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DISSERTATION

Influence of a high-fat diet on the expression of clock genes and genes related to the fat metabolism and LPS response in human monocytes

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1. Introduction

1.1 The circadian system

Almost all life forms, from eubacteria to human, exhibit circadian rhythms in various aspects of behavioral, physiological, and biochemical processes in synchrony with the rotation of the earth, presenting evolutionary survival advantage for them to adapt to the external environment, especially the day-night cycle (Dunlap et al., 1999; Cermakian et al., 2000). Importantly, ample evidence shows that interfering with the circadian system in humans and animals results in disruption of physiology, including sleep disorders, depression, bipolar disorder, seasonal affective disorder, obesity, hyperleptinemia, hyperlipidemia, hepatic steatosis, hyperglycemia, cardiovascular and immune system dysfunction (Alberti et al., 2005; Kohsaka et al., 2007; Knutson et al., 2008, Green et al., 2008; Duez et al., 2008; Scheer et al., 2009).

Since the identification of the *period* gene on the X chromosome (Konopka et al., 1971) , cloning of the first clock genes in the fruit fly, *Drosophila melanogaster* (Bargiello et al., 1984; Reddy et al., 1984) and the discovery of the first mammalian circadian clock gene in 1997 (Antoch et al., 1997; King et al., 1997; Tei et al., 1997), other crucial circadian clock genes and the complex network of transcriptional-translational loops through which these clock genes interact have been discovered sequentially, contributing the molecular basis to understand the mechanism of the circadian clock system.

Due to the experimental framework in the past years, it has been clear that the relationship between circadian clock genes and metabolism is not simple or linear, in fact, they are tightly interlocked as a complex network. On one hand, circadian clocks regulate metabolic processes, and on the other hand, metabolic signals feed back into circadian systems.

1.2 The circadian clock in mammals

1.2.1 The suprachiasmatic nucleus (SCN) as the “master pacemaker”

It is obvious that many aspects of mammalian behavior and physiology display daily rhythmicity, such as sleep-wake cycle, hormone secretion, feeding behaviour, locomotor activity, energy metabolism, body temperature, immune function, and digestive activity (Schibler et al., 2002).

Several interesting studies carried out by separating humans and animals temporally from the social world and light/dark cycle, show that mammalian daily rhythms can still continue with a period of around 24 hours, and it can persist for a long term, even years (Aschoff et al., 1984; Kerman et al., 1999). This implicates the existence of endogenous circadian clocks in mammals and in addition, shows that they are self-sustained.

Anatomically, the principal circadian clock in the brain is localized in the suprachiasmatic nucleus (SCN), a tiny region consisting of about 15000 neurons in the hypothalamus (Sahar et al., 2009). It interacts with many other regions of the brain and is situated directly above the optic chiasm bilateral to the third ventricle. This central clock is regarded as the "master pacemaker", which keeps "slave oscillators" throughout the body in synchronization to co-organize the physiological functions (Saini et al., 2011). Neurons in the SCN contain neurotransmitters and different peptides, including vasopressin, vasoactive intestinal peptide, transforming growth factor-α (TGF-α) and prokineticin-2 (PK2) (Dibner et al., 2010), which are secreted in a circadian manner. Due to its important role in physiological processes, damage to the SCN can result in systematic disorders, including the sleep-wake cycle, hormonal levels, locomotor activity, and feeding behavior (Rosenwasser et al., 2005). The lesion and transplantation studies provide insight into understanding the important role of the SCN. In the case of sleep, mice develop partial sleep loss or the length and timing of sleep episodes become erratic and it also causes sleep disorders in humans. In addition, after transplantation of the SCN, the SCN-lesioned animals recover behavioral circadian rhythm and the restored locomotor rhythmicity matched that of the donors. (Lehman et al., 1987; Ralph et al., 1990; Weaver et al., 1998).

