, f: p = 0.272). For complete overview of the results of the RM-ANOVA on all time-points, see Fig 1-3 and S3 Table.

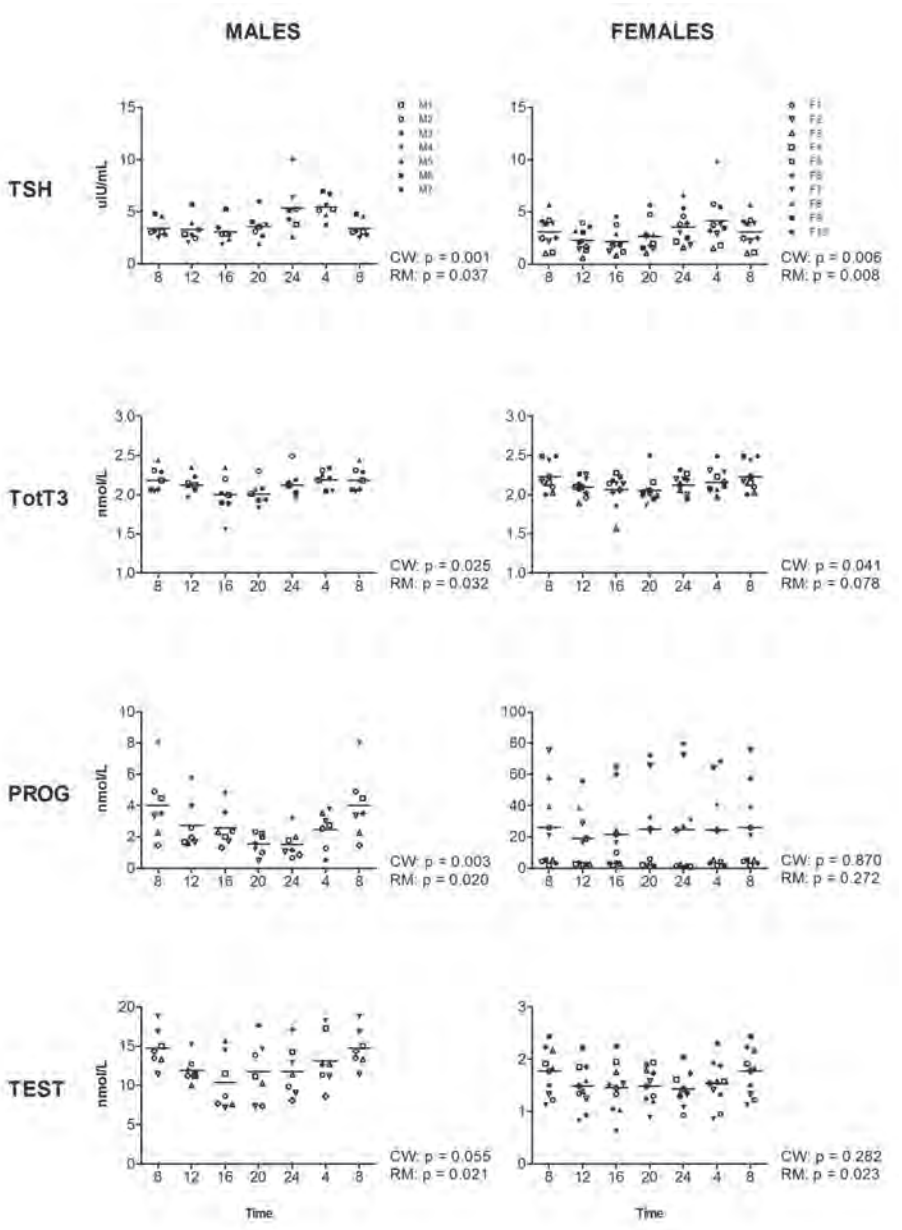

Figure 1. Levels of TSH, TotT3, PROG, and TEST during the day, measured at four-hour intervals for male (left panels) and female (right panels) volunteers. Time indicates clock time. Values at 8-hr were double plotted to help visualize daily patterns. Data represent mean ±sd.

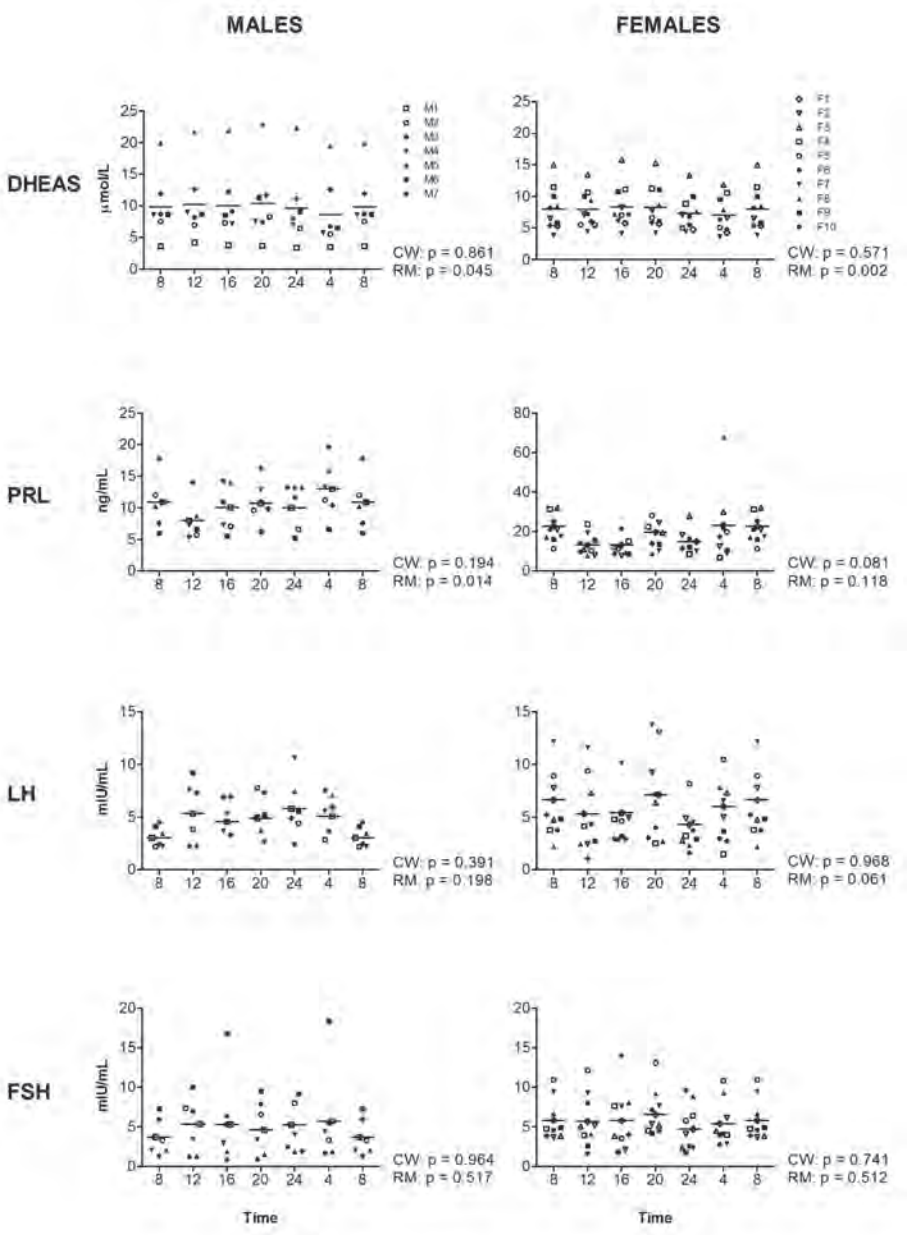

Figure 2. Levels of DHEAS, PRL, LH, and FSH during the day, measured at four-hour intervals for male (left panels) and female (right panels) volunteers. Time indicates clock time. Values at 8-hr were double plotted to help visualize daily patterns. Data represent mean ±sd.

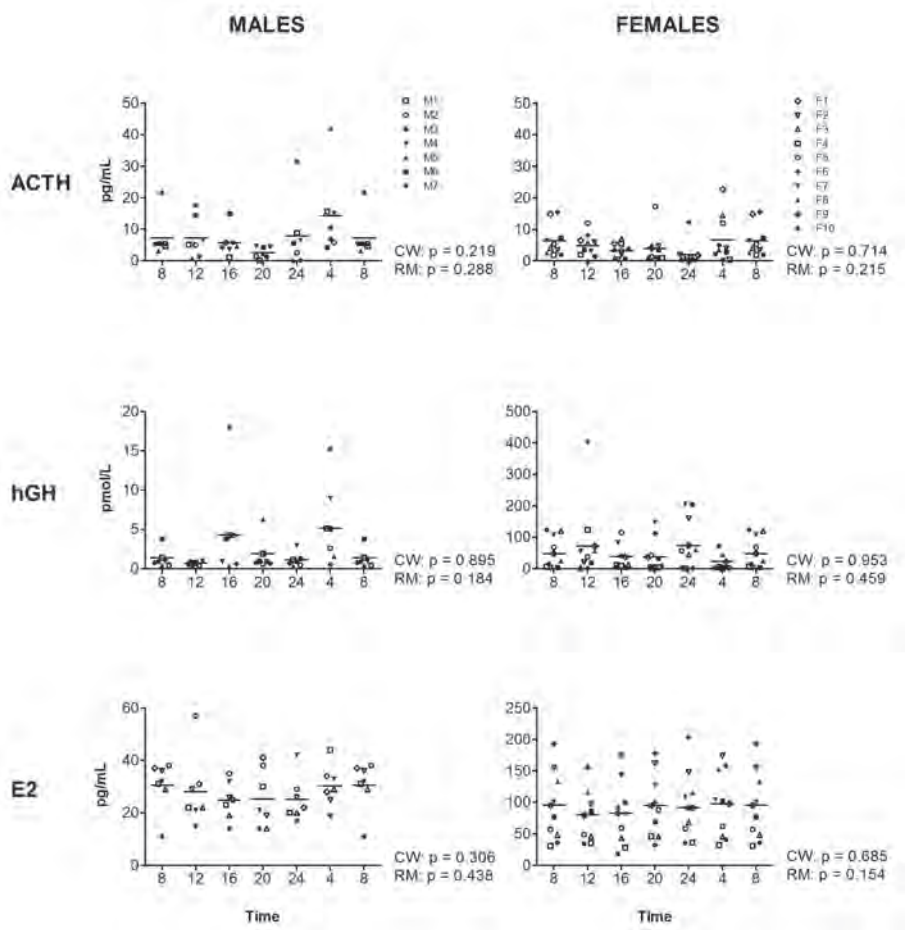

Figure 3. Levels of ACTH, hGH, and E2 during the day, measured at four-hour intervals for male (left panels) and female (right panels) volunteers. Time indicates clock time. Values at 8-hr were double plotted to help visualize daily patterns. Data represent mean ± sd.

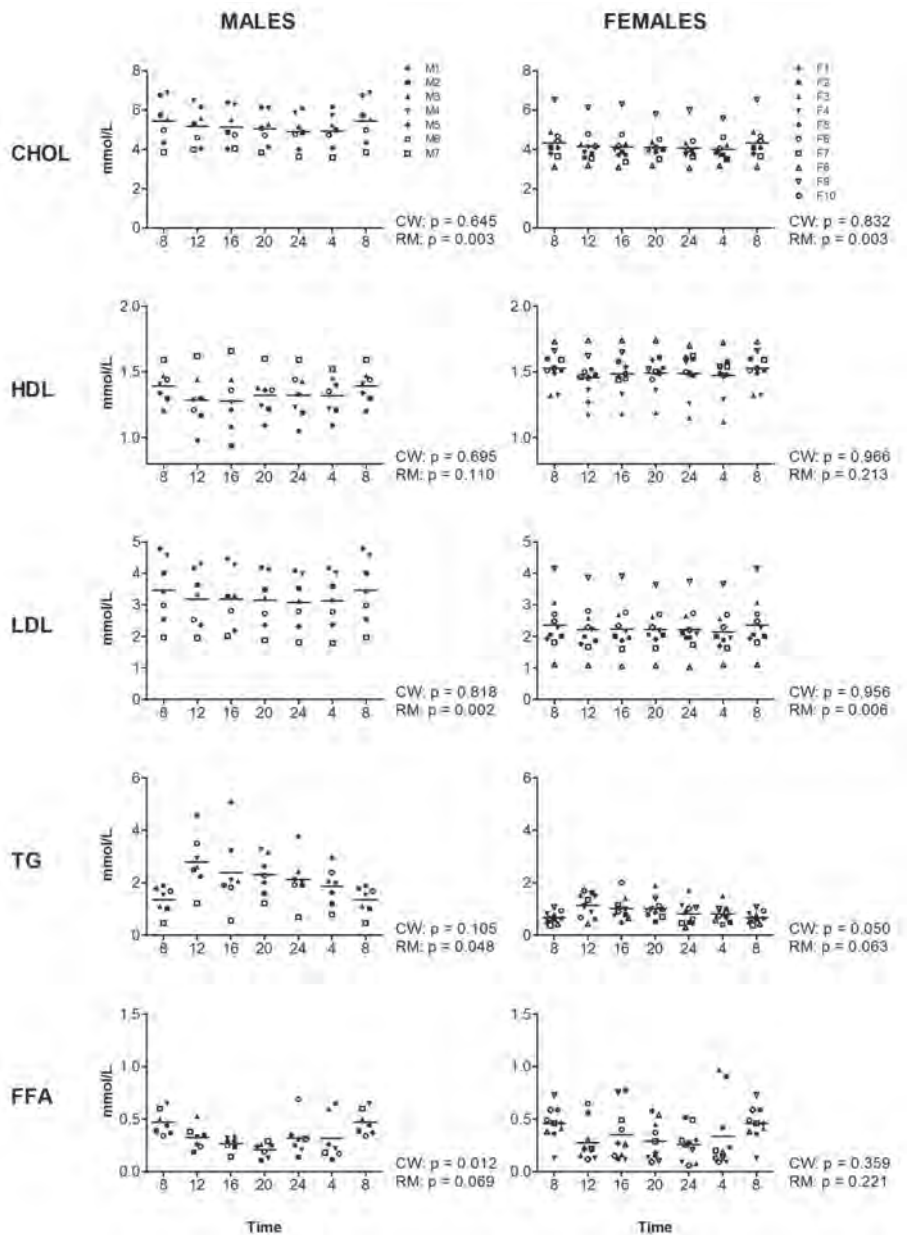

Figure 4. Levels of different lipids during the day, measured at four-hour intervals for male (left panels) and female (right panels) volunteers. Time indicates clock time. Values at 8-hr were double plotted to help visualize daily patterns. Data represent mean ±sd.

Lipids

Five types of lipids were examined; free fatty acids (FFA), triglycerides (TG), total cholesterol (CHOL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) (Fig. 4). CircWave analysis indicated that FFA levels follow a cosine curve in males ($p = 0.012$, amplitude 74.91%), but not significantly in females ($p = 0.359$, amplitude = 80.70%). The levels of FFA were highest at the beginning of the active period in males (CT = 08:31). A trend towards a cosine curve fit was observed for TG in females ($p = 0.050$, amplitude = 50.33%), but not in males ($p = 0.105$, amplitude = 70.36%) (Fig. 4). Visual inspection of the levels of FFA and TG in Fig. 4 implies that the differences between males and females for these markers is likely related to the variation observed within the groups, since the highest levels are observed at similar time-points for both genders. For complete overview of all markers, see Fig. 4 and S4 Table.

Despite the limited presence of significant cosine curve fits observed among the different lipid markers, for several markers RM-ANOVA shows significant effects of time: for CHOL and LDL in both genders (CHOL: m: $p = 0.003$, f: $p = 0.003$; LDL: m: $p = 0.002$, f: $p = 0.006$), for TG in males and a trend was present in females (m: $p = 0.048$, f: $p = 0.063$) (S4 Table). The levels of FFA show a trend towards an effect of time in males (m: $p = 0.069$, f: $p = 0.221$). For HDL, no significant effect was observed in any gender (m: $p = 0.110$, f: $p = 0.213$). The highest level of most lipids including FFA, LDL, and CHOL was observed at the beginning of the active phase, the highest level of TG in males was observed during the middle of the day (Fig. 4).

In summary, for all lipid markers, except HDL significant or trends towards diurnal variation are observed.

Classical circadian markers

The levels of cortisol were investigated in all participants (n=17), clock genes *PER1* and *BMAL1* were investigated in a subset (n=6, females) to gain insight in classical circadian markers in uncontrolled conditions (Fig. 5). Cortisol levels show a significant cosine curve fit, are highest at the beginning of the day (m: CT = 07:47; f: CT = 07:29), and the rhythm has a relative large amplitude (m: $p < 0.0005$, amplitude = 129.11%; f: $p < 0.0005$, amplitude = 161.63%) (Fig. 5, upper panels). The levels of *PER1* and *BMAL1* mRNA show a significant cosine curve fit as well (*PER1* $p = 0.002$, amplitude = 80.15%; *BMAL1* $p = 0.035$, amplitude = 35.55%). Levels

of *PER1* were highest during the morning (CT = 8:07), while levels of *BMAL1* were highest during the afternoon (CT = 14:12). For complete overview of statistics see S5 Table.

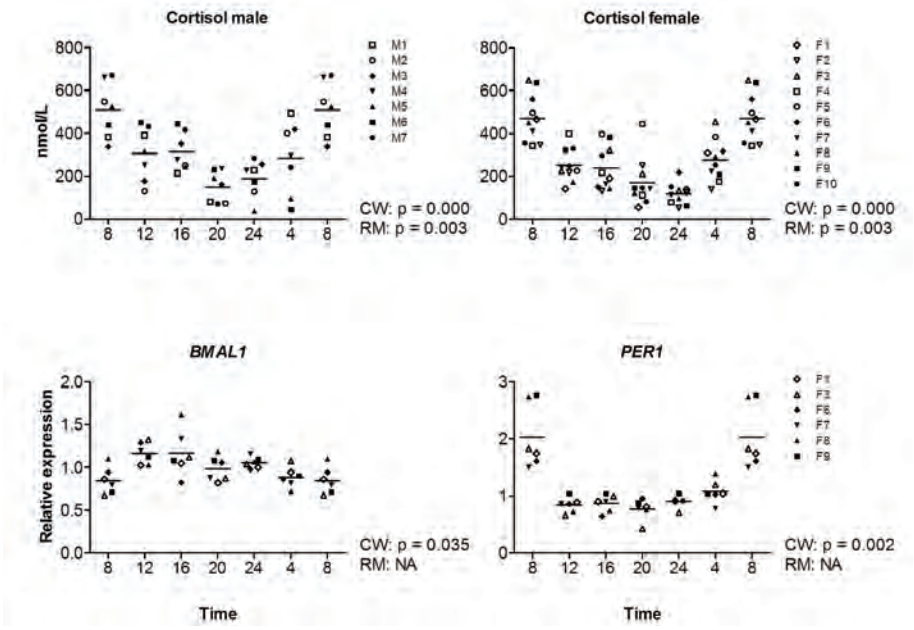

Figure 5. Around the clock levels for classical circadian markers cortisol, *PER1* and *BMAL1*. Upper panels show cortisol levels in male (left panels) and female (right panels) volunteers, determined in serum. mRNA expression of *BMAL1* and *PER1* in buffy coats are depicted in the lower panels (females subset n=6). All were measured at 4 hour intervals, where time indicates clock time. Values at 8-hr were double plotted to help visualize daily patterns. Data represent mean ±sd.

DISCUSSION

The aim of the present study was to investigate diurnal variation in a set of hormones, metabolic markers, and classical circadian markers in serum, plasma and peripheral blood mononuclear cells (PBMCs), in a routine non-fasted molecular epidemiology-like setting, with no restrictions on sleep or diet.

Hormones

Markers without diurnal variation

For markers without diurnal variation, taking the time-point of sampling into account is not required. This study observed no diurnal variation or trends towards

diurnal variation in any gender in four of the eleven hormonal markers investigated, ACTH, hGH, E2, and FSH. Previous published studies performed in a different type of setting than the present study, namely a controlled (laboratory) settings do show diurnal variation in ACTH levels in males , with the highest level being observed at CT 4.00 h (Li et al., 2013) or CT 7.00 h (Nicolau and Haus, 1989), whereas no diurnal was observed in females (Nicolau and Haus, 1989). Interestingly, in the present study, levels were also highest at CT 4.00, indicating similar patterns. However, in our study larger amount of variation is expected than a controlled laboratory setting potentially causing the diurnal variation of ACTH undetectable within the inter-individual variations. Since during day time-points, when sampling is usually performed in a cohort study, variation in ACTH levels is minimal. Thus, diurnal variation seems not to be a major issue for ACTH in a molecular epidemiology setting. However, considering the previous studies on ACTH and that in a large cohort setting power will be increased, diurnal variation might become larger than the inter-individual variation and caution is required. In addition, if subjects are investigated which possibly have a circadian disturbance (e.g. night shift workers), variation in levels of the markers during night time-points should be taken into account as well (see recommendations section for suggestions).

For hGH, former reports in controlled settings have shown diurnal variation in males (Mazzoccoli et al., 2011, Nicolau and Haus, 1989). However, it is important to note that it is known that hGH is released in pulses with levels varying up to 4 times (Riedel et al., 1995). Hence, in the present study with sampling intervals of 4 hours these pulse releases might interfere with the detection of diurnal variation. Indeed, in the present study at several time-points large variations in the levels of hGH are observed, particularly in males. This indicates that hGH is a difficult marker to measure in a non-time-controlled cohort setting, since possible differences are easily masked by the large amount of variation present for this marker.

For estradiol, the absence of diurnal variation in the present study is in line with previous studies that reported the absence of diurnal variation in females (Brambilla et al., 2009, Mortola et al., 1992), although diurnal variation is observed in estradiol levels in elderly females (mean age 77 years) (Nicolau and Haus, 1989). Together, these data indicate that in a cohort study with non-elderly adults, diurnal variation of estradiol is not an issue.

For FSH, conflicting data have been published previously. In females, the absence of a diurnal variation was reported in studies using (mildly) controlled settings (Klingman et al., 2011, Nicolau and Haus, 1989), while a third study reported diurnal variation in females during different phases of the menstrual cycle, with the highest levels of FSH being observed during the afternoon (Mortola et al., 1992). For males, conflicting results have been reported as well: a study by Spratt et al. showing the absence of diurnal variation in males (Spratt et al., 1988), while Nicolau et al., reported the presence of a diurnal variation in elderly males (Nicolau and Haus, 1989). Possibly, age has a role in the diurnal variation of FSH. However, the present study indicates that in a routine setting, time of day is not a major source of variation for FSH levels.

Markers with diurnal variation

For eight of the twelve investigated hormonal markers, diurnal variation was observed in at least one gender. Markers with the most robust diurnal variation in both genders were TSH and T3. This is in line with previous findings for these markers (Hirschfeld et al., 1996, Klingman et al., 2011, Mazzoccoli et al., 2011, Nicolau and Haus, 1989). For DHEAS, a previous study reported circadian rhythmicity in males and females (Nicolau and Haus, 1989), however, in the present study the rhythm of DHEAS could not be fitted to a cosine curve. The levels of DHEAS differed among the time-points and the pattern of expression (e.g. highest levels in the afternoon) is comparable to the previously published results, confirming diurnal variation of DHEAS.

For testosterone, diurnal variation was not robust, but significant changes were observed when the time-points were compared in both genders. Diurnal variation in testosterone is in line with previous findings (Diver et al., 2003, Nicolau and Haus, 1989, Ostrowska et al., 1998, Plymate et al., 1989, Spratt et al., 1988, Brambilla et al., 2009). The study by Spratt et al. showed that the robustness of the diurnal variation of testosterone was largely dependent on the frequency of sampling.

For several of the other markers, the robustness of diurnal variation varied among genders. Diurnal variation was often more pronounced in males. Since in the present study menstrual period was not assessed, nor was the use of contraceptives, this might explain the less robust results in females. For example, progesterone showed diurnal variation in males, but not females. For females, it has been shown

that the rhythmicity of progesterone varies with the menstrual period, including the timing of the highest levels (Fujimoto et al., 1990, Nicolau and Haus, 1989, Veldhuis et al., 1988). Interestingly, 5 females show high progesterone levels, which are indicative of the luteal phase. Of these females, two show a significant or trend (p-value between 0.05 and 0.1) towards an individual cosine curve fit (#6: p=0.0059 and #7: p=0.05222). These results suggest that indeed variation in menstrual period, and possibly contraceptives use, interfere with measurements of progesterone in a cohort setting.