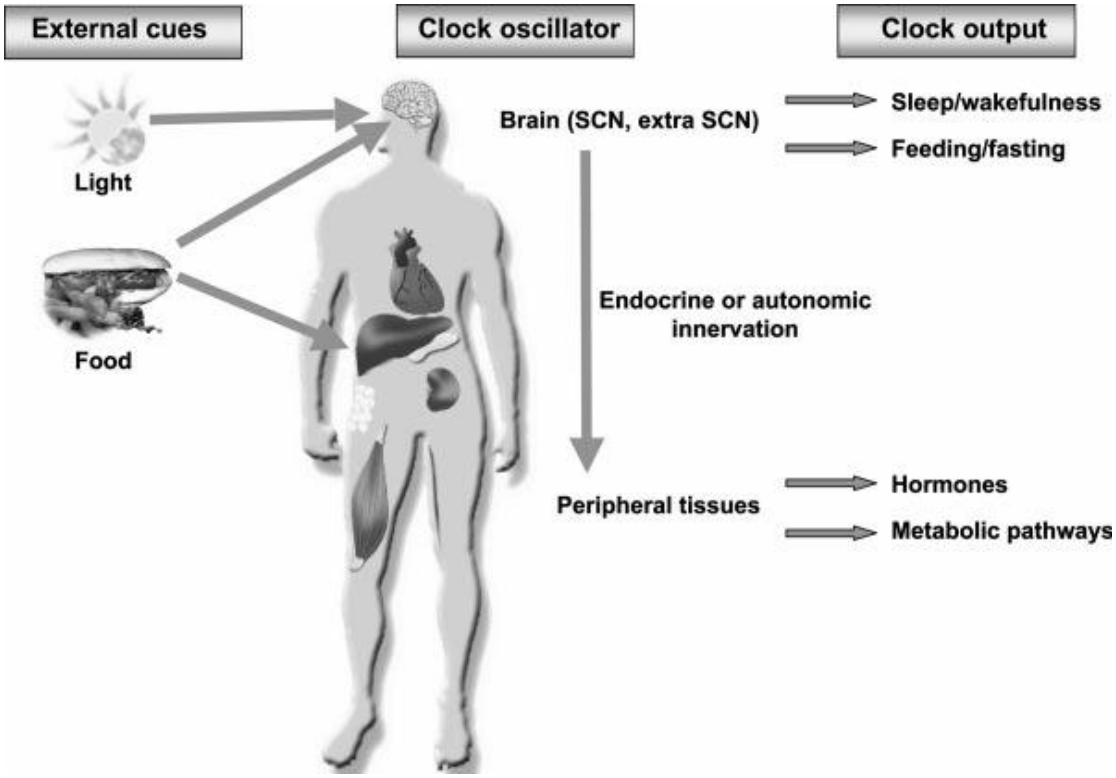


Figure 1.1: Entrainment of internal circadian clocks by environmental cues: The light-dark cycle is the predominant external cue for resetting the master pacemaker, while feeding and endocrine signals are the main cue for the peripheral oscillators. The SCN keeps peripheral clocks in synchronization via humoral and neural signals, and autonomic and behavioural cues. The circadian rhythmicity of sleep-wake cycle, hormone secretion, feeding behaviour, locomotory activity and energy metabolism reflects the existence of an internal circadian clock. Figure adapted from Maury et al., 2010.

1.2.2 Light can entrain the SCN

As the period of internal oscillations is approximately 24 hours, ranging from 20 to 28 hours in different species, thus, the inborn clocks need external cues to be synchronized with the rotation of the earth, whose period is exactly 24 hours. External cues reset the circadian system daily and therefore enable the individual to predict timing of dawn or dark, feeding, temperature changes and daily challenges. This is considered as an evolutionary advantage for the life forms. The external cue is (Zeitgeber) also known as a time giver, referring to all the environmental signals that keep endogenous clocks synchronized with the solar time. The principal external cue for the master pacemaker is the light-dark cycle (Quintero et al., 2003). It is well-known that the sleep-wake cycle has a close link to the light. When animal models and humans are kept totally in darkness for a short term, their sleep-wake cycle is pushed forward or back, because under free-

running conditions, circadian rhythms of the subjects only depend on their endogenous period (Golombek et al., 2010).

In mammals, the SCN receives light signals by specialized photosensitive ganglion cells in the retina, via the retino-hypothalamic tract (RHT) (Cermakian et al., 2002; Freedman et al., 1999; Gooley et al., 2001), and keeps subsidiary clocks of peripheral tissues in synchrony via neural-humoral signals (Morse et al., 2002; Schibler et al., 2002; Yamazaki et al., 2000; Yoo et al., 2004). Then, neurons in the ventrolateral SCN (vlSCN) start light-induced gene expression, including *Per1* and *Per2* (Hastings et al., 2004). However, this induction depends on when it happens. At circadian late night, while levels of *period* genes expression are low and start to rise, the induction will accelerate the process, and hence push forward the oscillator. In contrast, during circadian early night, when mRNA levels of *Per* genes are falling down, this induction will delay the oscillator (Hastings et al., 2007).

1.2.3 The peripheral clocks in mammals

The characteristics of peripheral clocks demonstrated in recent studies are self-sustained, cell-autonomous, tissue-specific and widely expressed in organisms; the components of clock exist not only in the brain, but also in many peripheral tissues. Cycling of the clock genes was demonstrated in the explanted livers, lung and skeletal muscles of the transgenic rats (Yamazaki et al., 2000). Moreover, the persistent cycling of clock gene and clock-controlled gene expression also occurs in isolated mammalian kidney, heart, adipose tissue and even in cultured cell lines (Balsalobre et al., 1998; Kriegsfeld et al 2003). These critical findings suggest that the inborn clocks do exist in peripheral tissues, thus isolated organs and cultured cell lines maintain self-sustained physiological rhythms.

It is well-known that the oscillators in many peripheral organs are independent, they participate in lipid metabolism, glycometabolism, protein metabolism and clock-controlled transcription. The question is how do they co-organize in synchronization? The fact that they have the ability to be entrained by external cues, and also that peripheral oscillators could be sensitive to metabolites or endocrine and behavioral cues generated by the SCN, may allow the peripheral clocks to be kept in synchronization.

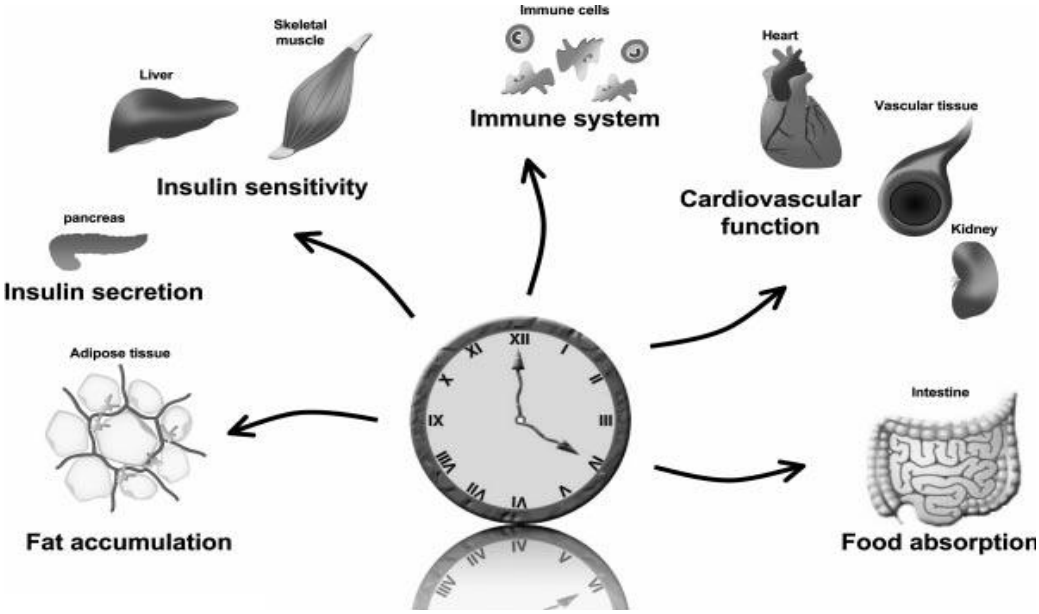


Figure 1.2: Peripheral clocks serve important roles in mammals: Microarray studies have shown that about 5-25% of the transcription in mammalian liver, heart, skeletal muscle, adipose and other tissues display circadian oscillations. Peripheral oscillators are core components of the physiological and metabolic processes, including fat accumulation, food digestion, hormonal secretion, immune system and cardiovascular function. Figure adapted from Maury et al., 2010.