For the final two markers, LH and PRL, only mild amounts of diurnal variation were observed. For LH previous studies in females have shown that rhythmicity is dependent on the menstrual cycle and that peak timing differs among women (Klingman et al., 2011, Mortola et al., 1992, Veldhuis et al., 1988), which likely explains the results of the present study. For prolactin, in the present study only a trend towards a cosine curve fit was observed in females and effects of time (by RM-ANOVA) in males. This is in line with previous studies that have shown diurnal variation of prolactin in females in controlled settings (Birketvedt et al., 2012, Fujimoto et al., 1990), and higher levels of prolactin during the night in males (Miyatake et al., 1980). Together, these results indicate that for prolactin time of day should be taken into account (see recommendations section for suggestions).

Lipids

Of the 5 lipid markers, only HDL showed no diurnal variation (no cosine curve fit and no effect of time by RM-ANOVA). Previously, it has been shown that a large subset of lipids shows diurnal variation (for examples see references: Miida et al., 2002, Chua et al., 2013, for review see reference: Gooley and Chua, 2014), however, the rhythmicity of these markers is highly variable among individuals (Chua et al., 2013). Furthermore, it is known that many lipids are directly regulated by the endogenous circadian clock (Dallmann et al., 2012, Ang et al., 2012, Gooley and Chua, 2014), lipid metabolism is related to sleep (Davies et al., 2014, Ollila et al., 2012), and that levels of FFA, TG and LDL are dependent on meal-timing (Yoshino et al., 2014, Niklowitz et al., 2006) The results of the present study, diurnal variation in LDL, TG, FFA and total cholesterol are thus in line with previous findings. These results show that for these markers time of day needs to be taken into account (see recommendations section below for suggestions). Previously, diurnal variation has been observed for HDL in a controlled setting (Miida et al., 2002), however, our

study indicates that this variation does not exceed the inter-individual variation in a routine setting.

Classical circadian markers

In chronobiology research, several markers are often used to study circadian rhythm. Hence, these can be named as 'classical circadian markers'. One of these markers is cortisol, which is often used to study circadian rhythm in humans. In addition, components of the molecular biological clock can be used to study circadian rhythm, i.e. 'clock genes'. Since these genes are part of the core clock mechanism in cells, their rhythm reflects the circadian rhythm in these cells. We show that the 'classical circadian markers' investigated in this study (cortisol and the clock genes *PER1* and *BMAL1*) have a robust cosine curve fit in our study population, which resembles a routine non-fasted molecular epidemiology setting. Our findings for cortisol (rhythmic expression with high levels during the early morning), are in line with previous literature. Multiple studies, in a range of different settings, have reported similar findings (for review see reference (Gamble et al., 2014), for examples see references (Kusanagi et al., 2008, Takimoto et al., 2005, Teboul et al., 2005)).

In our study *PER1* expression fits to a cosine curve with the highest levels in the morning, which is also consistent with previous studies. For example, James et al. observed rhythmic *PER1* expression with peak levels on average 2:36 h after waking (±1:47 h) in PBMCs (James et al., 2007), Kusanagi et al. observed rhythmic *PER1expression* with peak expression on average at 7:42 h in PMBCs (Kusanagi et al., 2008), and Takimoto et al. reported peak levels at 06:00 h in whole blood cells (Takimoto et al., 2005). For *BMAL1*, previous studies have indicated that rhythmicity of *BMAL1* gene expression in PBMCs is highly variable amongst individuals (Teboul et al., 2005, James et al., 2007). For example, *BMAL1* expression has been reported to be high during the middle of the day (comparable to our study) (James et al., 2007, Teboul et al., 2005), during the night (Takimoto et al., 2005), during the evening (Teboul et al., 2005), or not being rhythmic (Kusanagi et al., 2008). Teboul et al. suggested that this variation might be related to chronotype, since in their study two groups of individuals could be distinguished: peak expression of BMAL1 during the middle of the day (12:30 hr) or during the evening (21:45 hr).
Together, our results shows that thediurnal variation, including the acrophase of the fitted cosine curves, of several classical markers in our study is comparable to

previous studies using (mildly) controlled settings (James et al., 2007, Kusanagi et al., 2008, Takimoto et al., 2005, Teboul et al., 2005).

Concluding remarks and reccomendations

The present study showed that markers for which diurnal variation (circadian rhythms and/or time-of-day-effects) is not an issue in a routine setting are ACTH, FSH, E2 and HDL. For all other tested markers diurnal variation was observed, which exceeded the inter-individual variation in a routine setting. It is important to note that we tested this in a small test group (n=17). In a large cohort with numerous subjects, levels of inter-individual variation might decrease due to larger sample sizes, making the contribution of diurnal variation relatively larger. Hence, for some of the markers without detectable diurnal variation in the present study, diurnal variation might play a role in larger studies. Nevertheless, our study provides an indication for which markers diurnal variation needs to be taken into account in a routine setting.

Taking diurnal variation into account can be done in several ways depending on the study design and marker characteristics (such as dependent on food intake, sleep/wake cycle etc.). For example, measurements can be taken at a single time-point or if sampling at a single time-points is not possible, registration of sampling time and their relevant time-points (e.g. timing of food intake) is an alternative method to take into account diurnal variation. Apart from a research setting, several of the markers in our study are measured in a clinical setting as well. In general, a substantial amount of variance is incorporated in reference levels for diagnostic purposes. However, for biomarkers with large diurnal variation it might be beneficial to take multiple samples during the day or define reference levels that take into account time-of-day.

Our finding that most markers tested show diurnal variation is in line with recent studies showing that many genes are regulated by the circadian clock. For example, a recent study has shown that 43% of the protein coding genes shows diurnal variation in at least one organ in mice (Zhang et al., 2014a), as a consequence one could expect diurnal variation in many serum or plasma protein levels as well. This large set of markers with diurnal variation indicates the need for investigating diurnal variation before setting up studies measuring markers in routine and controlled settings, since time- of- day likely matters for many more markers than the ones investigated in the present study.

CHAPTER 6

ATTENUATION OF CIRCADIAN RHYTHMICITY IN HEPATIC GENE EXPRESSION UPON CHRONIC ALTERNATING LIGHT CYCLE EXPOSURE

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In preparation

ABSTRACT

Disturbance of circadian rhythms in shift workers results in increased health risks, including obesity and cancer. Previously, we have shown that mice gain more weight when chronically exposed to conditions that cause circadian rhythm disturbance (CRD). The aim of the present study was to understand the mechanism(s) underlying the observed overweight phenotype in mice. As the liver is a key metabolic organ and regulator of energy metabolism, liver transcriptome analysis enables the identification of disturbed processes involved in the obese phenotype. Mice were exposed to a weekly alternating light-dark schedule for 18 weeks and 7 days after the last shift gene expression was analyzed over a 24-hour period to allow identification of more permanent affected processes upon long-term CRD. Overall, the effects on gene expression were minor compared to previous studies where the transcriptome was studied directly after impingement on the circadian system. However, an overall attenuation of circadian rhythmicity was found in hepatic gene expression, associated with a wide variety of processes, including cell cycle control and DNA damage processes. The largest decrease in amplitude was found in genes involved in the balance between lipid and glucose metabolism: *Ppard*, *Pdk4*, *Slc16a5* and *Igfbp-1*. In conclusion, metabolic flexibility might be compromised by long-term exposure to CRD, resulting in increased weight gain in mice. Further research should elucidate the relevance of acute versus more permanent disturbed processes for adverse health outcomes to develop evidence based preventive measures.

INTRODUCTION

The circadian system imposes 24 hour rhythmicity on behavior, physiology and metabolism and allows us to anticipate daily recurring changes in our environment. This internal timekeeping system is composed of a light-entrainable central clock in the suprachiasmatic nucleus (SCN) located in the brain, and peripheral clocks in all other tissues in the body. On a molecular level, circadian rhythms are generated by a set of clock genes that act in an auto-regulatory transcriptional-translation feedback loop (TTFL) and confer rhythmicity to biological output processes via clock-controlled genes (Lee et al., 2001). Various expression profiling studies have shown that, depending on the tissue, up to 10% of the transcriptome is under circadian control (Akhtar et al., 2002, Bozek et al., 2009, Panda et al., 2002, Storch et al., 2002). Accordingly, it is not surprising that long lasting circadian rhythm disturbance (CRD), e.g. by chronically alternating light cycles, result in a loss of homeostasis in addition to the acute disturbances as shown in several studies (Archer et al., 2014a, Barclay et al., 2012, Moller-Levet et al., 2013).

As a consequence of chronic circadian rhythm disturbance (CRD), long-term shift workers are at risk for various adverse health effects. Various studies indicated an association between social jet-lag, shift work and increased body weight, although conflicting data exist (Kubo et al., 2011, Nabe-Nielsen et al., 2011, Roenneberg et al., 2012, Parsons et al., 2014). These human studies, however, are hampered by difficulties with exposure assessment and confounding factors, such as unhealthy food intake. Animal studies can limit the influence of these factors, as specifically designed CRD exposure protocols allow exclusion of or strict control over confounding factors. Previously, we have shown that chronically alternating light cycles cause an increased breast cancer risk and body weight gain in breast cancer predisposed mice (Van Dycke et al., 2015). This model provides a unique tool to further analyze mechanisms that underlie the relationship between shift work and cancer and increased body weight.

Shift work involves a multitude of aspects, including internal desynchronization, melatonin suppression, sleep disruption, lifestyle disturbance and decreased vitamin D levels (Fritschi et al., 2011). A number of these aspects, including circadian desynchrony, lifestyle disturbance and timing of food intake, have been proposed to underlie the relationship between shift work and increased body weight (Fonken

et al., 2010, Salgado-Delgado et al., 2010). Unravelling the mechanisms that link shift work with adverse health effects is a requisite for developing evidence-based interventions that minimize health risks. Especially, understanding the underlying metabolic changes could provide targets for preventive measures, since food intake can be adjusted.

Transcriptomics studies provide a useful tool to investigate a wide variety of processes and several studies show that CRD affects animal and human gene expression profiles (Archer et al., 2014a, Barclay et al., 2012, Moller-Levet et al., 2013). In human laboratory studies, mistimed sleep and insufficient sleep cause the majority of the rhythmically expressed mRNAs in the blood transcriptome to become arrhythmic (Archer et al., 2014a, Moller-Levet et al., 2013). Timed sleep restriction in mice was shown to disrupt global liver gene expression, with many genes associated with lipid and glucose metabolism processes (Barclay et al., 2012). Furthermore, in these studies the exposure period was limited to weeks, whereas shift workers are involved in irregular working hours for many years. Although these human and animal studies show (semi)acute effects on both circadian and non-circadian biological processes, directly or shortly after impingement on the circadian system, they fail to provide insight into the constitutively disturbed processes underlying long-term health effects such as obesity and cancer. Therefore, to gain more knowledge on processes involved in chronic CRD we performed a mouse study in which we chronically altered light cycles and focused on changes in longer term expression patterns, which may explain the observed weight gain phenotype.

METHODS

Experimental set-up

Animal experiments were performed in compliance with national legislation, including the 1997 Dutch Act on Animal Experimentation, and were approved by the institute's Animal Experimentation Ethical Committee. Food and water were available *ad libitum* throughout the whole experiment.

The experimental set-up of the study has been described previously (Chapter 2) (Van Dycke et al., 2015). In short, breast cancer-prone female *p53*$^{R270H/+}$*WAPCre* mice

(Wijnhoven et al., 2005) in an FVB genetic background were kept under normal LD conditions or chronically exposed to a weekly alternating 12:12 hour light-dark cycle. After 18 inversions, on day 7 of the last shift, animals were sacrificed at 4 hour time intervals over a 24-hour period (n=4 animals per time point). Blood and tissues were collected for further analysis. Due to premature animal loss, 21 animals per condition were available for further analysis. In an additional group of mice (n=5 per group), a radio transmitter (Physio Tel, TA11 TA-F10; Data Sciences, St. Paul, MN) was implanted in the peritoneal cavity to record locomotor activity and core body temperature every ten minutes (Van Dycke et al., 2015).

Micro-array analysis

Total RNA was extracted from RNA*later* (Invitrogen, Grand Island, NY, USA) protected liver tissues using the miRNeasy Mini Kit (Qiagen Benelux, Venlo, The Netherlands). RNA concentrations were measured using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, the Netherlands).

RNA was processed for gene expression analysis at the Microarray Department of the University of Amsterdam, the Netherlands, using Agilent SurePrint G3 Mouse GE 8x60K arrays (Amstelveen, the Netherlands). This type of microarray contains 55,681 probes for biological features such as mRNAs and ncRNAs. RNA amplification and labeling were carried out according to the manufacturer's protocol. Experimental samples (each corresponding to RNA isolated from one individual mouse) were labelled with Cy3 and the common reference sample (made by pooling equimolar amounts of RNA from experimental samples) was labelled with Cy5. Slides were scanned with an Agilent G2565CA DNA microarray scanner, followed by automated feature extraction, resulting in a table containing individual probe signal intensities for both dyes.

Data analysis

The raw data were subjected to a set of quality control checks to ensure comparable signal average and distribution. Raw microarray data for gene-coding probes were normalized in R (www.r-project.org) using a four step approach (Pennings et al., 2011): (1) natural log-transformation, (2) quantile normalization of all scans, (3) correcting the sample spot signal for the corresponding reference spot signal and

(4) averaging data from replicate probe spots. The normalized data of 55,681 probes were further analyzed in R and Excel (Microsoft Corporation, USA). Probe to gene annotation data was downloaded from NCBI. Significantly regulated probes were annotated to the corresponding genes for further biological interpretation. To this end, when multiple probes corresponding to the same gene were significant, they were counted as one gene in further analysis; probes that did not correspond to genes according to the current NCBI database were excluded from further analysis. To identify differentially expressed genes for chronic CRD exposed animals compared to controls, we performed a one-way ANOVA with Qlucore Omics Explorer (Qlucore AB, Lund, Sweden) in which $p<0.001$ was considered statistically significant. CircWave Batch v5.0 software (Hut, R., www.euclock.org/results/item/circ-wave-batch.html) was used to analyze circadian rhythmicity of gene expression under regular LD conditions and after prolonged exposure to weekly LD-inversions. Enrichment of selected gene lists was analyzed with MetaCore GO Processes from GeneGo, Inc. (http://www.genego.com/). Gene expression data were imported into Metacore using their NCBI Entrez GeneID as identifier; therefore only genes annotated with a GeneID in the current NCBI database were used for pathway enrichment analysis. To ensure biological relevance, only processes including between 10 and 1000 genes were taken into account. GO Processes were considered significantly enriched with a $p<0.001$. Gene Set Enrichment Analysis allowed us to identify significant up- or downregulation of enriched gene sets ($P<0.05$ and $FDR<0.25$) (Mootha et al., 2003, Subramanian et al., 2005).

RESULTS

Attenuation of circadian rhythmicity in gene expression

The alternating light cycle protocol to chronically disturb the circadian rhythm (CRD protocol) used in this study has previously been shown to shorten tumor latency times, increase body weight and affect sleep timing (Van Dycke et al., 2015). Although the weight gain phenotype is already apparent at week 6, group differences only became significant after week 24. Given the overweight phenotype, we analyzed possible transcriptional changes in the liver. Since we were interested in processes that are involved in the observed overweight phenotype early on, we analyzed gene expression 18 weeks after start of the exposure, before the differences in body weight gain were significant. Clock and clock-controlled

genes, as well as temperature rhythms, were re-entrained 7 days after the final light-dark shift whereas corticosterone serum levels showed a bimodal rhythm upon chronic CRD (Van Dycke et al., 2015). This re-entrainment of the majority of these classical parameters allowed investigation of more permanent disturbed genes and processes.

The observed re-entrainment of temperature and clock gene expression, in contrast to altered serum corticosterone rhythms suggest that chronic CRD results in internal desynchronization. Therefore, we determined circadian rhythmicity of all genes using CircWave, for the control light-dark (LD group) and chronic CRD groups separately. Significant circadian gene expression ($p<0.001$) was observed for 594 genes in LD controls and only in 133 genes in chronic CRD exposed animals. The overlap between the two groups was limited to 33 genes, including several clock or clock controlled genes (for a complete overview, see Supplemental Table S1). Interestingly, 561 genes lost significant circadian expression after chronic CRD based on p-value. However, closer examination of the expression profiles revealed that oscillation was not completely abolished for these genes (Fig. 1). Rather, the majority of these genes showed a lower amplitude oscillations, as compared to LD controls (Fig. 1) and the average amplitude of all 561 genes was significantly decreased in chronic CRD exposed animals (Fig. 2a, Mann Whitney test, $p < 0.001$). This lower amplitude explains the lack of significant circadian rhythmicity in gene expression. Furthermore, a limited effect on phase was found (Fig. 2b). These findings are indicative of a re-entrained, yet attenuated circadian rhythmicity in gene expression upon prolonged exposure to weekly LD-inversions.

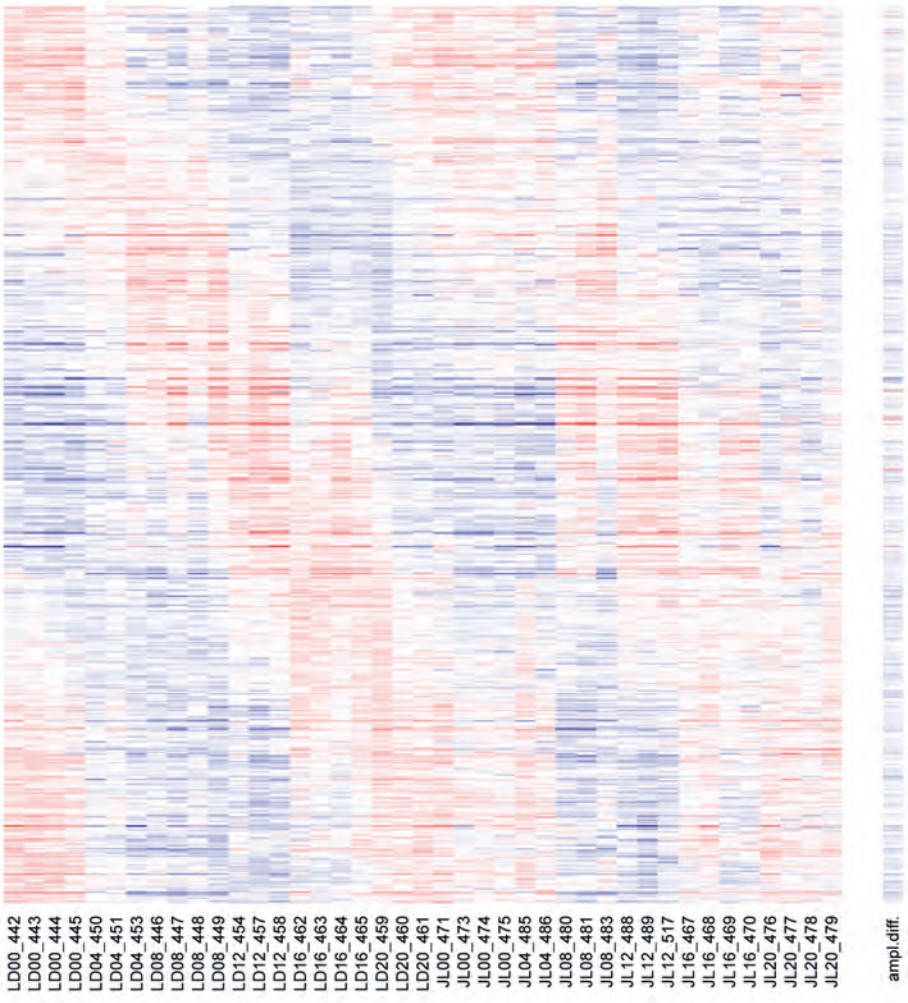

Figure 1. Heatmap of 561 genes that lost significant circadian rhythmicity in gene expression after prolonged exposure to CRD, according to CircWave analysis. It is clearly shown that the circadian rhythm of these genes is not abolished, however the amplitude is decreased in the majority of these genes.

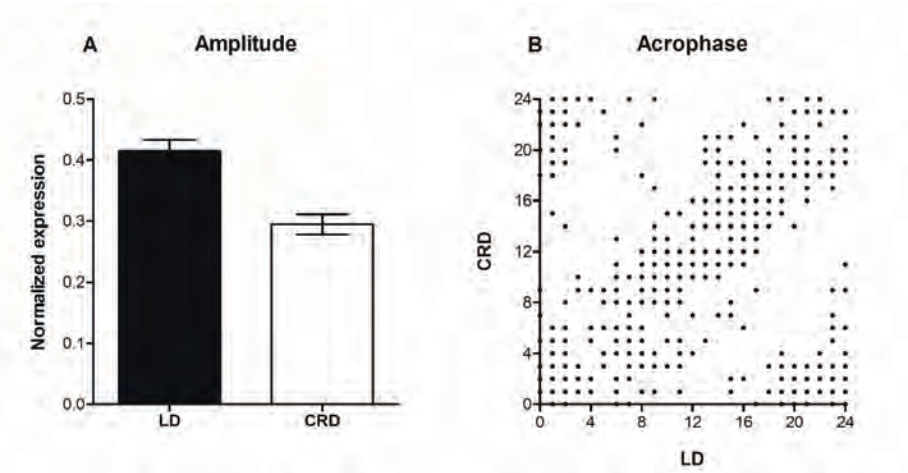

Figure 2. Overview of circadian parameters of genes with significant circadian gene expression under regular LD conditions, lost upon chronic CRD. **A.** The amplitude of these genes was significantly reduced in chronic CRD animals compared to controls **B.** Acrophase was comparable between no large phase shifts were found. All indicative of an attenuated circadian gene expression resulting from chronic exposure to CRD.

Mice exposed to weekly LD-inversions showed a larger increase in relative body weight compared to the animals kept in a stable LD cycle (Van Dycke et al., 2015). To gain insight in mechanisms underlying the observed increased weight gain, we determined enrichment of the 561 genes that lost significant circadian gene expression upon chronic CRD exposure compared to LD conditions in biological processes. Chronic CRD caused attenuation of circadian rhythmicity in genes involved in 237 processes ($p < 0.001$, >10 and <1000 genes in process). The largest groups of processes involve hormonal regulation, response to exogenous compounds and metabolism, comprising both lipid and glucose metabolism (Fig. 3). In addition, the affected genes were associated with circadian rhythm, cell cycle, and signaling processes (Fig. 3). A complete list of significantly enriched processes is given in Supplemental Table S2.

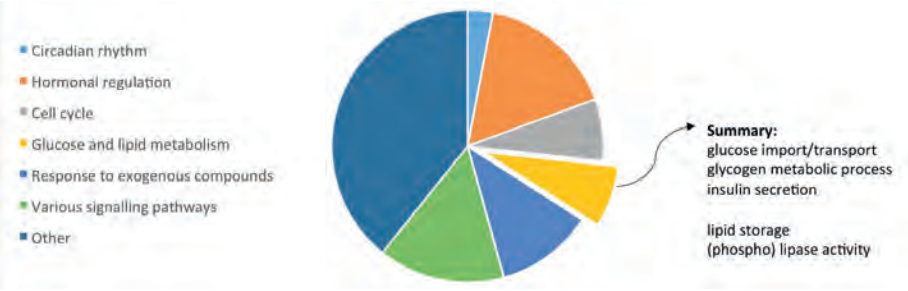

Figure 3. Significantly enriched processes for genes with attenuated circadian rhythmicity in gene expression upon chronic CRD exposure, arranged in themes. In relation to the observed overweight phenotype it is interesting to see decreased amplitude in genes involved in glucose and lipid metabolism processes, especially in glucose transport and storage and also lipid storage.

In addition to chronic CRD affected biological processes, we investigated the top 20 rhythmically expressed genes with the largest relative decrease in amplitude (Supplemental Table S3) in more detail. Since we study gene expression changes 7 days after the last light-dark shift, only minor differences might be expected due to re-entrainment. Within this group of most affected genes regarding amplitude, several metabolic related genes still showed an altered rhythm, including *Ppard*, *Pdk4*, *Slc16a5* and *Igfbp-1* (Fig. 4). Under LD conditions, both the nuclear hormone receptor *Ppard* and isoenzyme *Pdk4* peak at the dark to light transition. Chronic CRD exposure appears to lower peak mRNA expression of these genes, and delay acrophase to early light phase. *Igfbp-1*, which binds IGF and thereby regulates IGF action, shows both alteration of phase and attenuation of the rhythm. Under normal LD conditions, *Igfbp-1* gene expression peaks at the dark to light transition, whereas chronic CRD caused a slightly advanced and lower peak expression. Moreover, the circadian rhythmicity of monocarboxylate transporter *Slc16a5* gene expression is completely abolished in chronic CRD exposed animals.

Disturbance of non-circadian genes and processes

In addition to disturbance of circadian rhythmicity of gene expression, chronic disturbance of the circadian system might also affect non-circadian processes. Using one-way ANOVA, we compared the overall expression of each gene of CRD mice to that of the LD controls, not taking into account time-of-day. The latter was accomplished by combining all time-points per group, comparing the average expression of all CRD exposed mice to the average expression of all LD controls. Surprisingly, we found only 18 genes to be differentially expressed ($p < 0.001$) in CRD exposed animals, as compared to controls (Supplemental Table S4), with

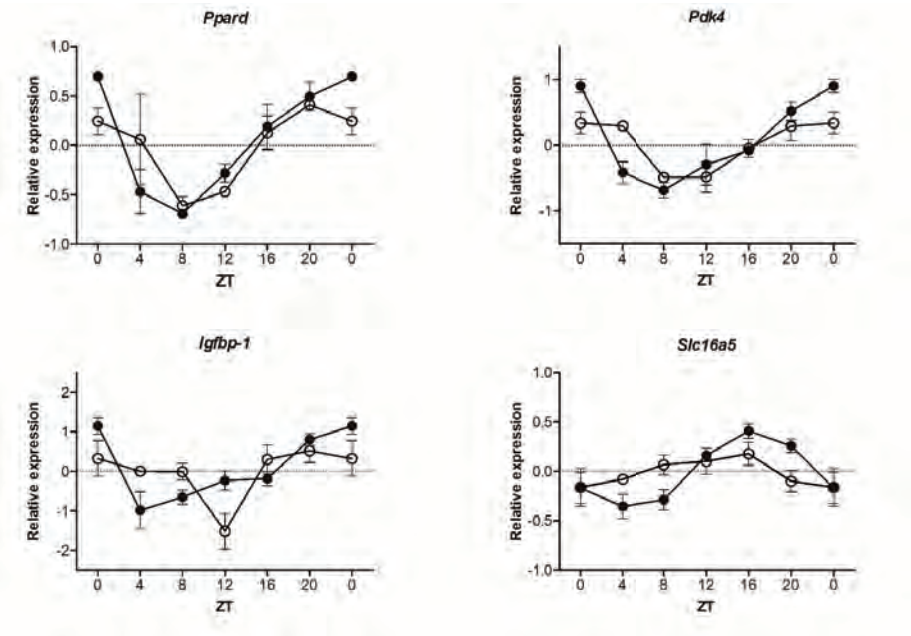

Figure 4. Altered circadian gene expression of *Ppard*, *Pdk4*, *Igfbp-1* and *Slc16a5*, involved in the balance between glucose and lipid metabolism. *Ppard* and *Pdk4* both show a phase delay and decreased amplitude upon chronic CRD (open symbols) compared to LD controls (closed symbols). CRD caused a slight advance and decrease of peak *Igfbp-1* gene expression, with a distinct trough at the light to dark transition. For *Slc16a5* the rhythm is completely abolished in CRD exposed animals.

minor differences in average gene expression. This number is limited, and does not allow pathway analysis. Therefore, Gene Set Enrichment Analysis (GSEA) was used, to identify subtle differences in GO processes or canonical pathways, again using all time-points per group combined. In the GSEA analysis, the complete gene expression dataset is used to study significant overrepresentation of up or down regulated genes between CRD exposed animals and LD controls in gene sets representing GO Processes and other online databases (C2 gene sets). GSEA identified 36 upregulated processes or pathways, mainly involved in DNA replication, transcription, translation and metabolism (Supplemental Table S5). The majority of the 75 downregulated processes identified represented immune, cell cycle and proliferation processes (Supplemental Table S5). Interestingly, also downregulation of several catabolic processes was observed. This analysis indicates that although no major differences in average gene expression between CRD animals and LD controls were found, genes involved in a wide variety of processes are affected by CRD.

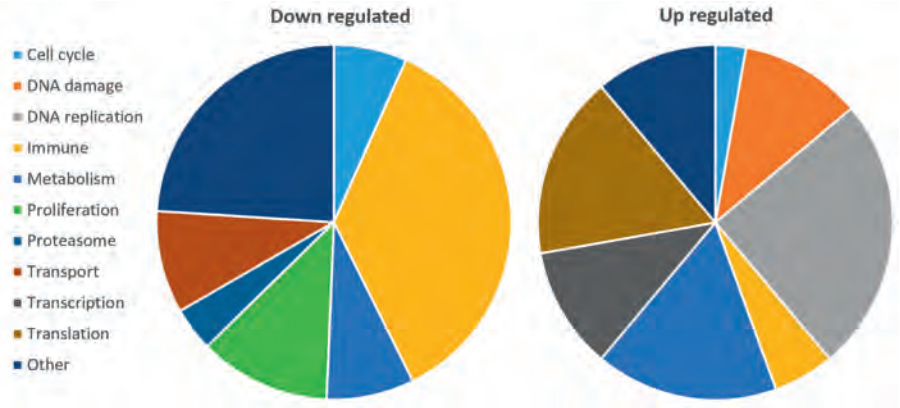

Figure 5. Using Gene Set Enrichment Analysis significant overrepresentation of regulated genes between CRD exposed animals and LD controls in GO processes or the C2 gene sets is studied. The majority gene sets with significant overrepresentation of down regulated genes is involved in immune processes, whereas the up regulated genes are mainly involved in DNA replication.

DISCUSSION

Due to the growing 24/7 economy, shift work will become increasingly part of our society and will, therefore, have an incremental effect on public health outcomes. Understanding mechanisms underlying the adverse health effects originating from chronic circadian rhythm disturbance (CRD) is of prime importance to develop preventive measures for shift work. An experimental set-up inducing CRD, along with a mouse model at risk for the development of breast cancer risk, weight gain and other morbidities, provides a unique tool to identify such underlying disturbed processes (Kubo et al., 2011). The major advantage of this model is the ability to control the exposure and to exclude confounding factors, which both hamper human observational studies. Importantly, we aimed to find constitutively disturbed processes upon long-term exposure to CRD. As such, we studied the hepatic transcriptome of mice exposed to 18 weeks of CRD compared to LD controls, 7 days after the last shift. We focused on identifying disturbances in both circadian and non-circadian genes and processes.

Studying rhythmically expressed genes, we observed weaker rhythmicity in hepatic gene expression upon chronic CRD exposure, affecting a wide variety of molecular processes. This could be indicative for an overall loss of homeostasis, resulting from constant re-entrainment to the chronically alternating light cycle. Although this

finding is generally in line with previous studies that have shown a loss of circadian rhythmicity in the human blood transcriptome resulting from insufficient sleep (Moller-Levet et al., 2013) and mistimed sleep (Archer et al., 2014a), our findings are less pronounced. This can be explained by the fact that we monitored effects only 7 days of recovery after the last shift. This resulted in re-entrainment of classical circadian parameters such as clock gene expression and core body temperature. The loss of significant circadian gene expression is mainly due to a decrease in amplitude, indicating that overall circadian gene expression is also re-entrained, but rhythmicity is attenuated. In short-term experiments as performed by others (Barclay et al., 2012), melatonin, clock genes and clock controlled genes were found to be disturbed, directly after impingement on the circadian system.

In relation to the weight gain phenotype observed in mice under CRD conditions, we found that genes with attenuated circadian expression upon CRD were enriched for processes involved in mainly glucose but also lipid metabolism. Under normal conditions both carbohydrate and lipid metabolism show distinct time-of-day dependent rhythms (Bailey et al., 2014). The attenuation in glucose metabolism processes is in line with recent findings that the circadian system is largely responsible for daily rhythms in glucose tolerance and that separately circadian misalignment reduces glucose tolerance (Morris et al., 2015). Furthermore, GSEA analysis revealed down regulation of catabolic processes, which shows that energy metabolism is also affected in non-circadian genes.

The largest decrease in amplitude was found in genes involved in the balance between lipid and glucose metabolism: *Ppard*, *Pdk4*, *Slc16a5* and *Igfbp-1*. The nuclear receptor *Ppard* has been shown to be a key regulator of *Pdk4* (Degenhardt et al., 2007). This direct regulation could explain the similar changes in circadian expression of both genes. A similar decrease in amplitude of *Pdk4* expression was observed in animals kept on a high fat diet (Eckel-Mahan et al., 2013), and animals that underwent timed sleep restriction (Barclay et al., 2012), both indicative of an interaction between circadian clock and metabolism. Furthermore, *Pdk4* plays an important role in the metabolic flexibility under various nutritional conditions (Zhang et al., 2014b). In response to starvation *Pdk4* mRNA and protein levels in liver increase (Wu et al., 2000). The conversion of pyruvate into Acetyl-CoA is inhibited by *Pdk4* as it inactivates the pyruvate dehydrogenase complex, maintaining blood glucose levels under fasting conditions (Jeoung and Harris, 2010). The observed

circadian rhythm in *Pdk4* expression under LD conditions correspond with daily rhythms in feeding and fasting. Attenuated rhythms in *Pdk4* resulting from chronic CRD exposure might result in limited ability to regulate blood glucose levels.

Little is known about the function of *Slc16a5*, and mainly described as a transporter for diuretics (Murakami et al., 2005). However, other monocarboxylate transporters are involved in energy balance processes (Lengacher et al., 2013). The Insulin-like Growth Factor-1 (IGF-1) axis, including *Igpbp-1*, is likely involved in glucose metabolism, as it can acutely regulate glucose levels (Katz et al., 2002). The combination of decreased amplitudes in *Pdk4*, *Ppard*, *Igfbp-1* and *Slc16a5* and increased body weight gain upon long-term exposure to CRD suggests an imbalance between nutritional status and lipid and glucose metabolism, resulting in obesity. Future studies should specify this imbalance and investigate which interventions could help balance nutritional intake and metabolism to prevent obesity. For example studying the metabolome could indicate which metabolic processes have increased or decreased activity based on the metabolites found to be linked to these processes.

In conclusion, chronic CRD results in weaker circadian rhythmicity of a wide variety of circadian and non-circadian processes, including processes that could be related to the body weight gain phenotype observed in shift workers. Attenuation in circadian rhythmicity of both glucose and lipid metabolism processes and genes involved in their balance provide a preliminary target for intervention. However, the chronic effects are minor compared to the acute effects shown by previous transcriptome analyses (Barclay et al., 2012, Moller-Levet et al., 2013). Taken together, based on these observations one could speculate that the weakened circadian rhythmicity 7 days after the last shift is only a remainder of more severe disturbance at earlier time points e.g. 1 or 2 days after the shift. The relevance of acute and more permanent disturbance of biological processes for adverse health outcomes remains to be determined. Insights herein would provide important input for the discussion whether slow or fast rotating shift schedules should be employed. Additionally, in depth analysis of affected genes and processes and metabolome data should provide further insights in the role of the constitutively disturbed processes in the relation between CRD and increased weight gain.

CHAPTER 7

SUMMARY AND GENERAL DISCUSSION

SUMMARY

Working around the clock as a consequence of our growing 24/7 economy strains and disturbs the tightly regulated circadian system. In epidemiological studies, shift work was associated with many adverse health effects, such as cancer, obesity and cardiovascular disease. These observational human studies are hampered by confounding factors, difficulties with exposure assessment and take many years to complete. Yet, due to the similarities in carcinogenesis (Balmain and Harris, 2000) and the circadian system (Lowrey and Takahashi, 2011) between mice and human, mice provide a unique tool to study the relationship between shift work and cancer. Animal studies are less influenced by confounding factors and therefore provide the ability to study the causal relationship between chronic circadian disturbance and adverse health effects. Additionally, animal studies provide the ability to study the different aspects of shift work and to identify underlying mechanisms and potential biomarkers for circadian rhythm disturbance (CRD) to aid the development and study of preventive measures.

In **chapter 2** the effect of chronically alternating light cycles on breast cancer development and body weight was studied using a unique breast cancer-prone mouse model. We observed alterations in the classical circadian markers body temperature and serum corticosterone, preceding health effects. Whereas age-matched breast cancer prone Li-Fraumeni female mice, housed under normal light dark conditions, displayed a tumor latency time of 50.3 weeks, animals exposed to weekly light-dark (LD) inversions showed a markedly shortened tumor latency time of 42.6 weeks. In addition, CRD animals showed an increase in body weight. To our knowledge this is the first study providing experimental evidence for a causal relationship between circadian rhythm disturbance and breast tumorigenesis.
These findings warrant the identification of biomarkers that evaluate the presence of circadian rhythm disturbance (CRD) and health risk before adverse health effects occur. The use of whole genome gene expression analysis is a valuable approach to identify differentially expressed genes upon CRD in the tissue of interest and select genes that qualify as useful biomarkers. In **chapter 3,** we provided a proof of principle for a comparative transcriptomics approach to identify potentially blood detectable biomarkers for breast cancer using the Li-Fraumeni mouse model. By comparing gene expression profiles of tumor samples with different characteristics to control mammary gland tissue, we determined differentially

expressed gene across tumor subtypes. Subsequently, candidates were prioritized based on possible blood-detectability as determined by cross-referencing with literature databases. Finally, a set of 16 candidate markers for breast cancer was selected based on the expression in tumor compared to normal mammary gland.

As the transcriptomics approach proved to be able to select potentially blood-detectable biomarkers for breast cancer, we employed a similar approach to identify biomarkers for CRD. Although blood-detectable biomarkers for CRD, independent of time of day, can facilitate studies regarding preventive interventions, these biomarkers have not yet been identified. In **chapter 4**, we exposed female mice to 6 shifts in a clockwise (CW) and counterclockwise (CCW) rotating CRD protocol. Hepatic gene expression analysis using a classification approach resulted in a consensus set of 15 genes, able to predict CRD with an accuracy ranging from 90% to 98%. Additionally, to find candidate markers that allow non-invasive detection of CRD, we applied a sequential approach selecting 9 potentially blood-detectable markers. One of these candidates, CD36 was found to be significantly increased in serum, although less pronounced than expected from the gene expression upregulation.

Future research into the adverse health effects of CRD will also require the measurement of disease related biomarker in large-scale cohort studies. **Chapter 5** provides a descriptive study of the daily variation of hormonal and lipid biomarkers in males and females, in a molecular epidemiology-like setting. Using two analysis methods: 1) Cosine fitting analysis and 2) RM-ANOVA, we found diurnal variation in many markers for at least one gender, which exceeded the inter-individual variation. Consequently, when investigating these markers one should take into account diurnal variation. Furthermore, since time-of-day likely matters for additional markers, these findings warrant the need for investigating diurnal variation before setting up studies measuring markers in routine and controlled settings.

In **chapter 6** we describe experiments where mice were exposed to weekly alternating light-dark cycles and the liver transcriptome was analyzed to identify disturbed processes involved in the body weight gain phenotype as observed in **chapter 2**. We found an overall attenuation of circadian rhythmicity in hepatic gene expression, which was less pronounced than previous studies investigating

the effect of insufficient or mistimed sleep on the blood transcriptome. Amplitude was particularly decreased in genes involved in the balance between lipid and glucose metabolism: *Ppard*, *Pdk4*, *Slc16a5* and *Igfbp-1*. Thus, compromised metabolic flexibility might underlie the body weight gain phenotype observed in **chapter 2** and the overweight phenotype in shift workers and therefore provide a preliminary target for intervention.

DISCUSSION

The existence of our 24-hour economy is irreversible and will require an increasing number of individuals to work at irregular hours. This might put them at risk for adverse health effects by constantly straining and disturbing the tightly regulated biological clock. The relationship between the circadian system and cancer has been studied extensively, both in epidemiological studies and animal experiments. Most epidemiological studies point towards an association between shift work and breast cancer, as well as other adverse health effects (IARC, 2010). Unfortunately, the complex mixture of exposures we call shift work hampers the accurate assessment of causality. Furthermore, large scale prospective studies are costly and labor intensive due to the use of classical circadian markers such as melatonin, requiring around the clock measurement. To date, animal studies have emphasized the relationship between the circadian system and cancer. Overall, they have shown that a partially or completely non-functional circadian system result in increased tumor growth. However, studies are lacking that combine relevant models for CRD exposure and adverse health effects such as breast cancer to provide experimental proof for the shift work cancer connection. Notably, there is a gap between epidemiological and animal studies concerning the experimental proof that shift work causes an increased cancer risk. In this thesis we aimed to elucidate the causal relationship between circadian rhythm disturbance and breast cancer development and to provide candidate biomarkers and targets for interventions for future studies.

Causality between CRD and breast cancer and obesity

The findings described in **chapter 2** show that chronic circadian rhythm disturbance (CRD) induced by chronically alternating light schedules decreased latency times of tumor development and increased body weight gain in a breast cancer-prone mouse model. Previous animal experiments, studying the relationship between CRD and cancer differed from this study in the combination of CRD induction methods and the breast cancer models used. For example, some studies addressed tumor growth rather than cancer initiation and progression by using xenograft models (Blask et al., 2003, Filipski et al., 2004) and chemically-induced tumor models (Hamilton, 1969, Shah et al., 1984). Additionally, 'unnatural' continuous bright light exposure and endogenous circadian rhythm disruption models were employed, which result in arrhythmicity (Anisimov et al., 2004, Wu

et al., 2011). To our knowledge, the study as described in **chapter 2** unequivocally shows a link between chronic CRD and breast cancer development, relevant for the human situation.

Shift work is a complex mixture of exposure and different aspects of these exposures have been suggested to underlie the observed cancer risk in individuals involved in shift work. Fritschi *et al.* proposed five mechanisms that should be considered for comprehensive investigation: (i) internal desynchronization, (ii) light at night (resulting in melatonin suppression), (iii) sleep disruption, (iv) lifestyle disturbances and (v) decreased vitamin D levels due to lack of sun light (Fritschi et al., 2011). In **chapter 2** the relevance of these hypotheses was tested and the findings suggest that internal desynchronization and sleep disruption are important in the etiology of CRD-associated health risks and pathologies. The other factors (melatonin suppression, lowered vitamin D levels and life style factors) could be excluded in our study and therefore appear to be less relevant in the shift work cancer relationship. Consequently, sleep disruption and internal desynchronization are targets for preventive measures.

Apparently, the process of carcinogenesis is induced by circadian rhythm disturbance and additional experiments could further elucidate which specific aspects of carcinogenesis is affected. It has been suggested that all aspects of the cellular response to DNA damage are controlled or influenced by the circadian clock (Sancar et al., 2010). Consequently, chronic disturbance of the circadian system is likely to affect these DNA damage control systems in place to prevent the accumulation of mutations, which eventually result in uncontrolled or neoplastic cell growth (Berenblum, 1975). Alternatively, the accumulation of mutations could be the result of increased exposure to endogenous DNA damaging agents. For example, the disturbance of metabolic processes by CRD could increase the amount of reactive oxygen species and consequently increase mutations. Based on the decreased latency time to tumor development observed in **chapter 2** one could hypothesize that the number of mutations increased faster under CRD conditions compared to LD conditions. This hypothesis can be addressed by evaluating the mutation frequency in vivo using a plasmid based transgenic mouse model (Dolle et al., 1996). Besides DNA repair processes, the immune system also plays an important role in breast tumorigenesis (Standish et al., 2008), and circadian disturbance has been shown to affect the immune response (Phillips et al., 2015),

increasing vulnerability for environmental stressors. Furthermore, epigenetic changes resulting from prolonged exposure to CRD could lead to increased tumor development, for example by silencing of tumor suppressor genes.

Besides the increased risk of cancer, epidemiological studies have also indicated an association between social jet-lag, shift work and body weight gain, although again conflicting data exist (Kubo et al., 2011, Nabe-Nielsen et al., 2011, Roenneberg et al., 2012, Parsons et al., 2014). Various mouse studies and the findings described in **chapter 2** have shown that circadian disturbance induced by continuous light, forced activity or a disrupted LD cycle causes an increase in body weight (Coomans et al., 2013, Oishi, 2009, Salgado-Delgado et al., 2008). In the liver, a key organ in glucose and lipid metabolism, approximately 10% of the transcriptome is rhythmically expressed, including metabolic processes (Akhtar et al., 2002, Panda et al., 2002). Furthermore, under normal conditions both carbohydrate and lipid metabolism show distinct time-of-day dependent rhythms (Bailey et al., 2014). We hypothesized that long-term CRD results in more permanent alterations in biological processes relevant for the observed phenotype.

In **chapter 6,** using the transcriptomics technique, we found an overall attenuation of circadian rhythmicity in gene expression 7 days after the last shift. The overall attenuation of circadian gene expression is similar, although less pronounced, in comparison with other studies using an omics approach to find the effects of disrupted sleep (Archer et al., 2014a, Barclay et al., 2012, Moller-Levet et al., 2013). The main difference between our study and others is the timing when the effects are observed. Where others studied circadian rhythmicity of gene expression directly after impingement on the circadian system, we studied the transcriptome 7 days after the last shift of long-term CRD exposure. One could speculate that the weakened circadian rhythmicity 7 days after the last shift is only a remainder of more severe disturbance at earlier time points e.g. 1 or 2 days after the shift. Follow-up studies should further investigate whether the number of genes that show an attenuation in circadian rhythmicity in gene expression and the magnitude of the amplitude decrease correlate with the magnitude of CRD and subsequent health effects.

The largest decrease in amplitude was observed in genes involved in the balance between glucose and lipid metabolism. Further research to find the exact

imbalance in metabolism could provide an interesting target for intervention, with timed nutritional intake. Previous animal studies have shown that timed feeding can indeed (partially) rescue tumor and obese phenotypes (Filipski et al., 2005, Fonken et al., 2010, Oike et al., 2015). In all cases food was restricted to the active phase and was shown to prevent dim light or jet lag induced body weight gain and accelerated xenograft tumor growth by chronic jet lag. The finding that advances and delays in meal-timing affect both circadian rhythms as well as metabolic parameters underlines the importance and potential of meal-timing (Yoon et al., 2012).

Evidence based preventive measures

Due to the high incidence of breast cancer and obesity an increased risk resulting from long-term shift work can have major public health implications. However, shift work is irreversibly linked to our 24/7 society and consequently evidence based interventions to prevent CRD and consequent health effects are needed. Currently advised preventive measures are versatile, including controlled light exposure, schedule adaptations, and behavioral and pharmaceutical interventions (Neil et al., 2014) and underlying philosophies and results are contradictive. Interventions using controlled light exposure are aimed at phase shifting the circadian rhythm and promoting adaptation to work at night. For example, by exposure to bright light during the night shift and wearing dark goggles on the commute home in the morning. This was shown to effectively increase day-time sleep (Boivin et al., 2012, Sasseville and Hebert, 2010) but also without effects on sleep (Budnick et al., 1995). In contrast, changing schedules from slow backwards rotating to fast forward rotation, are based on the principle of limited circadian rhythm adjustment (Knauth, 1996). Implementation of this fast forward rotation schedule has shown contradicting effects on sleep and metabolic markers (Boggild and Jeppesen, 2001, Harma et al., 2006, Viitasalo et al., 2008). A major limitation of these studies is the relatively short follow-up and lack of association with adverse health effects. It is important to acknowledge that the positive effects of interventions on the acute shift work related health complaints, such as sleepiness and gastrointestinal symptoms, do not necessarily represent preventive effects on long-term health effects, such as cancer and obesity.

The importance of preventive measures is also acknowledged by the Dutch government, which requested the Dutch Health council to advice on the availability

of preventive measures (Health Council of the Netherlands, 2015). Based on the afore mentioned review (Neil et al., 2014) and two additional reviews (Ruggiero and Redeker, 2014, Liira et al., 2014), it was concluded that there are no evidence based preventive measures to be recommended for shift workers to suppress long-term health effects. The council expresses the need for both experimental and observational studies that link suggested preventive measures with long-term health effects.

The CRD model in combination with the breast cancer-prone mouse model as used in **chapter 2**, are an ideal starting point to identify evidence based preventive measures that decrease long-term health risk. Firstly, because of the relative short period needed to evaluate the effect of these interventions on long-term health effects compared to human studies. And secondly, the ability to continuously measure activity and core body temperature using a telemetry system, enables the monitoring of circadian system adaptation. Cross-sectional studies or use of reporter mice for clock genes can evaluate whether peripheral clock also adapt, similar to locomotor activity and core body temperature rhythms. (Chaves et al., 2011). Hereby, providing experimental evidence whether interventions promoting adaptation or limiting adjustment of the circadian system are best suited to prevent disease. Furthermore, sleep patterns can be determined using mouse telemetry data as described previously for human wrist actimetry data (Juda et al., 2013b). This is important since sleep duration, timing and quality are often used to describe shift work-related strain (Akerstedt, 2003) or used as post-intervention measures (Neil et al., 2014, Vetter et al., 2015). Overall, specialized animals can be used to screen the variety of preventive measures proposed (Neil et al., 2014) and experimentally proven interventions can be studied in shift workers.

Biomarkers

As described above, many recommendations have been made to alleviate shift work related health effects. Although animal models can be used to identify interventions that are likely preventing adverse health effects, there is a need for scientific evaluation of these preventive measures in shift workers (Richter et al., 2010). Tools that measure the presence of CRD or evaluate the health risk could aid the evaluation of new interventions and provide individual, longitudinal exposure assessment in shift workers. In the context of shift work, two types of biomarkers can be differentiated: 1) biomarkers that report on the presence and magnitude of

circadian disturbance and 2) biomarkers that are associated with disease. The latter do not report on CRD, but can identify health risk which allows early intervention before adverse health effects occur.

Mouse models are frequently applied in biomarker discovery research (Kelly-Spratt et al., 2008). Similarities exist between breast cancer mouse models and human breast cancer, and these mouse models are therefore useful in biomarker discovery studies (Klein et al., 2007, Pitteri et al., 2008). In **chapter 3** the Li-Fraumeni mouse model mimicking human breast cancer development was used to provide proof of principle for the use of transcriptomics in the identification of new blood-based biomarkers for breast cancer. In this study, we used a genomics approach on mammary gland tumor tissue compared to find genes overexpressed in tumor tissue compared to control. Blood detectability was determined based on Gene Ontology 'extracellular' or UniProt 'secreted' annotation, or cross-reference with the Human Plasma Proteome project (Anderson et al., 2004). Additional selection based on actual expression in human breast tumor compared to normal mammary gland tissue yielded a set of 16 promising biomarkers for further validation. Importantly, circadian rhythmicity and food intake responsiveness should be excluded.

After validation, a panel of blood-based biomarkers for breast cancer as identified by this approach appeared a valuable add-on to currently available early detection methods for breast cancer screening. Using sensitive detection techniques, blood-based biomarkers can be detected in small aliquots of blood obtained by a finger prick (Pennings et al., 2014), which certainly is less invasive than undergoing mammography. Furthermore, blood-based biomarkers are not hampered by high density breast tissue, as is the case with mammography. This way, measurement of biomarkers is minimally invasive with relatively low costs, which makes them applicable for use in a large scale population screening programs. People at high risk of breast cancer, including shift workers, could be eligible for a blood test using such a panel of early detection biomarkers. Besides prevention, the most efficient way to reduce cancer mortality and morbidity is detection at an early stage, allowing effective therapeutic treatment. In the future, the breast cancer biomarker panel can even be combined with early detection markers for other tumor types in a multiplex assay.

Upon the proof of principle for a transcriptomics approach in the identification of blood-detectable biomarkers for breast cancer, as provided in **chapter 3**, a similar approach was taken to identify biomarkers for circadian rhythm disturbance in **chapter 4.** In the future, these markers are intended for the use in large cohort studies, such as the Nightingale Study (Pijpe et al., 2014) or the Doetinchem Cohort Study (Verschuren et al., 2008). To be eligible for use in such settings, these universal biomarkers should fulfill the following criteria: 1) independent of rotation schedule; 2) independent of time of day; 3) age-independent and 4) blood-detectable. Recently, transcriptomics- and metabolomics-based time of day independent biomarkers have been identified using jet lag exposed and clock mutant mice (Minami et al., 2009, Ueda et al., 2004), allowing detection of acute and transient effects of CRD, but not chronic effects. In **chapter 4** we identified an optimal CRD classification set based on hepatic gene expression and a selection of potentially blood detectable biomarkers, both focused on time-of-day independent detection of chronic CRD.

Although these selected blood-based biomarkers fulfill the criteria for use in large scale cohort studies, they should be further validated. Importantly, researchers currently using classical circadian markers need to be convinced of the relevance of these markers for CRD and their usefulness in relation to adverse health effects. Longitudinal measurements in both animal and human studies are required to evaluate the association of these biomarkers with CRD exposure duration. In addition to the relationship with exposure, the identified markers should be predictive for CRD associated health risk. Previous studies have shown that comparable chronic CRD protocols increased negative health effects in mice (Davidson et al., 2006, Filipski et al., 2004). Validation of the biomarkers in human studies are required to show whether this association with disease also applies to humans.

As described above, one could also opt for the measurement of disease associated biomarkers in a molecular epidemiology-like setting. Especially the combination of biomarkers for CRD and biomarkers for the adverse health effects could identify individuals at risk for disease development. Hormonal imbalance is often studied in large epidemiological settings since these are associated with high incidence cancer (such as breast cancer), and it is well known that metabolic markers might serve as important (early) indicators for metabolic disorders (Mester et al., 2010,

Stevens, 2005, Ferlay et al., 2015). In relation to shift work, metabolic markers such as cholesterol and triglycerides have been used to evaluate the advantageous effect of altered shift schedules (Orth-Gomer, 1982, Viitasalo et al., 2008). It is known that several disease related biomarkers show a diurnal rhythm in serum or plasma levels. However, most information on diurnal variation is derived from these controlled laboratory studies in which factors influencing diurnal variation (such as food intake and/or sleep/wake cycle) are controlled (for example see references (Klingman et al., 2011, Li et al., 2013, Jung et al., 2010, James et al., 2007, Davies et al., 2014, Ang et al., 2012, Dallmann et al., 2012)). With limited control over environmental factors in large scale cohort studies, it is important to have insight into the daily variation of these markers in a routine non-fasted molecular epidemiology-like setting, with no restrictions on sleep or diet.

In **chapter 5** we have shown that the majority of the hormonal and metabolic markers studied showed a daily variation (circadian rhythms and/or time-of-day-effects that does not fit a cosine curve) in at least one gender. A recent study also showed that 43% of the protein coding genes show diurnal variation in at least one organ in mice (Zhang et al., 2014a), this indicates that daily variation in serum or plasma proteins is likely. In large molecular epidemiological studies, it should be advised to study diurnal variation of markers of interest and take into account when present. Preferably, samples should be taken at one time point. Furthermore, time of sampling and relevant environmental factors, such as food intake, can be registered.

In shift workers, the measurement of markers with circadian rhythmicity is further complicated by variation caused by circadian disturbance, altered sleep timing and food intake and other shift work related factors. Consequently, when variation within groups is increased due to random sampling or disturbance by work schedule, larger group sized are required to increase power do detect differences between groups. To limit the additional variation, samples of biomarkers with diurnal variation in shift workers should best be taken on the last day shift. This way, individuals are least disturbed by work schedule and mostly recovered from previous shifts. Although this depends on chronotype, impingement on sleep wake rhythms by work hours and social responsibilities is limited. And it take several days for disturbed circadian rhythms to reentrain. Preferably, finger prick blood samples, urine samples and saliva samples, when applicable for the marker

of interest, could be taken at home on free days without the interference of work schedule.

Modelling shift work in animal studies

In this thesis, light schedules were altered to cause circadian rhythm disturbance. We have shown that altered light schedules in a mouse model for human breast cancer can provide important insights about health effects observed in human studies. Another important aspect of shiftwork is the activity at times of the day when we would normally be asleep. In humans, this consequently leads to altered meal timing and sleep disruption (Grundy et al., 2009, Lennernas et al., 1995). This can be mimicked by gently handling the animals and exposing them to new objects in their environment during normal sleeping hours, referred to as Timed Sleep Restriction (TSR) (Barclay et al., 2012). However, this labor intensive method is not compatible with long-term studies in large cohorts of animals. Currently, experiments are ongoing with specially developed 'shift work cages' in which the animals are gently, but continuously forced to be active at predefined time windows of the day (mimicking day, evening and night shifts). This set-up has been validated with similar sleep analyses as described in **chapter 2.** In combination with the Li-Fraumeni mouse model, the effect of altered sleep-wake cycle resulting from rotating shift work on breast cancer risk can be addressed.

Although the increased breast cancer risk is probably the most extensively studied cancer type associated with shift work, other types of cancer, such as colorectal and prostate cancer have been prospectively been associated with long-term shift work (Kubo et al., 2006, Schernhammer et al., 2003). Accurate exposure assessment and control for confounding factors is also limited for these types of cancer. Using mouse models for colorectal cancer (Karim and Huso, 2013) or prostate cancer (Ittmann et al., 2013), the effect of chronic CRD, either induced by chronically alternating light cycles or forced activity, on these cancer types can be studied. In addition to the cancer phenotype, other disease pathologies such as diabetes type 2 and obesity can be studies in animal models. Similarly to cancer, epidemiological research for these diseases is confounded by unhealthy habits of shift workers, which can be controlled for in experimental studies.

Future perspectives

The fact that shift work has become an irreversible part of our 24/7 society underlines the importance of unravelling which aspects of shift work are relevant for the observed health effects and how to develop interventions to prevent adverse effects. To further study the relationship between shift work and adverse health effects, it is of utmost importance to inventory what shift work entails and to study the relevance of these individual aspects. This requires the collaboration of researchers with expertise of the circadian system and animal models and researchers with the ability to provide detailed information on human shift work characteristics, to close the gap between animal studies and epidemiological shift work studies in humans. Detailed knowledge on light exposure, food intake, sleep timing, duration and quality and other life style or biological characteristics from shift workers can be used as input for laboratory animal studies. Cross-sectional studies in shift workers and day workers can identify the aspects that are specific for individuals working in shifts. Animal experiments allow the comparison of these individual aspects of shift work or combinations in relation to health effects in a relatively short time frame.

Alternatively, the findings from these animal studies can provide valuable input for the set-up of prospective human research. Upon validation, the candidate biomarkers identified in **chapter 4** are interesting alternatives for the classical markers previously used in human studies. Based on the findings in **chapter 6** and those of others (Filipski et al., 2005, Fonken et al., 2010, Oike et al., 2015) we hypothesized that the timing of food intake is important for both increased body weight and cancer risk. Therefore, prospective human studies would ideally register meal-timing in shift workers to associate this with obesity and cancer risk. Additionally, animal studies are in progress to identify the ideal timing of food intake in a simulated shift work setting in relation to long term health outcome. The most promising findings of such experiments can provide valuable input for food intake interventions to be validated and evaluated in cohort studies with shift workers.

CHAPTER 8

NEDERLANDSE SAMENVATTING
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NEDERLANDSE SAMENVATTING

In vrijwel alle organismen is een biologische klok aanwezig, van fruitvliegjes tot mensen. De biologische klok wordt gedreven door de master klok in de hersenen, de supra chiasmatische nucleus. Deze master klok houdt een ritme aan van ongeveer 24 uur, ook wel circadiaan ritme genoemd, en wordt op tijd gezet door licht. Lichaamstemperatuur en activiteit zijn voorbeelden van processen die door deze master klok geregisseerd worden en dus over de dag variëren. Daarnaast zijn er ook hormonen zoals melatonine en cortisocosteron die een ritme hebben van ongeveer 24 uur. Moleculair gezien bestaat de klok uit verschillende genen die via feedback mechanismen over de dag verschillend tot expressie komen.

Het werken in ploegendienst, als consequentie van onze groeiende 24/7 economie, belast en verstoort het strak gereguleerde biologisch ritme. In epidemiologische studies is het werken in ploegendienst geassocieerd met vele gezondheidsrisico's, zoals kanker, obesitas en cardiovasculaire aandoeningen. Deze observationele humane studies worden gehinderd door verstorende factoren, moeilijkheden met het vaststellen van de blootstelling en nemen daarnaast vele jaren in beslag. Door de sterke overeenkomsten in het carcinogenese en circadiane proces tussen muis en mens bieden muizen een unieke mogelijkheid om de relatie tussen het werken in ploegendienst en kanker beter te onderzoeken. Dierstudies worden niet beïnvloed door verstorende factoren en bieden daardoor de mogelijkheid om de causale relatie tussen chronische circadiane verstoring en lange termijn negatieve gezondheidseffecten te bestuderen. Bovendien kunnen de afzonderlijke aspecten van ploegendienstarbeid onderzocht worden. Door de toepassing van moleculair biologische technieken kunnen ook de onderliggende mechanismen en potentiele biomarkers geïdentificeerd worden, welke worden gebruikt voor de ontwikkeling en studie van preventieve maatregelen.

In **hoofdstuk 2** wordt beschreven wat het effect is van chronische jetlag op borsttumorontwikkeling en lichaamsgewicht, gebruikmakend van een unieke muizen met aanleg voor borstkanker. Dit muismodel heeft een mutatie in de borstklier die ook bij vrouwen met borstkanker voorkomt, hierdoor ontwikkelen deze muizen borsttumoren die vergelijkbaar zijn met humane borsttumoren. Onder normale licht-donkercondities ontwikkelden deze vrouwelijke, kankergevoelige muizen een borsttumor na gemiddeld 50.3 weken. Wanneer dit type muis werd

blootgesteld aan een wekelijkse omkering van het licht-donkerritme ontwikkelden borsttumoren significant eerder, al na gemiddeld 42.6 weken. Bovendien lieten ze een toename in lichaamsgewicht zien. Voor zover bekend is dit de eerste studie die experimenteel bewijs levert voor de causale relatie tussen verstoring van het circadiane ritme en borsttumor ontwikkeling.

Deze bevindingen maken het noodzakelijk om biomarkers te identificeren die de aanwezigheid van circadiane verstoring evalueren voordat negatieve gezondheidseffecten zich openbaren. Hiervoor is het waardevol om de expressie van alle genen te vergelijken tussen een controlegroep en een groep die is blootgesteld aan circadiane verstoring (CRD). De genen die verschillend tot expressie komen, kunnen als basis dienen voor de selectie van bruikbare biomarkers. Het tegelijkertijd in kaart brengen van de expressie van alle genen noemen we transcriptoomanalyse. In **hoofdstuk 3** hebben we een proof-of-principle gegeven voor het gebruik van transcriptoomanalyse om bij de muis biomarkers te identificeren die potentieel in bloed te detecteren zijn. Door de genexpressieprofielen van borsttumoren te vergelijken met controle borstklierweefsel vonden we genen die differentieel tot expressie kwamen in borsttumoren. Vervolgens werden kandidaat biomarkers gerangschikt op basis van potentiële detecteerbaarheid in bloed door vergelijking met literatuur databases. Uiteindelijk werden de 16 meest humaan relevante kandidaat markers geselecteerd.

Tot op heden waren er geen in bloed detecteerbare biomarkers voor CRD beschikbaar die onafhankelijk van het tijdstip van de dag gemeten kunnen worden. Deze biomarkers zijn nodig om studies naar preventieve maatregelen te faciliteren. Omdat transcriptoomanalyse bruikbaar bleek om biomarkers te identificeren die potentieel in bloed detecteerbaar zijn, werd een vergelijkbare aanpak gebruikt voor het vinden van biomarkers voor CRD. In **hoofdstuk 4** onderwierpen we vrouwelijke muizen aan 6 lichtverschuivingen met de klok mee (CW) of tegen de klok in (CCW). Genexpressie analyse van de lever resulteerde in een set van 15 genen, waarmee we in staat waren om de aanwezigheid van circadiane verstoring te voorspellen met een nauwkeurigheid van 90% tot 98%. Daarnaast hebben we een stapsgewijze aanpak gebruikt om kandidaat markers te vinden voor niet-invasieve detectie van CRD. Met deze aanpak selecteerden we 9 potentiële, in bloed detecteerbare markers. Eén van deze markers, CD36 was ook in muizen serum significant verhoogd na CRD.

Toekomstig onderzoek naar de negatieve gezondheidseffecten van CRD vereist ook het meten van ziektegerelateerde biomarkers in grote cohort studies. Het is voor een aantal van deze markers bekend dat ze een circadiaan ritme vertonen. Echter is dit vaak onderzocht onder streng gecontroleerde condities, die niet representatief zijn voor cohort studies. **Hoofdstuk 5** geeft een beschrijving van de dagvariatie van humane hormonale en lipide biomarkers in een setting met beperkte controle over de condities die de variaties beïnvloeden. We vonden een dagvariatie in veel markers die groter was dan de interindividuele variatie. Daarom zou men bij het bestuderen van deze markers de dagvariatie in ogenschouw moeten nemen. Bovendien is het aannemelijk dat het moment van de dag ook van belang is voor additionele markers. Dit vereist dat voor het opzetten van nieuwe studies met een beperkte controle over de meetcondities, vooraf de dagvariatie van de te meten biomarkers bepaald wordt.

In de studie beschreven in **hoofdstuk 6** zijn muizen blootgesteld aan wekelijks alternerende licht donker cycli. Van deze dieren is het transcriptoom van de lever geanalyseerd om verstoorde processen te identificeren die ten grondslag liggen aan het toegenomen lichaamsgewicht, zoals geobserveerd in de dierstudie beschreven in **hoofdstuk 2**. Een algehele afname van de circadiane ritmiek in lever genexpressie werd gevonden, die minder uitgesproken was dan gevonden in eerdere studies. De voornaamste afname in amplitude werd gezien in genen betrokken bij de balans tussen vet en glucose metabolisme: *Ppard*, *Pdk4*, *Slc16a5* and *Igfbp-1*. Het is dus mogelijk dat verstoorde metabole flexibiliteit ten grondslag ligt aan het lichaamsgewichttoename fenotype gezien in **hoofdstuk 2** en het overgewicht in ploegendienstmedewerkers en daarom dus een preliminair aanknopingspunt voor interventie is.

De bevindingen beschreven in dit proefschrift tonen aan dat ploegendienstarbeid negatieve invloed heeft op de gezondheid. Omdat ploegendienstarbeid door de 24/7 economie onlosmakelijk verbonden is aan onze maatschappij, vereist dit preventieve maatregelen om de gezondheid van ploegendienstmedewerkers te waarborgen. Op dit moment zijn er geen wetenschappelijk bewezen interventies beschikbaar, mede doordat langdurig onderzoek nodig is om de effectiviteit op lange termijn gezondheidseffecten te besturen. De ontwikkeling van deze interventies kan gefaciliteerd worden door biomarkers die in een vroegtijdig stadium ziekte of circadiane verstoring kunnen meten. Met behulp van

transcriptoomanalyse is in dit proefschrift de basis gelegd voor de identificatie van deze biomarkers die onafhankelijk van het tijdstip van de dag gemeten kunnen worden. Bovendien kunnen diermodellen op korte termijn waardevolle informatie opleveren over de werkzaamheid van interventies bij mensen. Daarnaast hebben we laten zien dat het voor ziektegerelateerde biomarkers belangrijk is om rekening te houden met de dagvariatie bij het opzetten van humane studies. Ten slotte hebben we metabole flexibiliteit geïdentificeerd als eerste aanknopingspunt voor de benodigde interventies.

CURRICULUM VITAE

Personal information

Name	Kirsten Catharina Gabriëlla Van Dycke
Date of birth	14 November 1986
Place of birth	Arnhem

Education

2007 – 2010	MSc. Biomedical Sciences, Radboud University Nijmegen
2004 – 2007	BSc. Biomedical Sciences, Radboud University Nijmegen
1998 – 2004	VWO, Nature & Health, Roelof van Echten College Hoogeveen

Research Experience

2010-2015 PhD project
Department of Genetics, Erasmus MC, in collaboration with the National Institute for Public Health and the Environment, Prof. G.T.J. van der Horst and Prof. H. van Steeg
Working around the clock: Adverse effects of circadian rhythm disturbance

2010 Thesis Consultancy profile
Dutch Health Council, Dr. H. van Dijk
The role of consensus in policy advising by the Health Council of the Netherlands

2009 Master thesis Health Technology Assessment
McGill University Health Center, Canada, Prof. J. Brophy
Cost-effectiveness of high-sensitivity C-reactive protein screening followed by targeted statin therapy in asymptomatic individuals

2008 Master thesis Toxicology
National Institute for Public Health and the Environment, Dr. M. Luijten
Effects of exposure route on gene expression profiles in the mouse

2007 Bachelor thesis Health Technology Assessment
Comprehensive Cancer Centre East (IKO), Dr. M. de Boer
Cost-effectiveness of adjuvant systemic therapy in patients with early stage breast cancer and micrometastases in the sentinel lymph node

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van Kerkhof LW*, **Van Dycke KC***, Jansen EH, Beekhof PK, Ruskovska T, Velickova N, Kamcev N, Pennings JL, van Steeg H, Rodenburg W. (2015) Diurnal variation of hormonal and lipid biomarkers in humans. PLoS One 18;10(8)
*joint first authors

Van Dycke KC, Rodenburg W, van Oostrom CT, van Kerkhof LW, Pennings JL, Roenneberg T, van Steeg H, van der Horst GT. (2015) Chronically alternating light cycles increase breast cancer risk in mice. Curr Biol 25(14):1932-7

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Rodenburg W. (2015) Biomarkers for circadian rhythm disruption independent of time of day. PLoS One 18;10(5)

Van Dycke KC*, Nijman RM*, Wackers PF, Jonker MJ, Rodenburg W, van Oostrom CT, Salvatori DC, Breit TM, van Steeg H, Luijten M, van der Horst GT. (2014) A day and night difference in the response of the hepatic transcriptome to cyclophosphamide treatment. ArchToxicol 89(2):221-31
*joint first authors

Pennings JL*, **Van Dycke KC***, van Oostrom CT, Kuiper RV, Rodenburg W, de Vries A. (2012) Biomarker discovery using a comparative omics approach in a mouse model developing heterogeneous mammary cancer subtypes. Proteomics 13:2149-57
*joint first authors

de Boer M, Adang EM, **Van Dycke KC**, van Dijck JA, Borm GF, Seferina SC, van Deurzen CH, van Diest PJ, Bult P, Donders AR, Tjan-Heijnen VC. (2012) Cost-effectiveness of adjuvant systemic therapy in low-risk breast cancer patients with nodal isolated tumor cells or micrometastases. AnnOncol 10: 2585-91

PHD PORTFOLIO

Name PhD student: Kirsten Van Dycke	PhD period: 2010-2014
Erasmus MC Department: Genetics	Promotor(s): G.T.J. van der Horst & H. van Steeg
Research School: MGC graduate school	Supervisor: W. Rodenburg

1. PhD training

	Year	Workload (Hours)
General courses		
- Laboratory animal science	2011	120
- Literature discussion	2011	20
- Writing in English for Publication	2013	60
Specific courses (e.g. Research school, Medical Training)		
- CBS Chronobiology Summerschool 2012	2012	40
- Begin R-cursus	2011	4
- Transgenesis, Gene Targeting and in vivo Imaging	2011	40
- Analysis of Microarray Gene Expression data	2011	40

1. PhD training		
	Year	**Workload (Hours)**
Seminars and workshops		
- GZB meetings + work discussions	2010-2014	250
- Center for Timing Research meeting 2011	2011	8
- Leopoldina Symposium (poster presentation)	2012	20
- Center for Timing Research meeting 2012 spring (1-minute presentation)	2012	10
- Center for Timing Research meeting 2012 autumn (oral presentation)	2012	12
- MGC PhD student Workshop Düsseldorf (poster presentation)	2012	40
- MGC PhD student Workshop Luxembourg (oral presentation)	2013	50
- PhD days Nederlandse Vereniging voor Toxicologie (NVT) (poster presentation)	2013	20
- MGC Symposium Rotterdam (oral presentation)	2014	16
- Center for Timing Research meeting 2014 (oral presentation)	2014	12
(Inter)national conferences		
- 12th European Biological Rhythms Society (ERBS) meeting Oxford	2011	40
- Society for Research on Biological Rhythms (SRBR) meeting Big Sky, Montana (oral presentation)	2014	50

2. Teaching		
	Year	**Workload (Hours)**
Other		
- Supervising Junior Science Program	2012	80
- Supervising Bachelor thesis Hogeschool Utrecht	2012-2013	100

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APPENDIX 1

A DAY AND NIGHT DIFFERENCE IN THE RESPONSE OF THE HEPATIC TRANSCRIPTOME TO CYCLOPHOSPHAMIDE TREATMENT

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ABSTRACT

Application of omics-based technologies is a widely used approach in research aiming to improve testing strategies for human health risk assessment. In most of these studies, however, temporal variations in gene expression caused by the circadian clock are a commonly neglected pitfall. In the present study, we investigated the impact of the circadian clock on the response of the hepatic transcriptome after exposure of mice to the chemotherapeutic agent cyclophosphamide (CP). Analysis of the data without considering clock progression revealed common responses in terms of regulated pathways between light and dark phase exposure, including DNA damage, oxidative stress, and a general immune response. The overall response however was stronger in mice exposed during the day. Use of time-matched controls, thereby eliminating non-CP responsive circadian clock controlled genes, showed that this difference in response was actually even more pronounced: CP-related responses were only identified in mice exposed during the day. Only minor differences were found in acute toxicity pathways, namely lymphocyte counts and kidney weights, indicating that gene expression is subject to time of day effects. This study is the first to highlight the impact of the circadian clock on the identification of toxic responses by omics approaches.

INTRODUCTION

Human health risk assessment still requires the use of many laboratory animals to identify hazard and estimate risk of adverse health effects induced by environmental and pharmaceutical chemicals. Toxicogenomics approaches are applied to develop and validate risk profiles of biomarkers, identified by profiling the changes in transcript, protein and/or metabolite levels in *in vivo* exposed animals. The combined use of 'omics' technology and bioinformatics tools across platforms and species further supports the identification of common patterns in the toxic response, as well as the establishment of mechanism-based predictive biomarker sets. However, a commonly neglected pitfall of this approach is that temporal or daily variations in gene expression, as well as protein and metabolite levels, and as a consequence the time of day of exposure, may affect the outcome of a toxicogenomics experiment.

To anticipate the day-night cycle, dictated by the Earth's 24-hour rotation around its axis, most if not all life forms have developed a circadian clock (from the Latin circa diem, "about a day") with a near 24-hour periodicity. This internal timekeeping system imposes day-night rhythms on behavior, physiology and metabolism: *e.g.* sleep-wake cycle, body temperature, blood pressure, energy metabolism and hormone levels. In this way, organisms adjust specific body functions to their special physiological needs during the 24-hour day (Lowrey and Takahashi 2011; Mohawk et al. 2012). In mammals, the circadian system consists of a master clock in the brain, and peripheral clocks in virtually all other cells and tissues. The master clock, comprised of the neurons of the suprachiasmatic nuclei (SCN), is sensitive to retinal light stimuli, allowing the clock to keep phase with the light-dark cycle. In turn, the SCN synchronize peripheral clocks through hormonal and neural factors (Balsalobre et al. 1998; Reppert and Weaver 2002; Weaver 1998; Welsh et al. 2010).

Circadian rhythmicity is a cell-autonomous property. Rhythms are generated by a molecular oscillator, consisting of clock genes and proteins that are organized in positive and negative transcription translation feedback loops (TTFL) and cyclically switch each other on and off (Lee et al. 2001). Briefly, in the positive limb of the TTFL, a heterodimer of the basic loop-helix-loop/PAS (bHLH/PAS) domain containing transcription factors *circadian locomotor output cycles caput* (CLOCK) and *aryl hydrocarbon receptor nuclear translocator-like* (BMAL1) drives the transcription of the *Period* (*Per1* and *Per2*) and *Cryptochrome* (*Cry1* and *Cry2*) genes. In the negative

limb, the PER and CRY proteins shut down transcription of their own genes by forming a heterodimeric complex that translocates to the nucleus and represses CLOCK/BMAL1 driven transcription (Ko and Takahashi 2006; Lee et al. 2001; Reppert and Weaver 2001). This molecular clock is coupled to output processes via a series of clock-controlled genes, including transcription factors, cyclic expression of which further relays rhythmicity to a wider set of genes (Kumaki et al. 2008; Miller et al. 2007; Ueda et al. 2002; Yan et al. 2008). Indeed, various expression profiling studies have shown that, depending on the tissue, up to 10% of the transcriptome is under circadian control, with mRNA expression peaks distributed throughout the circadian cycle (Akhtar et al. 2002; Bozek et al. 2009; Panda et al. 2002; Storch et al. 2002).

The liver plays a key role in most metabolic processes, especially in detoxification of xenobiotics. In this tissue, clock controlled transcription factors cause rhythmic expression of members of the cytochrome P450 (CYP) family and other phase I and phase II enzymes involved in this detoxification (Gachon et al. 2006). Adding to circadian control over xenobiotic metabolism (both activation and inactivation) is the diurnal variation in uptake and renal clearance of toxic substances (Levi and Schibler 2007). Circadian variation in the absorption, metabolism and excretion of toxic substances will ultimately determine the actual concentration to which the cells and tissues are exposed (Paschos et al. 2010). As such, the time of day of exposure to substances such as drugs, environmental chemicals and other xenobiotics may affect the severity of the toxic response, a phenomenon referred to as chronotoxicity.

To obtain insight into how the circadian clock affects the outcome of a transcriptomics experiment, we have performed an *in vivo* study in which mice were exposed to cyclophosphamide (CP) during the day or during the night. CP is a chemotherapeutic and immune suppressive agent that is widely used for treatment of several types of cancer, blood and bone marrow and tissue transplant rejections, and autoimmune disorders (Colvin 1999). As a prodrug, CP requires metabolic activation by cytochrome P450 enzymes (Hales 1982; Hill et al. 1972; Ludeman 1999). The human cytochrome P450 enzymes CYP2B6, CYP2C9, and CYP3A4/5 convert CP into the active metabolite 4-hydroxycyclophosphamide, which is in tautomeric equilibrium with aldophosphamide. A small proportion of aldophosphamide spontaneously decomposes to the genotoxic and carcinogenic

metabolite phosphoramide mustard and the toxic byproduct acrolein (Hales 1982; Kern and Kehrer 2002; King and Perry 2001). As shown here, analysis of the hepatic gene expression profiles of mice exposed to CP during the day or during the night revealed a remarkable time of day of exposure difference in the magnitude of the response. These results highlight the involvement of the circadian clock in genotoxic stress responses and indicate the importance to consider the circadian system when performing toxicological risk analyses studies.

METHODS

Animal experiments

Three-week-old male C57BL/6 mice were purchased from Harlan Laboratories (Horst, The Netherlands) and housed at the animal facilities of the National Institute for Public Health and the Environment (RIVM). Animals were kept under a regular 12-hr light: 12-hr dark cycle and food and water were provided *ad libitum*. All animal experiments were conducted in in accordance with national legislation and were approved by a local ethical committee on experimental animals.

For the microarray study, 8-week-old mice received a single *i.p.* injection of either 300 mg/kg cyclophosphamide (CP, CAS 6055-19-2, Sigma-Aldrich) or the vehicle (PBS) at two different Zeitgeber times, ZT8 and ZT20 (ZT0 = lights on). Animals (n=4 per time point) were sacrificed by cervical dislocation at 0.5, 1, 2 and 4 hours after CP injection (T0.5, T1, T2 and T4), and at 0 and 4 hours after vehicle injection (C0 and C4). The left lobe of the liver was isolated and stored using RNA*later* (Invitrogen, Grand Island, NY, USA) according to the manufacturer's protocol.

For the acute toxicity experiment, three dose groups were used: 30, 100 and 300 mg/kg CP. Eight-week-old mice (n=5 per group) received a single *i.p.* injection of CP or the vehicle (PBS) at ZT8 or ZT20 and were sacrificed by orbital bleeding under Ketamine/Xylazine anesthesia at 24 hours and 72 hours after exposure. Blood was collected in both EDTA and gel collection tubes. Bone marrow was aspirated from femur bones. The left lobe of the liver and the kidneys were removed and collected in formalin.

Microarray analysis

RNA was extracted from all liver samples using the miRNeasy Mini Kit (Qiagen Benelux, Venlo, The Netherlands). RNA concentrations were measured using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, the Netherlands).

RNA was hybridized to Affymetrix HT Mouse Genome 430 PM Array plates (Affymetrix Inc., Santa Clara, CA, USA) at the Microarray Department of the University of Amsterdam, the Netherlands. In short, labeled cRNA samples were prepared with the GeneChip 3" IVT express kit (Affymetrix) as described in the Affymetrix GeneChip HT 3" IVT Express Technical Manual (Affymetrix), using 200 ng of purified total RNA as template for the reaction. The array images were acquired using a GeneChip HT Array Plate Scanner (Affymetrix) and analyzed with Affymetrix HT software suite including expression console software (Affymetrix).

Data analysis

The raw data were subjected to a set of quality control checks. This quality check excluded the presence of significant hybridization and experimental blocking effects. All arrays passed quality control and were annotated according to de Leeuw *et al*. (de Leeuw et al. 2008) and expression values were calculated using the robust multi-array average (RMA) algorithm (Affy package, version 1.22.0; (Irizarry et al. 2003)), available from the Bioconductor project (http://www.bioconductor.org) for the R statistical language (http://cran.r-project.org). After normalization, 35,225 transcripts were used for Principal Component Analysis (PCA; produced in R, version 2.13.1), including non-annotated transcripts and without cut-off for fold change. All 35,225 transcripts were statistically analyzed for differential expression using a mixed linear model with coefficients for each experimental group (Smyth 2004; Wolfinger et al. 2001). For each regime, a contrast analysis was applied to compare each exposure time point with the vehicle control time point C0, and to compare exposure time point T4 with the time-matched vehicle control C4. For hypothesis testing, a permutation-based Fs test was used (Cui et al. 2005). False discovery rate (FDR) correction per contrast was performed according to Storey and Tibshirani (Storey and Tibshirani 2003), with an FDR<0.05 considered as statistically significant. The gene expression results have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (Accession No. GSE46035).

Enrichment of selected gene lists was analyzed with MetaCore Pathway Maps from GeneGo, Inc. (http://www.genego.com/). Gene expression data were imported into Metacore using their NCBI Entrez GeneID as identifier; therefore, only genes annotated with a GeneID in the current NCBI database were used for pathway enrichment analysis. Pathways were considered significantly enriched with an FDR<0.01. Gene Set Enrichment Analysis allowed us to identify significant up- or downregulation of enriched gene sets (P<0.05 and FDR<0.25) (Mootha et al. 2003; Subramanian et al. 2005).

Histology, blood and bone marrow analysis

Paraffin-embedded, formalin fixed liver and kidney sections were stained with Hematoxylin and Eosin (H&E) for histopathological evaluation. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were determined with an auto-analyzer (Cobas Fara, Roche Diagnostics, Woerden, the Netherlands), using dedicated kits from Roche Diagnostics. EDTA blood and bone marrow cells, aspirated from femur bones with impulse cytophotometer solution (composed as described previously in (Tonk et al. 2010)), were analyzed automatically using the ADVIA 2120 (Siemens, Deerfield, USA). Bone marrow cytospin slides (Shandon Cytospin 2, Thermo Fisher Scientific, Waltham, USA) were fixed with methanol, stained according to May Grunwald and Giemsa and analyzed according to OECD Test Guideline 474, Mammalian Erythrocyte Micronucleus Test (OECD). In short, 2000 polychromatic erythrocytes (PCEs) were scored per animal for the incidence of micronucleated PCEs (MNPCEs). To determine myelotoxicity, 500 polychromatic and normochromatic cells (NCE) were scored (PCE/NCE ratio).

All parameter values are represented as means ± SD, where appropriate. Statistical analyses were performed using GraphPad Prism version 6.02 for Windows, (GraphPad Software, La Jolla, California, USA). Comparisons between groups were performed with one-way ANOVA or two-way ANOVA followed Dunnet's test for dose groups and Sidak's test for differences between ZTs. P-values smaller than 0.05 were considered statistically significant.

RESULTS

Gene expression analysis - general impression

Male C57BL/6 mice, kept under a 12-hr light/12-hr dark cycle, were exposed to cyclophosphamide (CP, 300 mg/kg body weight *i.p.*) at two different times during the day, i.e. ZT8 and ZT20. Zeitgeber Time (ZT) is a standard experimental time based on the period of the Zeitgeber, which under aforementioned light regime equals a normal 24-hr day, and in which ZT0 represents "lights on" and ZT12 "lights off". At 0.5, 1, 2 and 4 hours after CP treatment (T0.5, T1, T2 and T4), and 0 and 4 hours after vehicle treatment (C0 and C4), animals were sacrificed and livers were collected for gene expression analysis. To obtain a general impression of the changes in transcription upon exposure to CP at different times of day, we first visualized the overall response using Principal Component Analysis (PCA) (Figure 1). This analysis revealed two distinct clusters, reflecting exposure at the light (ZT8, left half panel) and dark (ZT20, right half panel) phase, with the response becoming stronger over time after exposure (C0, T0.5, T1, T2 and T4 top down). In addition, differences in gene expression between the various time points appeared to be more pronounced after exposure during the light phase (see Figure 1, left panel). From these results, especially those for C4-T4, we conclude that time of day of exposure has a significant impact on the overall response to CP exposure.

Analysis of differentially expressed genes and pathway enrichment

We subsequently analyzed the gene expression data following a commonly used toxicological approach, with ZT8-C0 and ZT20-C0 (time-zero) as the control time points. Differences in hepatic gene expression between CP-treated animals and the vehicle controls were calculated using a one-way ANOVA with an FDR<0.05. This analysis resulted in an increasing number of differentially expressed genes (DEGs) over time, with the highest number of DEGs observed 4 hours after exposure: 5,837 and 2,942 DEGs (including non-annotated genes) at ZT8-T4 and ZT20-T4, respectively (Figure 2).

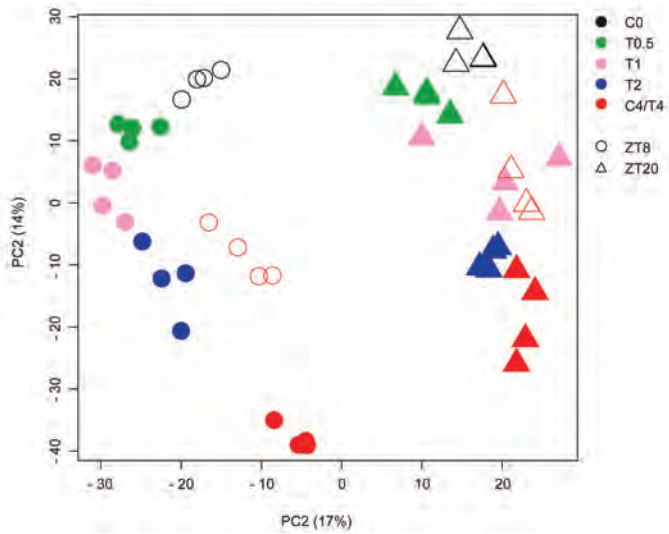

Figure 1. Principal Component Analysis using data of all genes. This analysis revealed two distinct clusters, representing the differential response of the genome to cyclophosphamide exposure at different times of day, ZT8 (circles) and ZT20 (triangles). The response was stronger over time after exposure (C0 to T4, top down); gene expression differences between various time points were more pronounced upon light phase exposure (left half panel). Open and closed symbols represent vehicle control and exposed samples, respectively.

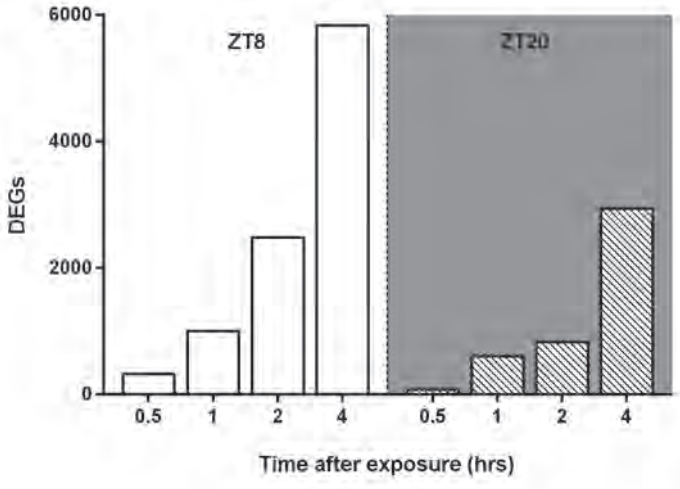

Figure 2. Differentially expressed genes time-zero analysis. The number of differentially expressed genes, including non-annotated genes (DEGs; FDR<0.05) in the liver of CP-treated animals compared to those of vehicle controls obtained at C0. The number of DEGs increases over time, both for animals exposed during the light (ZT8, open bars) and the dark phase (ZT20, dashed bars). However, the number of significantly regulated genes is two- to four-fold lower upon exposure at ZT20 compared to animals exposed at ZT8.

Despite identical exposure, two to four times as many DEGs were found when animals were exposed at ZT8, as compared to exposure at ZT20. To investigate whether the observed differences in the number of significantly regulated genes also resulted in a different biological response, enrichment analyses of MetaCore Pathways was performed. Gene Set Enrichment Analysis (GSEA) was used to obtain an impression of up or down regulation of pathway groups. Since we observed the highest number of significantly expressed genes 4 hours after exposure, we focused on this time point. For these analyses, 4,089 and 2,084 DEGs (excluding non-annotated genes) were used as input for ZT8 and ZT20, respectively. CP exposure at ZT8 caused differential regulation of 125 pathways (Supplementary Table S1a), most of which are consistent with the known effects of CP: genotoxicity, oxidative stress, and immune modulation (Hussain et al. 2013; Rehman et al. 2012; Tripathi and Jena 2008). We found induction of the ATM/ATR pathway and downregulation of pathways involved in cell cycle regulation, which are typically associated with a DNA damage response invoked by the alkylating properties of CP. Besides DNA damage, CP clearly induced oxidative stress as identified by the overrepresentation and upregulation of intracellular signaling cascades such as NF-**k**B, JAK/STAT, AKT/PI3K and MAPK pathways, all known to be activated by oxidative stress. In addition, cell adhesion and cytoskeleton pathways were enriched, probably in response to oxidative stress induced protein degradation. The liver is predominantly an organ of innate immunity, favoring a general defense response to stressors. Induction of several cytokine pathways, such as IL-1 and IL-6, is indicative of this general immune or stress response. This response is most likely coming from liver sinusoidal endothelial cells (LSEC) and Kupffer cells, which are known to be more sensitive to CP than hepatocytes (DeLeve 1996).

Exposure at ZT20 resulted in a far lower number of significantly responding pathways (*i.e.* 52 versus 125, see Supplementary Table S1b), which nonetheless share a strong overall similarity to those observed upon exposure at ZT8. Apparently, the response of the hepatic transcriptome is markedly weaker after CP treatment at ZT20. Interestingly, a DNA damage response, clearly visible at ZT8, was not significantly activated at ZT20 when using significantly regulated genes as input (Supplementary Table S1b). The observed differences in pathway regulation between the two time points may be due to the difference in number of genes used as input for the enrichment analyses. We therefore matched the number of input genes for ZT20 with ZT8, and used the top 4,089 genes (ranked according

to FDR). This more balanced approach not only yielded an increased number of pathways (from 52 up to 188), but, more importantly, also revealed a pattern of responsive pathways that is similar to the one observed for ZT8 (Supplementary Table S1c and S1a, respectively). Overall, for both time points, the DNA damage, oxidative stress and immune response as described above could be identified using this approach. Taken together, these pathway analyses suggest that the response to CP exposure at ZT20 is comparable to the response observed at ZT8, but that the effects in terms of gene expression changes are much stronger at ZT8.

Time-matched analysis of differentially expressed genes and pathway enrichment

As evident from the PCA plot (Figure 1), the factor time of day of exposure resulted in a distinct expression profile, since the profiles of the control group at time point t=0 hours (C0) were markedly different from those at time point t=4 hours (C4), both for ZT8 and ZT20. This indicates that also in non-exposed animals gene expression patterns change over time, which is likely attributed to circadian clock progression. Accordingly, the commonly time-zero analysis (as presented in the previous section) identifies a mixture of (i) circadian clock controlled genes, (ii) CP responsive genes, and (iii) expression of circadian genes influenced by CP exposure. To take into account the circadian regulation of gene expression, we repeated the aforementioned analyses with time-matched controls, 4 hours after exposure (C4). As expected, this time-matched analysis resulted in a lower number of DEGs for both ZT8 and ZT20, due to exclusion of genes that are under control of the circadian clock and do not respond to CP. We cross-referenced DEGs for time-zero analysis and time-matched analysis with clock controlled genes from 5 different literature data sets (Akhtar et al. 2002; Hughes et al. 2010; Miller et al. 2007; Panda et al. 2002; Ueda et al. 2002). A loss of circadian regulated genes using the time-matched analysis was observed. Remaining differentially expressed genes are a mixture of CP responsive genes and circadian genes affected by CP exposure The number of DEGS, however, remained higher at ZT8 than at ZT20, *i.e.* 2,015 versus 179 DEGs (including non-annotated genes) (Supplementary Figure S1).

To illustrate the effect of time-matched analysis in comparison to the time-zero analysis, we examined the cytochrome P450 genes involved in the biotransformation of CP (Cyp2b10, Cyp2c29, and Cyp3a13; the mouse homologues of human CYP2B6, CYP2C9 and CYP3A4/5, respectively). Although, these genes

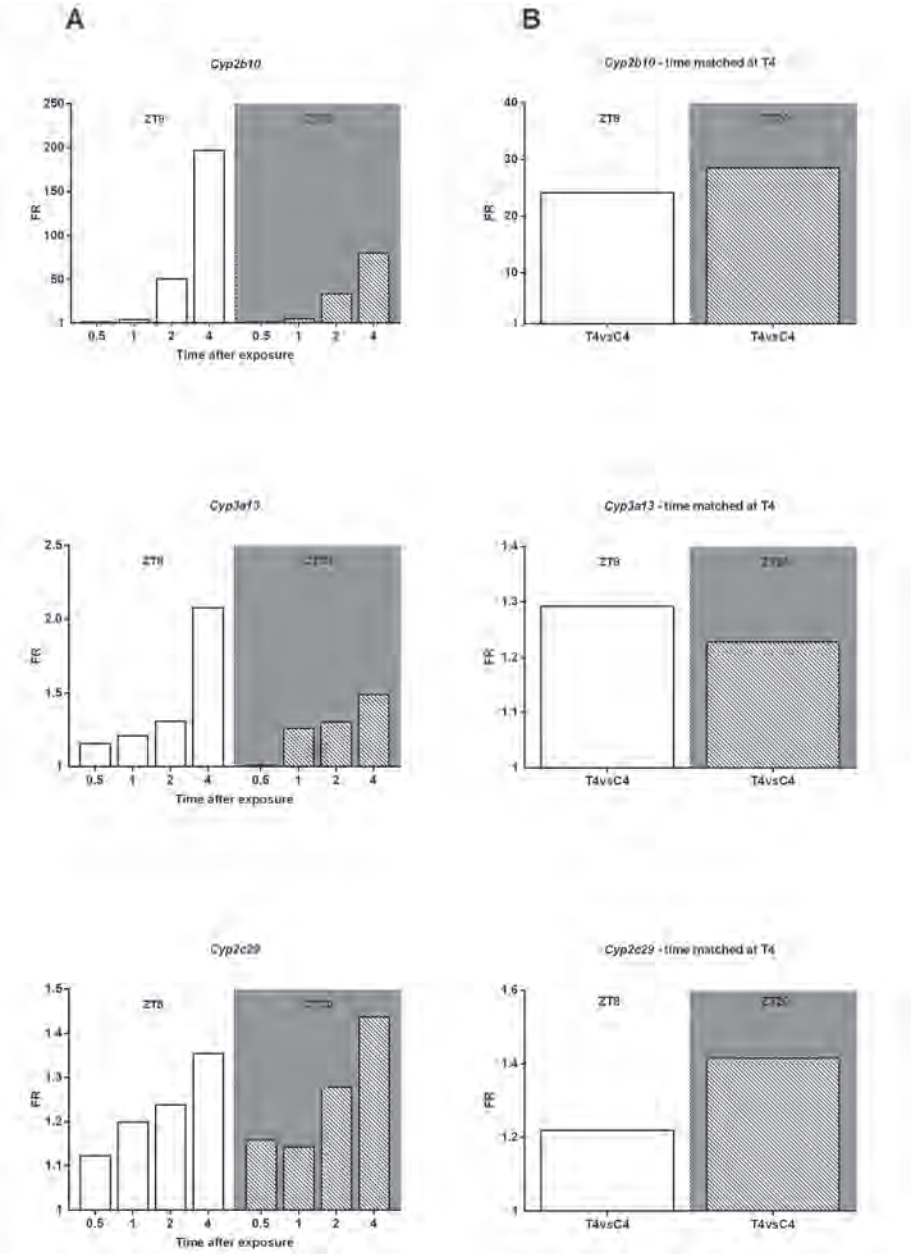

Figure 3. Cytochrome P450 gene expression upon CP exposure. Expression of cytochrome P450 genes encoding enzymes involved in the metabolism of CP (*Cyp2b10*, *Cyp2c29*, and *Cyp3a13*; the mouse homologues of *CYP2B6*, *CYP2C9* and *CYP3A4/5*, respectively) A. Fold Ratio (FR) of liver gene expression in CP-treated animals compared to that of vehicle control animals obtained at C0. All genes showed a time-dependent response to CP treatment from 0.5 until 4 hours after exposure. However, the magnitude of the response differed between light phase (ZT8) and dark phase exposure (ZT20). B. FR of liver gene expression

of CP-treated animals 4 hours after exposure compared to time-matched vehicle controls obtained at C4. Only a marginal difference in *Cyp2c29* gene expression induction seemed to be caused by time of day of exposure. *Cyp2b10* and *Cyp3a13* show no difference in gene expression between circadian phases upon CP exposure.

were significantly induced at both ZT8 and ZT20 when using time-zero controls, fold ratios (induction) clearly differed between ZT8 and ZT20 exposure (Figure 3A). However, time-matched analysis of the expression levels of these CP metabolizing cytochrome P450 genes revealed that induction levels were comparable at both circadian phases (Figure 3B). Other examples of genes for which a time-matched analysis affects fold ratio differences between ZT8 and ZT20 exposed animals, in comparison to time-zero analyses are shown in Supplementary Figure S2.

Analyses directed towards functional enrichment showed 79 significantly responsive pathways for treatment at ZT8 (1,353 annotated genes, FDR<0.01 for significant pathways; Supplementary Table S2a), of which 51 overlapped with those found at this treatment time in the previous C0 control based pathway analysis. The observed 79 pathways included pathways involved in DNA damage response, as well as oxidative stress related pathways such as NF-κB and MAPK signaling. However, cell adhesion and cytoskeleton remodeling pathways associated with oxidative stress induced protein damage were not as pronounced as compared to the analysis using C0 as a control. On the other hand, immune related pathways showed a comparable induction as previously found. We did not find any functional enrichment at ZT20, owing to the low number of input genes, *i.e.* 112 annotated genes. Use of an equal number (top 1,353; ranked according to FDR) of input genes resulted in only eight significantly enriched pathways that, not surprisingly, only marginally reflect a response typical for CP exposure (Supplementary Table S2c).

Thus, the use of time-matched controls uncovered marked differences in the transcriptomic response of the liver when exposed at ZT20 and ZT8. Exposure at ZT8 induced an unambiguous CP response, whereas the known CP effects could hardly be detected after exposure at ZT20.

Time-zero versus time-matched analysis of pathway enrichment

The pathway analyses described in the previous sections, using either time-zero (C0) or time-matched (C4) controls, were performed with a different number of input genes, which hampers a direct comparison of their effect. We therefore re-

analyzed enrichment of responsive biological pathways for the time-zero data set, using the same number of input genes as used for the time-matched analysis. Using the top 1,353 annotated genes, we still noticed a strong CP mediated toxic response after treatment at ZT8, as evident from the enrichment in DNA damage, oxidative stress and immune responsive pathways, while such response now appeared virtually absent when mice were treated at ZT20 (Supplementary Table S3a and S3b, respectively). A comparison of the number of significantly enriched MetaCore Pathways Maps, grouped per biological theme (Table 1) reveals that a reduced number of input genes for the time zero analysis results in a differential response at ZT8 and ZT20 that matches well with that observed when time-matched controls are used. Taken together, our study, especially our time-matched analysis, has shown that although CP exposure induces cytochrome P450 gene expression equally at ZT8 and ZT20, a robust transcriptional response of the liver is only visible at ZT8.

Table 1. Summary of MetaCore Pathway Maps enrichment

	C0 controls		C4 controls	
	ZT8	ZT20	ZT8	ZT20
Oxidative stress response	36	6	35	2
Immune response	17	1	20	0
DNA damage response	14	0	14	4
Other	2	0	0	2

Number of significantly enriched MetaCore Pathways Maps, grouped per biological theme. Top 1,353 genes (ranked based on FDR) were used as input for this analysis. Unless stated otherwise, pathways were grouped based on the main MetaCore Pathway biological function groups (Supplementary Tables S1b and S1c).

Acute toxicity effects

To correlate the observed changes in gene expression to more traditional toxicological endpoints, we conducted an acute toxicity experiment with a similar study design as used for the transcriptomics study. Animals were exposed to a single dose of 0, 30, 100 or 300 mg/kg CP at ZT8 or ZT20 and sacrificed 24 and 72 hours after treatment. Body and organ weights were collected, and white blood cell (WBC) counts and micronuclei formation were determined. In addition, we performed a histopathological analysis of the liver and kidneys. Adverse effects of CP became apparent by body weight loss at the highest doses of CP, irrespective

of whether animals were treated at ZT8 or ZT20 (Supplementary Figure S3). We also observed a slight, dose dependent decrease in kidney weight at 72 hours after exposure in the ZT8 group, whereas no such effect was seen in the ZT20 group (Supplementary Figure S4). Histopathology of the kidney, however, did not reveal any abnormalities in both groups. Likewise, we did not find any effect of CP exposure on the liver for all parameters examined. Liver weight loss was not observed (Supplementary Figure S5), and analysis of histopathology and serum levels of the toxicity serum marker enzymes ALT and AST did not reveal any abnormalities (data not shown).

However, CP exposure at ZT8 and at ZT20 resulted in a dose dependent decrease in the overall number of white blood cells (Supplementary Table S4). This is largely accounted for by a loss of lymphocytes, which showed a very similar response (Figure 4). Exposure to a low dose of CP at ZT8 caused a significantly stronger reduction in the number of lymphocytes after 24 hours than exposure at ZT20. This effect however was no longer observed 72 hours after exposure. In bone marrow, CP exposure resulted in a dose dependent induction of micronucleated polychromatic erythrocytes (MNPCEs), a well-accepted parameter for genotoxicity, except for the highest dose group (Supplementary Figure S6). Lack of micronuclei formation at this dose is most likely due to myelotoxicity as indicated by the decreased PCE/NCE ratio (Supplementary Figure S6). Despite the clear effect of CP exposure on micronuclei formation, we did not find a difference in micronuclei induction between the two ZT groups.

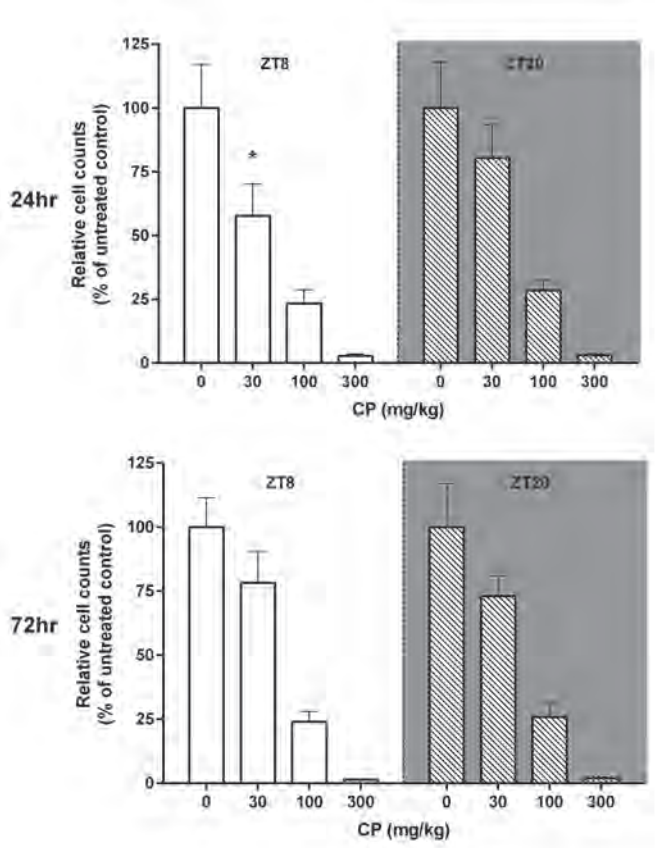

Figure 4. Lymphocyte counts in peripheral blood of CP exposed mice. Lymphocyte counts were measured 24 and 72 hours after exposure. Counts are depicted relative to vehicle controls per circadian phase, values represent mean ± sd. Exposure to 30 mg/kg CP at ZT8 significantly more reduced the number of lymphocytes at 24 hours than in the ZT20 exposed animal (Sidak's multiple comparisons test, $P<0.05$).

DISCUSSION

In toxicogenomic studies time of day of exposure of an animal to a (geno)toxic chemical is usually not taken into account. However, it has become evidently clear that the time of day of exposure can markedly influence the magnitude of the biological response, which is referred to as chronotoxicity (Levi et al. 2010; Levi and Schibler 2007), and which can be expected to translate into pronounced differences in the outcome of transcriptomics experiments (Destici et al. 2009). Likewise, with 10% of the active genes in a tissue being controlled by the clock,

one can envisage that the choice of the control time point (i.e. a time-zero control, taken at the start of exposure, or a time-matched control, taken when the animal is sacrificed) can be of influence. In the current study, we addressed the impact of the circadian clock on toxicogenomics experiments by exposing mice to a single dose of cyclophosphamide (CP) at defined time-points during the day (ZT8) and night (ZT20) and analyzing the response of the liver transcriptome.

Initially, following a commonly used toxicological approach that uses the zero time points as a control, we observed a more than two-fold stronger response of the liver transcriptome at ZT8 compared to ZT20, as evident from the number of significantly differentially expressed genes (5,837 versus 2,942 DEGs). Subsequent pathway analysis with the top 4,098 annotated genes revealed common CP toxicity associated responses (i.e. DNA damage response, oxidative stress and immune related pathways (Hussain et al. 2013; Rehman et al. 2012; Tripathi and Jena 2008)), independent of the time of exposure. As expected, when repeating the analysis using time-matched controls, we observed an overall reduction of the number of differentially expressed genes at both ZT8 (from 5,837 to 2,015 DEGs) and ZT20 (from 2,942 to 179 DEGs), partially because we filtered out the clock-controlled, CP non-responsive DEGs. Strikingly, this approach uncovered a massive impact of time of day effect of CP exposure on the hepatic transcriptome, being 10-fold more responsive at ZT8 than at ZT20 (2,015 versus 179 DEGs). Moreover, after subjecting the top 1,353 annotated genes to pathway enrichment analysis, the DNA damage, immune and oxidative stress responses, remained clearly visible at ZT8, but were markedly less intense (DNA damage response), or virtually absent (immune and oxidative stress response) at ZT20. Apparently, when time-matched controls are omitted, circadian clock progression during the experiment can partially blur the outcome of these experiments when DEGs are applied for functional analysis. However, the identified responsive pathways using an equal number of input genes as shown in Table 1 clearly show that the difference in liver responsiveness to CP can be identified using C0 and C4 controls.

Our transcriptomics data point towards an increased responsiveness of the liver to CP exclusively during the light phase. Upon exposure at ZT8, we observed a marked induction of CP toxicity related pathways, whereas virtually no such pathways were found for animals exposed at ZT20. Thus, the challenging question arises which (clock-controlled) mechanisms are underlying this day and night

difference in CP sensitivity. We have shown that CP exposure at ZT8 or ZT20 results in a comparable upregulation of *Cyp2b10*, *Cyp3a13* and *Cyp2c29* expression, which implies that the rate of CP metabolization does not depend on the time of drug administration. Indeed, a pharmacokinetics study of Gorbacheva and coworkers has shown that the formation and clearance of CP metabolites does not show time of day dependency [38]. Accordingly, the presence and absence of a CP-mediated toxic response at ZT8 and ZT20, respectively, is unlikely to originate from differences in the induction of DNA damage and rather relates to a differential magnitude of downstream events such as DNA damage signaling, DNA repair and apoptosis.

It has been suggested that all aspects of the cellular response to DNA damage are controlled or influenced by the circadian clock (Sancar et al. 2010). The CLOCK/BMAL1 complex positively regulates the expression of the *p21* gene, which plays a critical role in the G1/S checkpoint (Grechez-Cassiau et al. 2008) as well as the *p53* gene, involved in DNA damage initiated or intrinsic apoptosis pathways (Mullenders et al. 2009). Both *p21* and *p53* show low expression at the light dark transition in the Mouse1.OST dataset of the CircaDB database (http://circadb.org) (Pizarro et al. 2013). As *Bmal1* gene expression is also at its lowest around ZT12, it is tempting to speculate that this implies low expression of CLOCK/BMAL1 target genes at ZT8, which in turn results in a greater dynamic range for the CP response. This would fit with the observation that circadian clock deficient *Cry1/Cry2* double knockout mice (constitutive high expression of E-box genes) are more tolerant to CP, while *Clock* mutant and *Bmal1* knockout mice (constitutive low expression of E-box genes) appear to be more sensitive (Gorbacheva et al. 2005).

Various animal studies have demonstrated that the time of administration can cause changes in the biological outcome upon exposure to CP and some other chemotherapeutic agents (Blumenthal et al. 2001; Gorbacheva et al. 2005; Granda et al. 2001; Ohdo 2010). Compounds like celocoxib, doxorubicin and docetaxel were best tolerated during the light phase, around ZT7 (Blumenthal et al. 2001; Granda et al. 2001). Also for CP, wild type mice seemed to be more tolerant when exposed at the end of the light phase (i.e. ZT10-ZT14) than when exposed at the end of the dark phase (i.e. ZT18-ZT2), as demonstrated by a higher mortality rate (Gorbacheva et al. 2005). Remarkably, our transcriptomics data contrast these survival data in that the liver appears more sensitive to CP during the light phase. Upon exposure at ZT8, we observed activation of CP toxicity related pathways, whereas virtually no

such pathways were induced in the liver of animals exposed during the dark phase (ZT20). This increase in sensitivity is supported by the slightly lower lymphocyte counts in ZT8 exposed animals as compared to ZT20 exposed animals. In contrast, the increased responsiveness of hepatic gene expression could be an enhanced adaptive response to CP induced toxicity. As a result, ZT8 exposed animals are better primed to CP exposure. Using gene-expression, we cannot differentiate between increased sensitivity or enhanced adaptive response. Although we used the same mouse strain, a major difference between our study and the study by Gorbacheva and co-workers is the number of doses used. We employed a single dose, whereas studies by Gorbacheva *et al.* were done using multiple doses of CP. In addition, the time points used for the resting and the active phase were slightly different: we used ZT8/ZT20 instead of ZT10-14/ZT22-2 as used by Gorbacheva, respectively.

Despite the substantial changes in hepatic gene expression, we did not observe pathological changes in the liver of CP exposed mice. CP exposure has been reported to change the ultrastructural organization of the hepatocytes and SECs (Lushnikova et al. 2011). Probably, a single dose is not able to induce pathological changes or, alternatively, these changes only may become apparent at later time points. C57Bl6 mice have been reported to be less sensitive to CP-induced hepatoxicity than other mouse strains (Anton 1993). Other parameters, however, such as body weight, kidneys weights, white blood cell counts (WBC) and micronuclei induction were affected by CP exposure. CP is well-known for its toxic effects, including immunosuppression and DNA damage induction (Emadi et al. 2009), as also uncovered by our transcriptomics study. For example, CP has been reported to induce toxicity to B lymphocytes (Colvin 1999). Although in our study lymphocytes were not differentiated, the decrease in WBC could be contributed to the decrease in all lymphocytes. Overall, ZT8 exposed mice appeared to be somewhat more sensitive than ZT20 animals. These findings indicate that gene expression, in comparison to more traditional toxicity parameters, is more sensitive to time of day effects. Possibly, the observed differences in transcription do become apparent in apical end points at later time points.

In conclusion, this study highlights the importance of time of day in toxicity testing, especially in toxicogenomics studies. To our knowledge, exposures in *in vivo* rodent studies occur mainly during the day, which is comparable with ZT8

exposure in our study. For risk-assessment purposes, it is of the utmost importance to evaluate the worst-case scenario when estimating the risk of adverse health effects induced by environmental chemicals. For assessment of CP toxicity in mice, this seems to be exposure during the day. Whether ZT8 is the most sensitive time point, and whether this sensitivity to toxic effects of chemicals is higher in mice during the light phase in general, warrants further investigation. From a regulatory point of view, it would also be worthwhile to examine possible effects of the circadian clock on the outcome of *in vitro* toxicity tests. Furthermore, circadian sensitivity to pharmaceuticals might also be beneficial when used in a clinical setting, to minimize adverse effects and maximize efficacy, as has been shown for certain anti-cancer drugs (Levi et al. 2011). Thus, considering the circadian clock in toxicity testing might eventually lead to improved risk assessment and a more safe use of chemical substances, as well as to improved medicine use.

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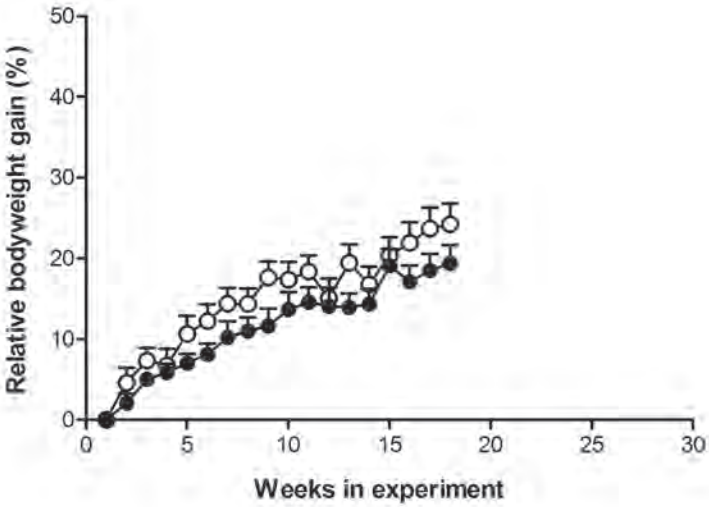
APPENDIX 2

SUPPLEMENTAL DATA

Supplemental data Chapter 2



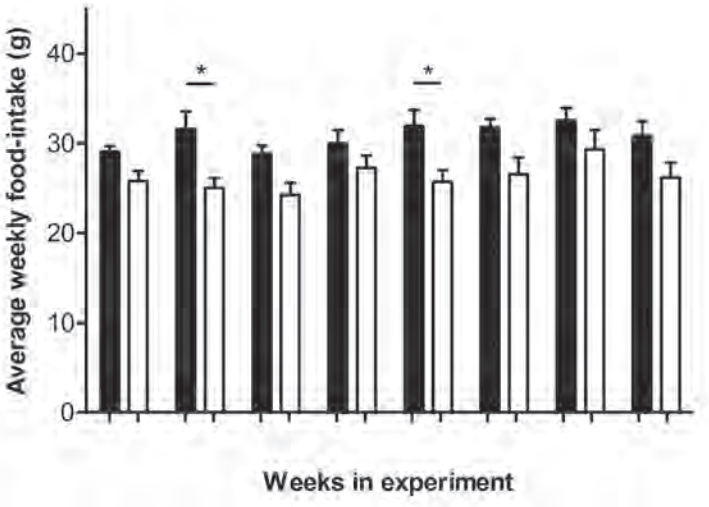


Figure S1. For the cross-sectional study **(a)** the differences in body weight increase between the groups was not significant (RM-ANOVA, group: $F(1, 40) = 2.255$, $p=0.1410$; time: $F(17, 680) = 53.74$, $p<0.0001$; interaction: $F(17, 680) = 1.046$, $p=0.4049$). The body weight gain is expressed relative to the body weights of animals at the start of the experiment (mean + SEM). **(b)** Food intake was slightly, but significantly (Two-way ANOVA, group: $F(7, 108) = 0.4663$, $p<0.0001$), lower in the animals kept under LD-inversion conditions (open bars) compared to the LD controls (closed bars) (*Sidak's posttest $p<0.05$).

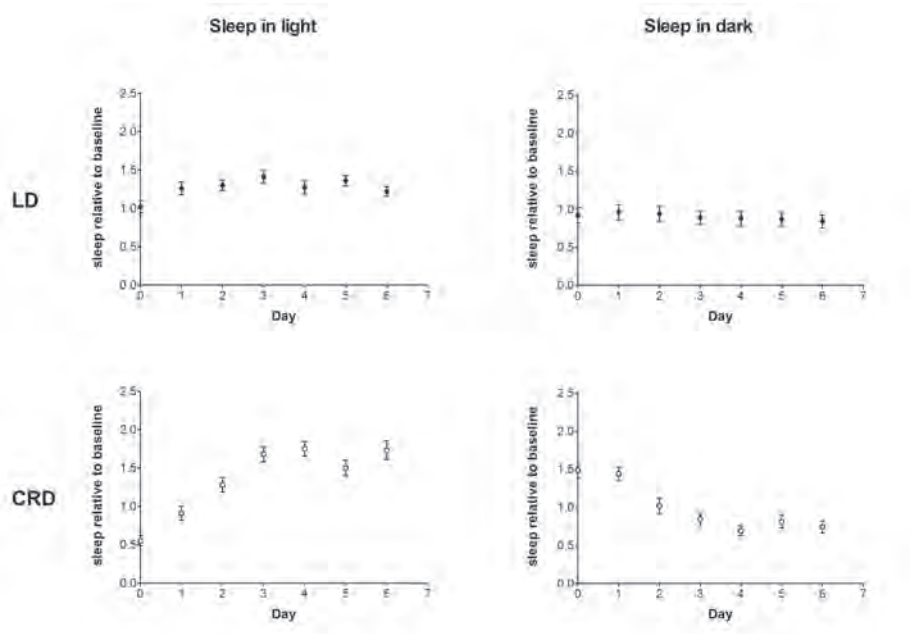
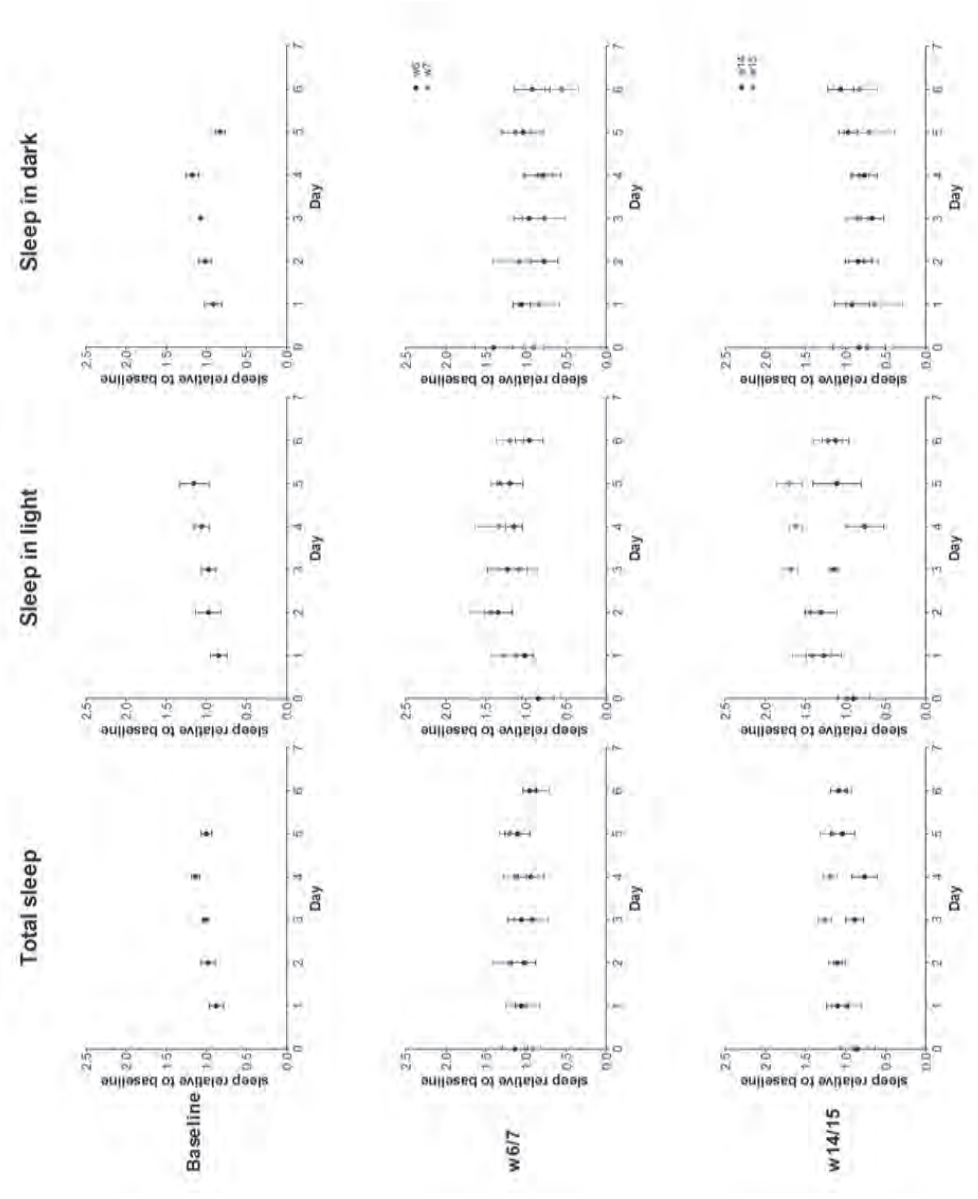


Figure S2. Left and right panels show sleep timing for LD controls at different days of the week and animals exposed to LD-inversions at different days after an LD-inversion, for light and dark phase, respectively. Day 0 corresponds to the day the light schedule inverses and day 1-6 indicate the number of days after the inversion. The timing of sleep continuously changes due to the alternating light schedule. Sleep in light instantly decreases at the day of the shift and subsequently increases the following days. Sleep in dark shows an inverse pattern. For separate analyses of the recorded weeks, see Figure S3a and Figure S3b.


Total sleep
Sleep in light
Sleep in dark
Baseline
w6/7
w14/15
sleep relative to baseline
Day
w6
w7
w14
w15

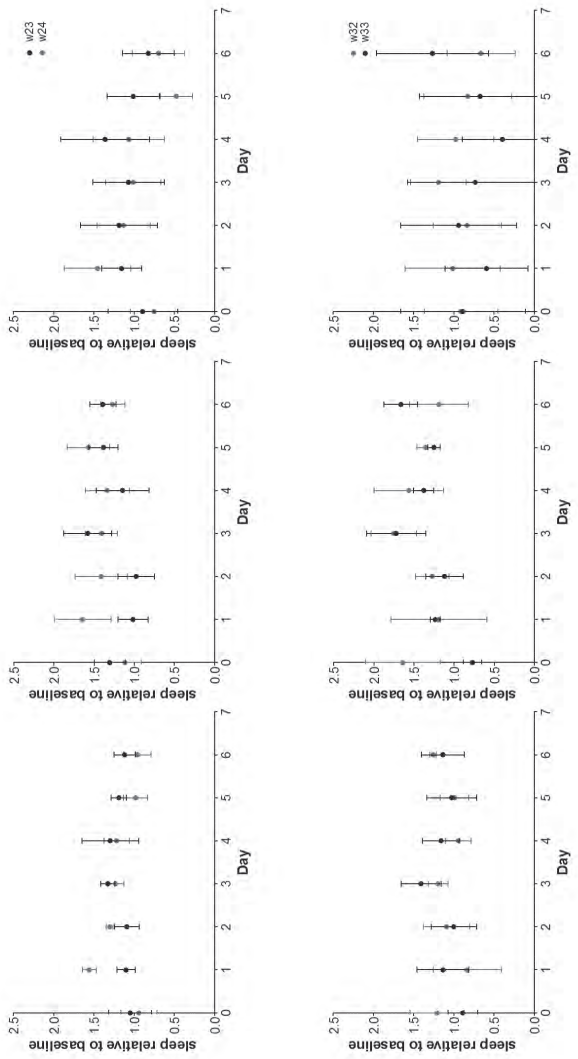
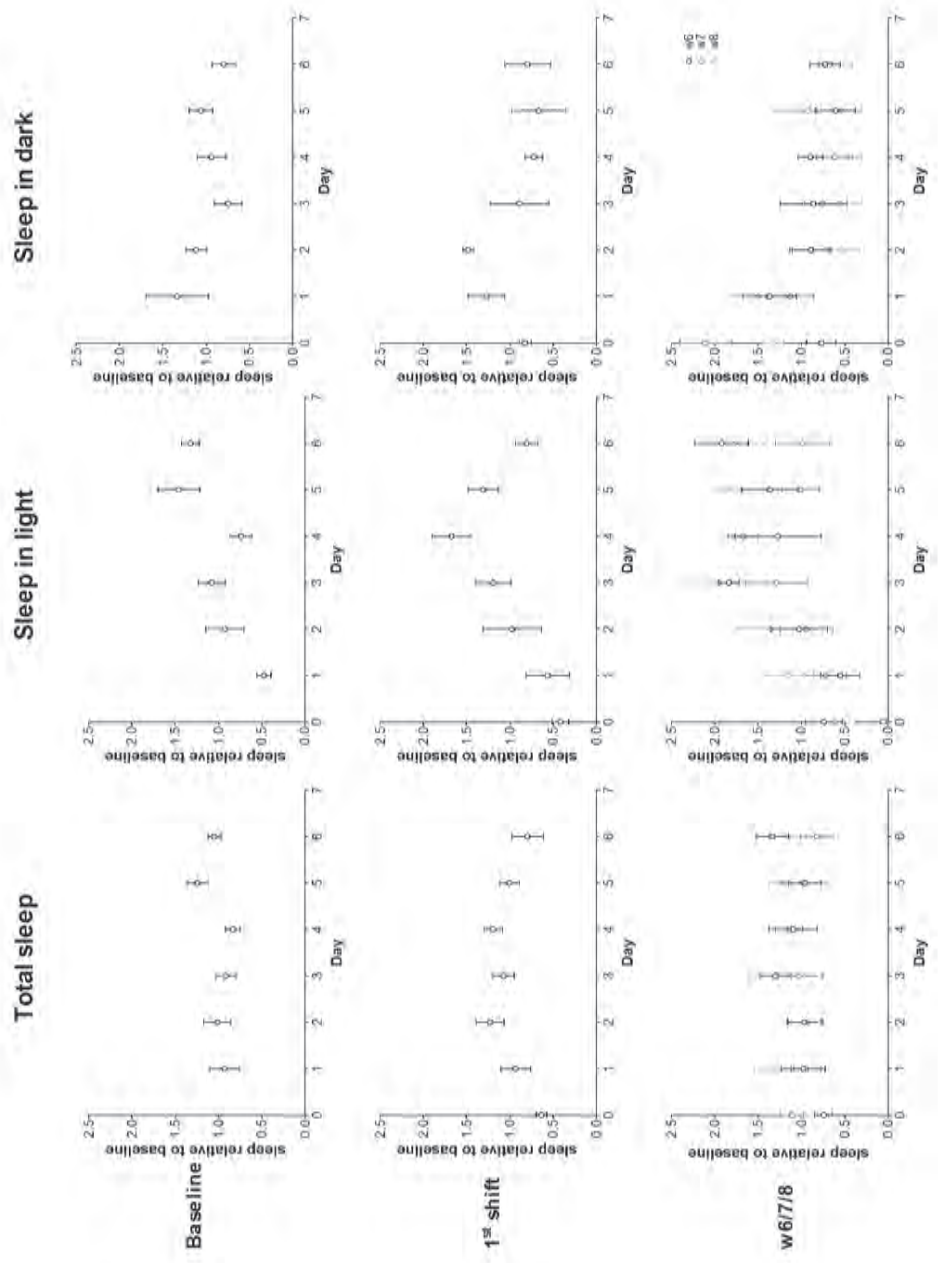


Figure S3a. Total sleep and sleep timing for LD controls at different days of the week.


Total sleep
Sleep in light
Sleep in dark
Baseline
1st shift
w6/7/8
sleep relative to baseline
Day
w6
w7
w8

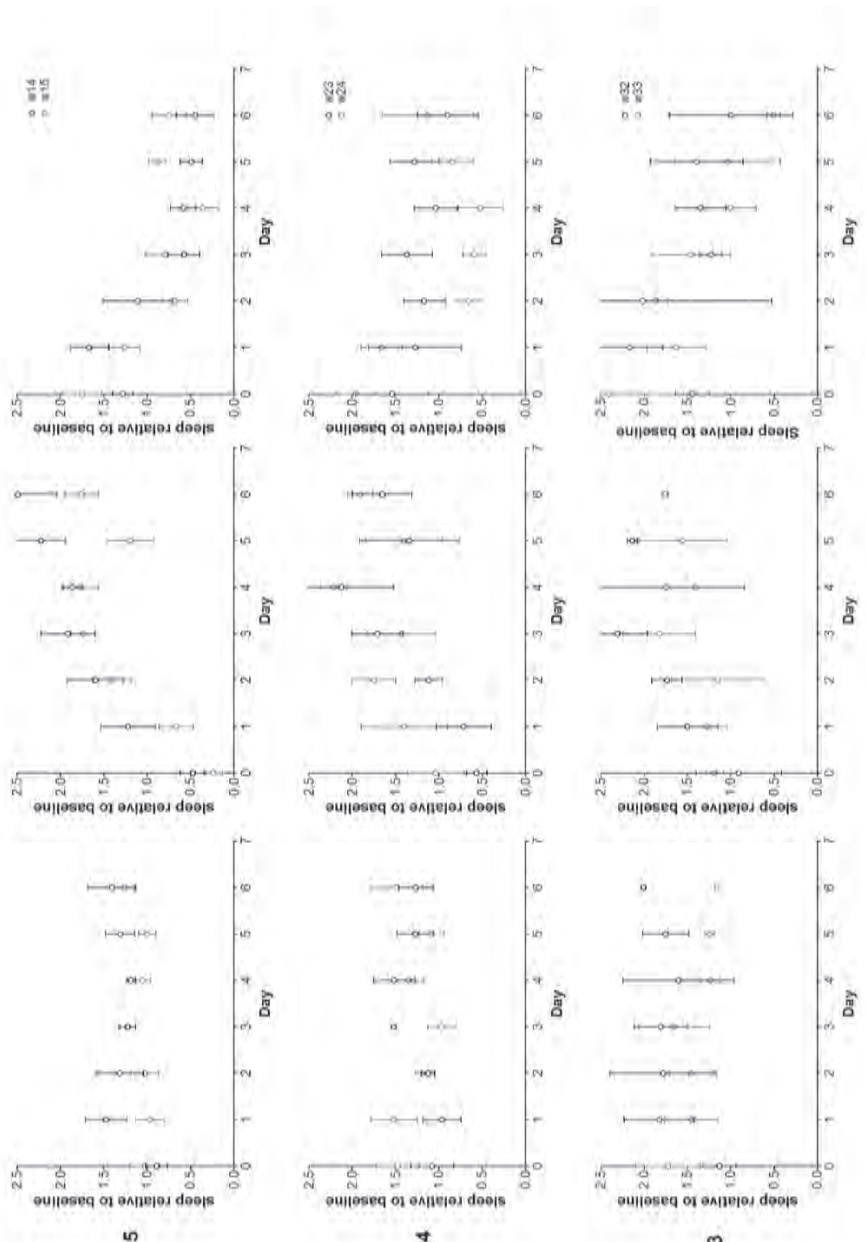


Figure S3b. Total sleep and sleep timing for animals exposed to weekly LD-inversions at subsequent days after an LD-inversion.

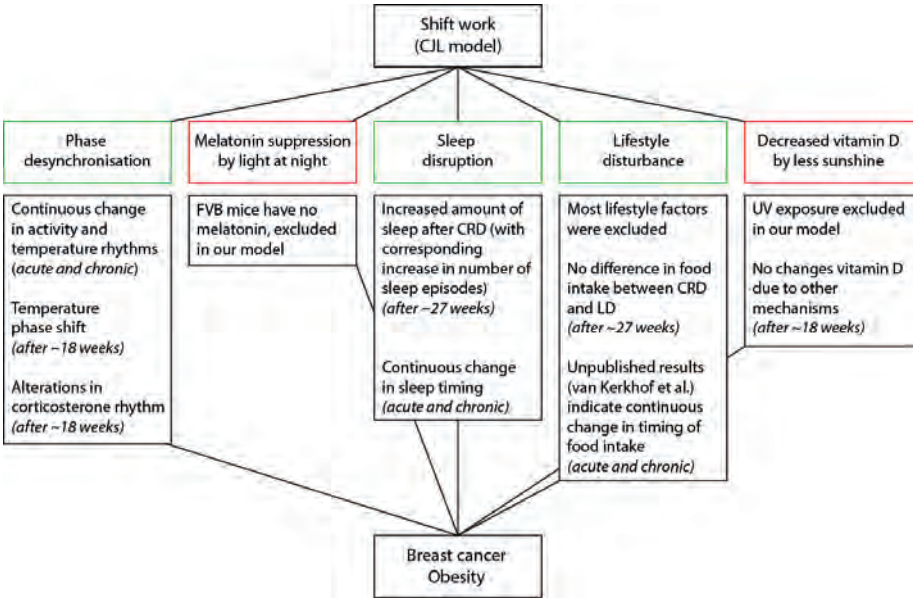


Figure S4. Hypotheses linking shift work with cancer (adapted from Fritschi et al., 2011). The present study underlines the importance of internal desynchronisation, sleep disruption and timing of food intake in the relationship between shift work and adverse health effects, especially breast cancer and obesity. Melatonin suppression and decreased vitamin D by less sunshine exposure were excluded in our model.

Table S1. Pathology data

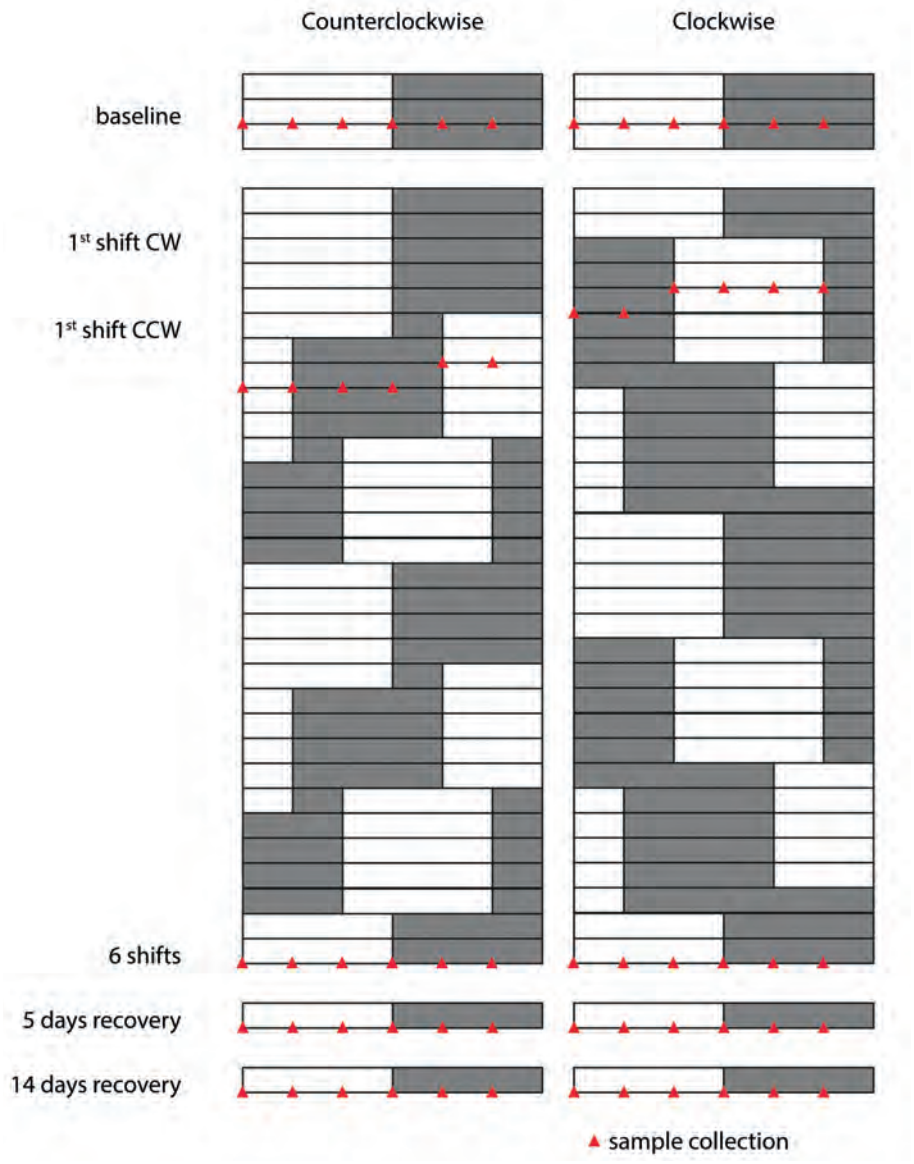
Tumor spectra		
	LD	LD-inversions
Total number of mice analyzed	20	21
Tumor-bearing mice	20	21
Mammary gland tumor-bearing mice	16	16
Mammary gland tumor[#]	18	17
Carcinoma	5	1
Fibrosarcoma/Carcinosarcoma	12	15
Intraepithelial neoplasia	1	1
Mammary gland hyperplasia	-	2
Lymphosarcoma	3	5
Other tumors	5	7

[#]Total number of mammary gland tumors per group, multiple tumors were found in some animals.

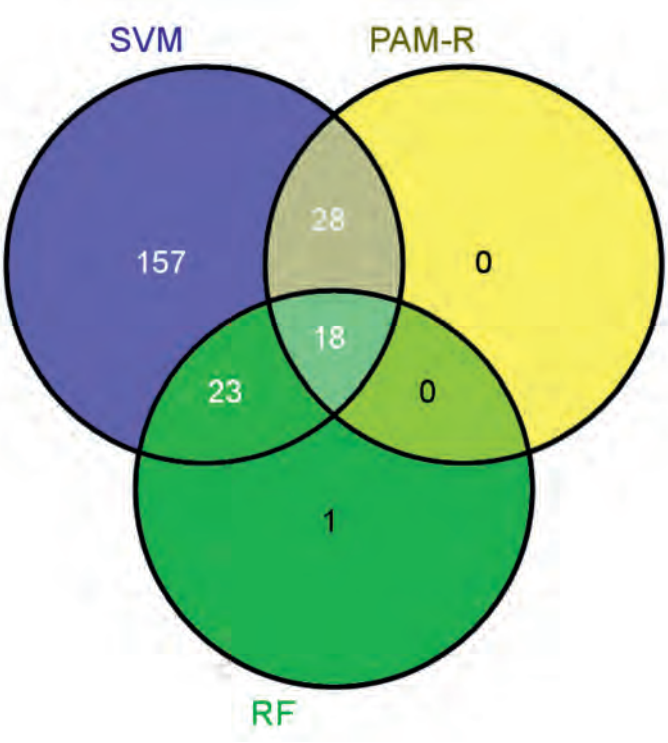
Supplemental data Chapter 3

The supplemental data of chapter 3 comprise three tables that are too extensive to be added in print. These tables are publically available via: http://onlinelibrary.wiley.com/doi/10.1002/pmic.201100497/abstract

Supplemental data Chapter 4



Supporting Information Figure S1. Schematic overview of the experimental set up and time of sample collection. White areas indicate lights on, grey areas indicate lights off.



Supporting Information Figure S2. Venn-diagram of selected hepatic gene expression biomarkers using the three classifier algorithms. The consensus gene set consists of the 15 genes overlapping between the three algorithms.

Supporting Information Table S1. Results of prediction of the three classifier algorithms, using the consensus gene set (A-C). A summary of the prediction accuracy and other prediction parameters is shown in (D).

A.		SVM		
		Non-CRD	CRD	
Experiment	Non-CRD	23	1	**24**
	CRD	0	24	**24**
		23	**25**	

B.		PAM-R		
		Non-CRD	CRD	
Experiment	Non-CRD	21	3	**24**
	CRD	0	24	**24**
		21	**27**	

C.		RF		
		Non-CRD	CRD	
Experiment	Non-CRD	21	3	**24**
	CRD	2	22	**24**
		23	**25**	

D.	SVM	PAM-R	RF
Accuracy	97.9%	93.6%	89.6%
Sensitivity	100%	100%	91.7%
Specificity	95.8%	87.5%	87.5%
Positive predictive value	96.0%	88.9%	88.0%
Negative predictive value	100%	100%	91.3%

Supporting Information Table S2. Ability of the consensus gene set to correctly classify phase-shifted animals as CRD after one shift, six shifts or five days recovery using the three classifier algorithms.

	Sensitivity		
	SVM	PAM-R	RF
1 shift	33.3%	29.2%	29.2%
6 shifts	87.5%	91.7%	83.3%
5 days recovery	83.3%	87.5%	75.0%

Supporting Information Table S3. Sequential approach gene selection. Supplemental Table S3 is too extensive to be added in print. This table is publically available via: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4436131/

Supplemental data Chapter 5

Supplementary Table S1. Participant characteristics. Age is provided in years (y), weight in kilograms (kg), waist circumference in centimeters (cm), total sleep duration between the first sampling time at 08:00 AM and the last sampling time at 04:00 AM in hours (hr), sleep timing of sleep episodes between the first sampling time at 08:00 AM and the last sampling time at 04:00 AM provided in clock time (CT).

Participant	Gender	Age (y)	Weight (kg)	Waist circumference (cm)	Total sleep duration (hr) between 08:00 and 04:00	Sleep timing (CT) between 08:00 and 04:00
F1	f	22	85	80	1.25	02:00-03:15
F2	f	22	52	68	5.42	13:30-14:30, 22:00-23:30, 00:45-3:40
F3	f	22	50	83	3.00	00:15-03:15
F4	f	22	49	69	3.00	00:15-03:15
F5	f	22	48	67	3.00	00:15-03:15
F6	f	22	50	69	3.00	00:15-03:15
F7	f	21	59	75	6.50	09:00-10:00, 17:00-19:00, 00:15-03:45
F8	f	21	55	77	4.75	16:15-18:00, 00:15-03:15
F9	f	21	82	93	0.00	none
F10	f	22	55	74	2.50	00:30-03:00
M1	m	41	105	123	4.00	14:00:15:30, 00:30-03:00
M2	m	24	100	110	3.00	00:30-03:30
M3	m	23	72	86	2.00	01:00-03:00
M4	m	21	70	83	5.00	20:30-23:30, 01:00-03:00
M5	m	21	88	85	3.00	00:15-03:15
M6	m	23	79	79	3.33	00:30-03:50
M7	m	21	75	90	3.67	00:10-03:50
Females						
Mean		**21.70**	**58.50**	**75.50**	**3.24**	
SD		**0.48**	**13.61**	**8.17**	**1.91**	
Males						
Mean		**24.86**	**84.14**	**93.71**	**3.43**	
SD		**7.22**	**13.90**	**16.35**	**0.94**	

Supplementary Table S2. Overview of methods used to determine parameters in plasma and serum. The intra assay variation (CV) was determined with three quality control samples (N=8) for the markers as determined on the auto-analyzer and with two quality control samples (N=5) for markers as measured on the immune-analyzer. The CVs of markers measured with the Luminex technique were obtained from the manufacturer.

	Technique	CV	Source
ACTH	Luminex (Millipore)	10.8	Plasma
CORT	Immune-analyzer (Access-2, Beckman-Coulter)	6.2	Serum
DHEAS	Immune-analyzer (Access-2, Beckman-Coulter)	3.9	Serum
E2	Immune-analyzer (Access-2, Beckman-Coulter)	6.3	Serum
FSH	Luminex (Millipore)	7.2	Plasma
hGH	Immune-analyzer (Access-2, Beckman-Coulter)	2.3	Serum
LH	Luminex (Millipore)	6.3	Plasma
PRL	Immune-analyzer (Access-2, Beckman-Coulter)	1.8	Serum
PRG	Immune-analyzer (Access-2, Beckman-Coulter)	10.8	Serum
TEST	Immune-analyzer (Access-2, Beckman-Coulter)	1.4	Serum
TotT3	Immune-analyzer (Access-2, Beckman-Coulter)	4.2	Serum
TSH	Immune-analyzer (Access-2, Beckman-Coulter)	1.4	Serum
FFA	Auto-analyzer (Unicel DxC 800, Beckman-Coulter)	4.4	Plasma
HDL	Auto-analyzer (Unicel DxC 800, Beckman-Coulter)	4.0	Plasma
LDL	Auto-analyzer (Unicel DxC 800, Beckman-Coulter)	7.2	Plasma
TG	Auto-analyzer (Unicel DxC 800, Beckman-Coulter)	4.3	Plasma
CHOL	Auto-analyzer (Unicel DxC 800, Beckman-Coulter)	4.5	Plasma

Supplementary Table S3. Overview of hormonal parameters in serum or plasma for **A.** CircWave analysis of circadian rhythms; **B.** Repeated-Measures ANOVA to determine daily variation. Amplitude is presented as the % of the median. Ct = Clock time.

A.	Circwave analysis					
	males			females		
Markers	**p-value**	**peak (CT)**	**amplitude**	**p-value**	**peak (CT)**	**amplitude**
ACTH	0.219	17:54	6.12%	0.714	17:22	3.71%
CORT	0.000	07:47	129.11%	0.000	07:29	161.63%
DHEAS	0.861	20:13	21.89%	0.571	18:23	21.36%
E2	0.306	06:51	24.66%	0.685	05:52	25.11%
FSH	0.964	02:03	40.47%	0.741	18:41	31.20%
hGH	0.895	04:13	581.23%	0.953	11:31	397.11%
LH	0.391	00:36	59.22%	0.968	07:15	45.25%
PRL	0.194	04:55	49.11%	0.081	06:20	97.09%
PRG	0.003	07:52	117.78%	0.870	06:17	44.70%
TEST	0.055	07:33	38.38%	0.282	07:38	22.75%
TotT3	0.025	06:18	10.14%	0.041	07:04	8.08%
TSH	0.001	01:54	75.89%	0.006	03:22	74.26%

B.	RM-ANOVA all time points			
	males		females	
Markers	**p-value**	**F-value**	**p-value**	**F-value**
ACTH	0.288	F (2.277, 13.66) = 1.375	0.215	F (3.037, 27.33) = 1.775
CORT	0.003	F (2.611, 15.67) = 7.614	0.000	F (3.466, 31.19) = 20.56
DHEAS	0.045	F (2.389, 14.33) = 3.694	0.002	F (2.943, 26.49) = 6.530
E2	0.438	F (2.760, 16.56) = 0.938	0.154	F (3.873, 34.85) = 1.792
FSH	0.517	F (1.990, 11.94) = 0.695	0.512	F (2.079, 18.71) = 0.687
hGH	0.184	F (1.465, 8.792) = 2.091	0.459	F (2.243, 20.18) = 0.838
LH	0.198	F (2.794, 16.76) = 1.746	0.061	F (3.491, 31.42) = 2.617
PRL	0.014	F (2.715, 16.29) = 4.984	0.118	F (1.865, 16.79) = 2.461
PRG	0.020	F (2.310, 13.86) = 4.974	0.272	F (2.204, 19.84) = 1.395
TEST	0.021	F (2.945, 17.67) = 4.221	0.023	F (2.760, 24.84) = 3.922
TotT3	0.032	F (2.461, 14.77) = 4.078	0.078	F (2.896, 26.06) = 2.570
TSH	0.037	F (1.266, 7.596) = 5.991	0.008	F (1.700, 15.30) = 7.305

Supplementary Table S4. Overview of lipid parameters in serum or plasma for **A.** CircWave analysis of circadian rhythms; **B.** Repeated-Measures ANOVA to determine daily variation. Amplitude is presented as the % of the median. Ct = Clock time.

A.	Circwave analysis					
	males			females		
Markers	p-value	peak (CT)	amplitude	p-value	peak (CT)	amplitude
FFA	0.012	08:31	74.91%	0.36	06:56	80.70%
HDL	0.695	07:38	9.20%	0.97	07:30	3.86%
LDL	0.818	08:07	12.02%	0.96	08:46	8.35%
TG	0.105	13:46	70.36%	0.05	14:13	50.33%
CHOL	0.645	08:47	11.44%	0.83	08:57	8.17%

B.	RM-ANOVA all time points			
	males		females	
Markers	p-value	F-value	p-value	F-value
FFA	0.069	F (2.442, 14.65) = 3.078	0.221	F (2.638, 23.75) = 1.589
HDL	0.110	F (2.708, 16.25) = 2.399	0.213	F (2.475, 22.28) = 1.641
LDL	0.002	F (2.875, 17.25) = 7.746	0.006	F (2.660, 23.94) = 5.670
TG	0.048	F (1.943, 11.66) = 4.012	0.063	F (2.846, 25.62) = 2.798
CHOL	0.003	F (2.381, 14.29) = 8.618	0.003	F (2.938, 26.44) = 6.020

Supplementary Table S5. CircWave analysis of circadian rhythms classical circadian markers in serum (CORT) or PBMCs (*PER1*, *BMAL1*). Amplitude is presented as the % of the median. Ct = Clock time.

	Circwave analysis					
	males			females		
Markers	p-value	peak (CT)	amplitude	p-value	peak (CT)	amplitude
CORT	0.000	07:47	129.11%	0.000	07:29	161.63%
PER1				0.002	08:07	80.15 %
BMAL1				0.035	14:12	35.55 %

Supporting information S6. Raw data files of data presented in this study.

Supporting information S6 is too extensive to be added in print. This table is publically available via: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4540433/

Supplemental data Chapter 6

Supplemental Table S1. Genes oscillating under both LD and CRD conditions

Symbol
Gm13889
Ypel2
Chka
Nedd4l
Asap2
Dbp
Dnmt3b
Mif4gd
1110067D22Rik
Arntl
Clpx
Rorc
Per3
St5
A530001N23Rik
Fgfr4
Wdr91
Larp1
Dnajb11
Gramd4
Slc45a3
Tenc1
Hsd17b7
Nr1d2
D7Wsu130e
Prkd3
Gm129
9830001H06Rik
Pdcd2l
Stx2
Lingo4
Dynll2
Hist1h4i

Supplemental Table S2. Significantly enriched processes based on 561 genes that lost significant circadian rhythmicity after CRD exposure.

Processes	Group
entrainment of circadian clock by photoperiod	Circadian rhythm
entrainment of circadian clock	Circadian rhythm
photoperiodism	Circadian rhythm
circadian regulation of gene expression	Circadian rhythm
regulation of circadian rhythm	Circadian rhythm
circadian rhythm	Circadian rhythm
rhythmic process	Circadian rhythm
epithelial cell proliferation involved in mammary gland duct elongation	Hormonal regulation
Sertoli cell proliferation	Hormonal regulation
mammary gland branching involved in pregnancy	Hormonal regulation
branch elongation involved in mammary gland duct branching	Hormonal regulation
negative regulation of gonadotropin secretion	Hormonal regulation
vagina development	Hormonal regulation
branching involved in prostate gland morphogenesis	Hormonal regulation
cellular response to follicle-stimulating hormone stimulus	Hormonal regulation
Sertoli cell development	Hormonal regulation
response to follicle-stimulating hormone	Hormonal regulation
androgen metabolic process	Hormonal regulation
cellular response to estradiol stimulus	Hormonal regulation
Sertoli cell differentiation	Hormonal regulation
cellular response to gonadotropin stimulus	Hormonal regulation
cellular hormone metabolic process	Hormonal regulation
regulation of endocrine process	Hormonal regulation
hormone-mediated signaling pathway	Hormonal regulation
response to testosterone	Hormonal regulation
negative regulation of peptide hormone secretion	Hormonal regulation
ovarian follicle development	Hormonal regulation
ovulation cycle process	Hormonal regulation
negative regulation of hormone secretion	Hormonal regulation
ovulation cycle	Hormonal regulation
female gonad development	Hormonal regulation
hormone metabolic process	Hormonal regulation
response to estradiol	Hormonal regulation
female sex differentiation	Hormonal regulation
development of primary female sexual characteristics	Hormonal regulation
regulation of peptide hormone secretion	Hormonal regulation
regulation of hormone levels	Hormonal regulation
male sex differentiation	Hormonal regulation
steroid metabolic process	Hormonal regulation
cellular response to hormone stimulus	Hormonal regulation
cellular response to peptide hormone stimulus	Hormonal regulation
regulation of hormone secretion	Hormonal regulation
response to estrogen	Hormonal regulation
response to peptide hormone	Hormonal regulation
response to steroid hormone	Hormonal regulation

Processes	Group
negative regulation of mitosis	Cell cycle
negative regulation of nuclear division	Cell cycle
DNA damage response, signal transduction by p53 class mediator	Cell cycle
transforming growth factor beta receptor signaling pathway	Cell cycle
signal transduction in response to DNA damage	Cell cycle
regulation of mitosis	Cell cycle
cellular response to transforming growth factor beta stimulus	Cell cycle
regulation of nuclear division	Cell cycle
response to transforming growth factor beta	Cell cycle
negative regulation of cell cycle process	Cell cycle
regulation of cell division	Cell cycle
negative regulation of cell cycle	Cell cycle
regulation of mitotic cell cycle	Cell cycle
regulation of cell cycle process	Cell cycle
mitotic cell cycle process	Cell cycle
mitotic cell cycle	Cell cycle
negative regulation of cell proliferation	Cell cycle
lipid storage	Glucose and lipid metabolism
positive regulation of phospholipase activity	Glucose and lipid metabolism
regulation of glucose import	Glucose and lipid metabolism
positive regulation of glucose import	Glucose and lipid metabolism
regulation of glycogen metabolic process	Glucose and lipid metabolism
positive regulation of lipase activity	Glucose and lipid metabolism
regulation of phospholipase activity	Glucose and lipid metabolism
positive regulation of glucose transport	Glucose and lipid metabolism
negative regulation of insulin secretion	Glucose and lipid metabolism
regulation of glucose transport	Glucose and lipid metabolism
regulation of lipase activity	Glucose and lipid metabolism
monosaccharide metabolic process	Glucose and lipid metabolism
regulation of insulin secretion	Glucose and lipid metabolism
glucose metabolic process	Glucose and lipid metabolism
hexose metabolic process	Glucose and lipid metabolism
regulation of lipid metabolic process	Glucose and lipid metabolism
lipid biosynthetic process	Glucose and lipid metabolism
coumarin metabolic process	Resp. to exogenous compound
phenylpropanoid metabolic process	Resp. to exogenous compound
flavonoid glucuronidation	Resp. to exogenous compound
flavonoid biosynthetic process	Resp. to exogenous compound
flavonoid metabolic process	Resp. to exogenous compound
cellular glucuronidation	Resp. to exogenous compound
glucuronate metabolic process	Resp. to exogenous compound
uronic acid metabolic process	Resp. to exogenous compound

Processes	Group
quinone metabolic process	Resp. to exogenous compound
catechol-containing compound biosynthetic process	Resp. to exogenous compound
catecholamine biosynthetic process	Resp. to exogenous compound
secondary metabolic process	Resp. to exogenous compound
response to dexamethasone	Resp. to exogenous compound
water-soluble vitamin metabolic process	Resp. to exogenous compound
xenobiotic metabolic process	Resp. to exogenous compound
cellular response to xenobiotic stimulus	Resp. to exogenous compound
vitamin metabolic process	Resp. to exogenous compound
response to xenobiotic stimulus	Resp. to exogenous compound
isoprenoid metabolic process	Resp. to exogenous compound
response to purine-containing compound	Resp. to exogenous compound
organic hydroxy compound biosynthetic process	Resp. to exogenous compound
response to ethanol	Resp. to exogenous compound
monocarboxylic acid metabolic process	Resp. to exogenous compound
response to alcohol	Resp. to exogenous compound
response to nutrient	Resp. to exogenous compound
response to drug	Resp. to exogenous compound
cellular response to nitrogen compound	Resp. to exogenous compound
ephrin receptor signaling pathway	Various signaling pathways
positive regulation of epidermal growth factor receptor signaling pathway	Various signaling pathways
positive regulation of ERBB signaling pathway	Various signaling pathways
ERK1 and ERK2 cascade	Various signaling pathways
cellular response to growth hormone stimulus	Various signaling pathways
cellular response to cAMP	Various signaling pathways
response to cAMP	Various signaling pathways
response to organophosphorus	Various signaling pathways
transmembrane receptor protein serine/threonine kinase signaling pathway	Various signaling pathways
regulation of stress-activated MAPK cascade	Various signaling pathways
regulation of stress-activated protein kinase signaling cascade	Various signaling pathways
activation of MAPK activity	Various signaling pathways
regulation of ERK1 and ERK2 cascade	Various signaling pathways
regulation of JNK cascade	Various signaling pathways
cellular response to fibroblast growth factor stimulus	Various signaling pathways
neurotrophin TRK receptor signaling pathway	Various signaling pathways
response to glucocorticoid	Various signaling pathways
response to fibroblast growth factor	Various signaling pathways
MAPK cascade	Various signaling pathways
neurotrophin signaling pathway	Various signaling pathways
response to corticosteroid	Various signaling pathways

Processes	Group
cellular response to peptide	Various signaling pathways
response to hypoxia	Various signaling pathways
response to decreased oxygen levels	Various signaling pathways
transmembrane receptor protein tyrosine kinase signaling pathway	Various signaling pathways
response to oxygen levels	Various signaling pathways
response to metal ion	Various signaling pathways
positive regulation of MAPK cascade	Various signaling pathways
cellular response to organonitrogen compound	Various signaling pathways
cellular response to growth factor stimulus	Various signaling pathways
response to peptide	Various signaling pathways
regulation of MAPK cascade	Various signaling pathways
response to growth factor	Various signaling pathways
response to extracellular stimulus	Various signaling pathways
positive regulation of intracellular signal transduction	Various signaling pathways
iron ion transmembrane transport	Other
positive regulation of myelination	Other
CDP-diacylglycerol biosynthetic process	Other
regulation of oligodendrocyte progenitor proliferation	Other
CDP-diacylglycerol metabolic process	Other
positive regulation of neurological system process	Other
regulation of transmission of nerve impulse	Other
negative regulation of heart rate	Other
positive regulation of bone remodeling	Other
positive regulation of bone resorption	Other
regulation of smooth muscle cell apoptotic process	Other
response to lead ion	Other
regulation of calcium ion import	Other
negative regulation of smooth muscle cell proliferation	Other
positive regulation of phospholipase C activity	Other
regulation of phospholipase C activity	Other
negative regulation of muscle cell apoptotic process	Other
negative regulation of heart contraction	Other
positive regulation of heart contraction	Other
regulation of muscle cell apoptotic process	Other
osteoclast differentiation	Other
transferrin transport	Other
positive regulation of multicellular organism growth	Other
positive regulation of fibroblast proliferation	Other
regulation of bone resorption	Other
ferric iron transport	Other
trivalent inorganic cation transport	Other

Processes	Group
cellular aldehyde metabolic process	Other
regulation of smooth muscle cell proliferation	Other
regulation of phosphatidylinositol 3-kinase activity	Other
activation of phospholipase C activity	Other
regulation of bone remodeling	Other
protein targeting to mitochondrion	Other
cellular ketone metabolic process	Other
establishment of protein localization to mitochondrion	Other
iron ion transport	Other
pigment metabolic process	Other
positive regulation of blood pressure	Other
negative regulation of peptide secretion	Other
protein localization to mitochondrion	Other
regulation of neurological system process	Other
regulation of fibroblast proliferation	Other
actin filament organization	Other
nucleoside monophosphate biosynthetic process	Other
coenzyme biosynthetic process	Other
regulation of cellular amine metabolic process	Other
regulation of heart rate	Other
transition metal ion transport	Other
mitochondrial transport	Other
cofactor biosynthetic process	Other
tissue remodeling	Other
exocytosis	Other
actin cytoskeleton organization	Other
actin filament-based process	Other
cofactor metabolic process	Other
cell-cell junction organization	Other
regulation of peptide secretion	Other
regulation of peptide transport	Other
phospholipid biosynthetic process	Other
coenzyme metabolic process	Other
activation of protein kinase activity	Other
positive regulation of ion transport	Other
organophosphate biosynthetic process	Other
regulation of neuron apoptotic process	Other
regulation of blood pressure	Other
regulation of actin cytoskeleton organization	Other
regulation of actin filament-based process	Other
regulation of homeostatic process	Other

Processes	Group
regulation of neuron death	Other
phospholipid metabolic process	Other
positive regulation of kinase activity	Other
positive regulation of transferase activity	Other
positive regulation of protein kinase activity	Other
secretion by cell	Other
regulation of cytoskeleton organization	Other
small molecule biosynthetic process	Other
organonitrogen compound biosynthetic process	Other
cellular metal ion homeostasis	Other
secretion	Other
metal ion homeostasis	Other
cellular cation homeostasis	Other
positive regulation of protein phosphorylation	Other
cation homeostasis	Other
cellular ion homeostasis	Other
regulation of protein serine/threonine kinase activity	Other
regulation of organelle organization	Other
negative regulation of cellular component organization	Other
regulation of kinase activity	Other
ion homeostasis	Other
regulation of protein kinase activity	Other
positive regulation of multicellular organismal process	Other
positive regulation of monocyte chemotaxis	Other
response to interleukin-15	Other
acute-phase response	Other
acute inflammatory response	Other
platelet degranulation	Other

Supplementary Table S3. Top 20 genes with largest decrease in amplitude.

Symbol	Diff. amplitude
Upp2	-0.5235
Sybu	-0.4985
Atxn7l1	-0.4623
Pdk4	-0.4521
Slc16a5	-0.4486
Ccrn4l	-0.4224
Nr1d1	-0.4044
Etnk2	-0.3957
Igfbp1	-0.388
Stbd1	-0.3809
Wfdc2	-0.3807
Serpina6	-0.3761
Aqp4	-0.3759
B130006D01Rik	-0.3631
Olfr1134	-0.362
Ppard	-0.3608
Cyp2s1	-0.3605
Wdr81	-0.3541
Mcm10	-0.3462
Itsn1	-0.3454

Supplementary Table S4. Genes differentially expressed in the CRD group compared to LD controls.

Symbol	p-value	FC
Nespas	2.96E-06	-0.1539
Gm128	6.37E-05	-0.3601
Rfx7	9.32E-05	0.1652
Defa-rs1	0.000136	0.1718
Adamts5	0.000168	-0.1777
Rhox4e	0.000192	-0.1678
Sprr1a	0.000235	-0.2050
Gm1123	0.000316	-0.1293
Mpped2	0.00036	0.1585
9630013A20Rik	0.00043	0.1606
Gm10295	0.00052	-0.1364
LOC100503435	0.000674	0.1059
1190028D05Rik	0.000778	0.1334
Olfr1248	0.000815	0.1640
Mixl1	0.000868	-0.1246
Glt25d2	0.000912	-0.2461
Gm10773	0.000912	0.1299

Supplemental Table S5. Overrepresentation of up or down regulated genes in GO processes or the C2 gene sets.

Upregulated	
REACTOME_MEIOSIS	Cell cycle
REACTOME_ACTIVATION_OF_ATR_IN_RESPONSE_TO_REPLICATION_STRESS	DNA damage
KEGG_MISMATCH_REPAIR	DNA damage
REACTOME_G2_M_CHECKPOINTS	DNA damage
PID_FANCONI_PATHWAY	DNA damage
REACTOME_DNA_STRAND_ELONGATION	DNA replication
KEGG_DNA_REPLICATION	DNA replication
REACTOME_EXTENSION_OF_TELOMERES	DNA replication
REACTOME_LAGGING_STRAND_SYNTHESIS	DNA replication
DNA_DEPENDENT_ATPASE_ACTIVITY	DNA replication
REACTOME_MEIOTIC_RECOMBINATION	DNA replication
REACTOME_TELOMERE_MAINTENANCE	DNA replication
REACTOME_ACTIVATION_OF_THE_PRE_REPLICATIVE_COMPLEX	DNA replication
REACTOME_CHROMOSOME_MAINTENANCE	DNA replication
REACTOME_INFLUENZA_VIRAL_RNA_TRANSCRIPTION_AND_REPLICATION	Immune
REACTOME_INFLUENZA_LIFE_CYCLE	Immune
GLUTATHIONE_TRANSFERASE_ACTIVITY	Metabolism
REACTOME_PHASE_II_CONJUGATION	Metabolism
REACTOME_GLUTATHIONE_CONJUGATION	Metabolism
KEGG_GLUTATHIONE_METABOLISM	Metabolism
REACTOME_CHOLESTEROL_BIOSYNTHESIS	Metabolism
REACTOME_METABOLISM_OF_PROTEINS	Metabolism
REACTOME_NONSENSE_MEDIATED_DECAY_ENHANCED_BY_THE_EXON_JUNCTION_COMPLEX	Transcription
REACTOME_METABOLISM_OF_RNA	Transcription
REACTOME_METABOLISM_OF_MRNA	Transcription
REACTOME_CLEAVAGE_OF_GROWING_TRANSCRIPT_IN_THE_TERMINATION_REGION_	Transcription
KEGG_RIBOSOME	Translation
REACTOME_SRP_DEPENDENT_COTRANSLATIONAL_PROTEIN_TARGETING_TO_MEMBRANE	Translation
REACTOME_PEPTIDE_CHAIN_ELONGATION	Translation
STRUCTURAL_CONSTITUENT_OF_RIBOSOME	Translation
REACTOME_TRANSLATION	Translation
REACTOME_3_UTR_MEDIATED_TRANSLATIONAL_REGULATION	Translation
TRANSFERASE_ACTIVITY_TRANSFERRING_ALKYL_OR_ARYLOTHER_THAN_METHYLGROUPS	Various
KEGG_ALZHEIMERS_DISEASE	Various
STRUCTURAL_MOLECULE_ACTIVITY	Various
REGULATION_OF_MUSCLE_CONTRACTION	Various

Downregulated	
REACTOME_APOPTOSIS	Cell cycle
REACTOME_REGULATION_OF_MITOTIC_CELL_CYCLE	Cell cycle
REACTOME_SCF_BETA_TRCP_MEDIATED_DEGRADATION_OF_EMI1	Cell cycle
REACTOME_AUTODEGRADATION_OF_THE_E3_UBIQUITIN_LIGASE_COP1	Cell cycle
REACTOME_APC_C_CDC20_MEDIATED_DEGRADATION_OF_MITOTIC_PROTEINS	Cell cycle
REACTOME_ADAPTIVE_IMMUNE_SYSTEM	Immune
REACTOME_INTERFERON_GAMMA_SIGNALING	Immune
REACTOME_CYTOKINE_SIGNALING_IN_IMMUNE_SYSTEM	Immune
REACTOME_INTERFERON_SIGNALING	Immune
REACTOME_INTERFERON_ALPHA_BETA_SIGNALING	Immune
REACTOME_SIGNALING_BY_THE_B_CELL_RECEPTOR_BCR	Immune
KEGG_ACUTE_MYELOID_LEUKEMIA	Immune
REACTOME_ACTIVATION_OF_NF_KAPPAB_IN_B_CELLS	Immune
KEGG_CHEMOKINE_SIGNALING_PATHWAY	Immune
KEGG_PRIMARY_IMMUNODEFICIENCY	Immune
REACTOME_ANTIGEN_ACTIVATES_B_CELL_RECEPTOR_LEADING_TO_ GENERATION_OF_SECOND_MESSENGERS	Immune
REACTOME_CROSS_PRESENTATION_OF_SOLUBLE_EXOGENOUS_ANTIGENS_ ENDOSOMES	Immune
BIOCARTA_IL2_PATHWAY	Immune
REACTOME_IMMUNOREGULATORY_INTERACTIONS_BETWEEN_A_LYMPHOID_ AND_A_NON_LYMPHOID_CELL	Immune
KEGG_B_CELL_RECEPTOR_SIGNALING_PATHWAY	Immune
REACTOME_CLASS_I_MHC_MEDIATED_ANTIGEN_PROCESSING_PRESENTATION	Immune
REACTOME_IL_2_SIGNALING	Immune
PID_TCR_PATHWAY	Immune
DEFENSE_RESPONSE	Immune
KEGG_T_CELL_RECEPTOR_SIGNALING_PATHWAY	Immune
BIOCARTA_NFAT_PATHWAY	Immune
REACTOME_DOWNSTREAM_SIGNALING_EVENTS_OF_B_CELL_RECEPTOR_BCR	Immune
REACTOME_ER_PHAGOSOME_PATHWAY	Immune
REACTOME_INTEGRIN_ALPHAIIB_BETA3_SIGNALING	Immune
PID_IL4_2PATHWAY	Immune
REACTOME_ANTIGEN_PROCESSING_UBIQUITINATION_PROTEASOME_ DEGRADATION	Immune
PID_INTEGRIN_A4B1_PATHWAY	Immune
REACTOME_CYTOCHROME_P450_ARRANGED_BY_SUBSTRATE_TYPE	Metabolism
PROTEIN_CATABOLIC_PROCESS	Metabolism
CELLULAR_PROTEIN_CATABOLIC_PROCESS	Metabolism
MONOOXYGENASE_ACTIVITY	Metabolism
BIOPOLYMER_CATABOLIC_PROCESS	Metabolism
REACTOME_SYNTHESIS_OF_BILE_ACIDS_AND_BILE_SALTS	Metabolism
PID_ERBB1_INTERNALIZATION_PATHWAY	Proliferation
PID_MET_PATHWAY	Proliferation
PID_PDGFRBPATHWAY	Proliferation

Downregulated Continued	
TRANSMEMBRANE_RECEPTOR_PROTEIN_SERINE_THREONINE_KINASE_ SIGNALING_PATHWAY	Proliferation
REACTOME_SIGNALING_BY_WNT	Proliferation
REACTOME_SIGNALING_BY_SCF_KIT	Proliferation
PID_AURORA_A_PATHWAY	Proliferation
KEGG_ERBB_SIGNALING_PATHWAY	Proliferation
PID_ECADHERIN_KERATINOCYTE_PATHWAY	Proliferation
KEGG_PROTEASOME	Proteasome
PID_PI3KCIPATHWAY	Proteasome
REACTOME_REGULATION_OF_ORNITHINE_DECARBOXYLASE_ODC	Proteasome
AMINE_TRANSPORT	Transport
AMINO_ACID_TRANSPORT	Transport
INTRACELLULAR_TRANSPORT	Transport
CYTOPLASMIC_VESICLE_PART	Transport
CYTOPLASMIC_VESICLE_MEMBRANE	Transport
AMINE_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	Transport
RNA_BINDING	Various
PID_TCPTP_PATHWAY	Various
MITOCHONDRION	Various
MEMBRANE_FRACTION	Various
CYTOSKELETON	Various
ENZYME_LINKED_RECEPTOR_PROTEIN_SIGNALING_PATHWAY	Various
PROTEIN_TRANSPORT	Transport
BIOCARTA_TPO_PATHWAY	Various
POST_TRANSLATIONAL_PROTEIN_MODIFICATION	Various
REACTOME_METABOLISM_OF_AMINO_ACIDS_AND_DERIVATIVES	Various
REACTOME_SIGNAL_TRANSDUCTION_BY_L1	Various
PHOSPHORYLATION	Various
KEGG_FOCAL_ADHESION	Various
MITOCHONDRIAL_PART	Various
ACTIN_POLYMERIZATION_AND_OR_DEPOLYMERIZATION	Various
REGULATION_OF_PROTEIN_METABOLIC_PROCESS	Various
CELL_FRACTION	Various
MICROTUBULE	Various
PROTEIN_TARGETING	Various