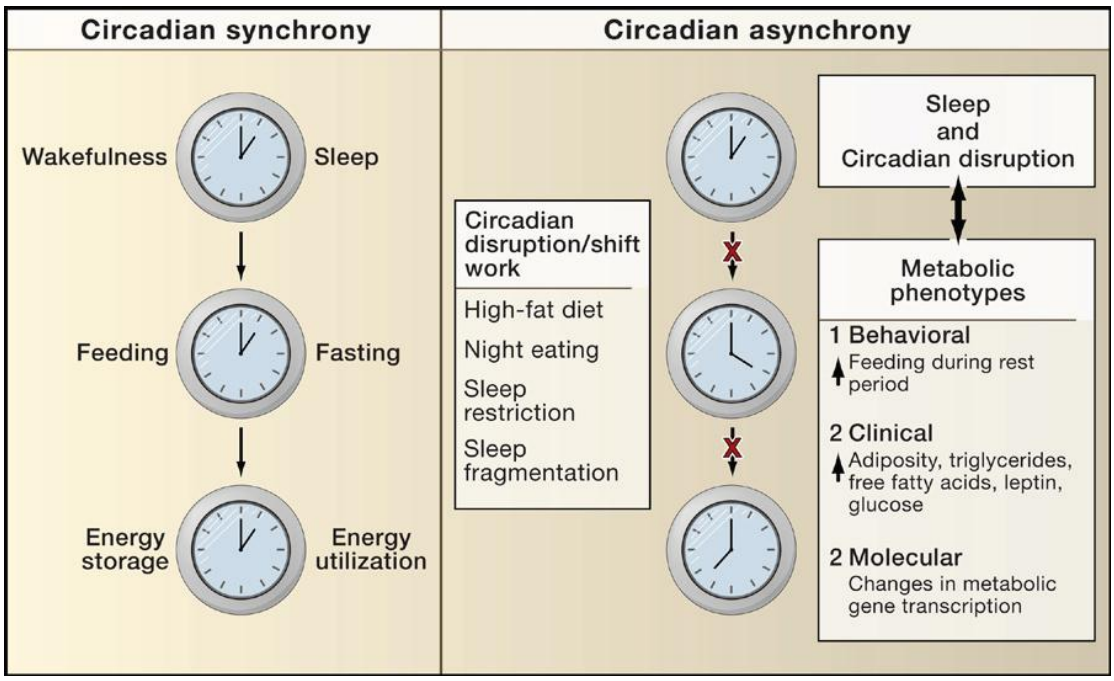


Figure 1.3: Close link between circadian timing system and health: The internal clock machinery makes the mammals sense when to sleep, feeding at a certain time of each day, and synchronize the energy storage or utilization inside the body. Shift workers and animal models provide evidence that disruption of clock

oscillations will result in many metabolic diseases such as obesity, type 2 diabetes, metabolic syndrome, digestive disorders and associated cardiovascular diseases. Figure adapted from Green et al., 2008.

1.2.4 Food can entrain peripheral circadian clocks

Although the peripheral clocks in skin are light-entrainable, most of the other oscillators in vivo don't respond to light and thus seem to be entrained by other stimuli (Campbel et al., 1998; Kawara et al., 2002). To date, it is clear that peripheral oscillators can be strongly affected by feeding cycles distinct from light-entrained oscillators of the master clock and ample evidence shows feeding is a predominant stimulus to entrain subsidiary clocks. For example, in the early study of rabbits under 12: 12 h light:dark conditions**,** while fed *ad libitum,* caecal contraction frequency and food intake rhythms were in phase and significantly correlated in the rabbits, while fed for only 4 h daily or fasting, no phase relationship between caecotrophy and caecal contraction could be recognized and it was no longer bound to the contraction frequency (Hornicke et al., 1984). Feeding restriction can reset the phase of peripheral oscillators but not the SCN (Damiola et al., 2000; Stokkan et al., 2001; Mistlberger et al., 2002). This concept was demonstrated by seemingly simple observations. The phase of *Per* genes (*Per1, Per2, Per3*), *Cry1* as well as *Dbp* and *Rev-erbα* in liver differed by 8-12h between mice fed during daytime and mice fed at night, however, the phase of circadian clock gene expression did not shift in the SCN (Damiola et al., 2000). Vollmers and his colleagues also reported that feeding significantly influence rhythmic gene expression, and that it drove circadian and ultradian transcription in mice liver (Vollmers et al., 2009).

The mechanism of how feeding entrains the peripheral clocks is not fully understood. It may relate to nutrients themselves (sterols, lipids, and carbohydrates), nutrient-sensing hormones, and nutrient-induced metabolites. Several cell culture experiments allow some insights into understanding the entrainment of clocks by food. Glucose, insulin, leptin and glucocorticoids were proved to induce changes of clock gene expression (Balsalobre et al., 2000a b; Hirota et al., 2002; Fu et al., 2005).

1.2.5 The molecular mechanism of circadian clock

The analysis of circadian genes in fruit fly *Drosophila melanogaster*, and later in mouse brought the realization that all organisms rely on the same basic molecular mechanism. Indeed, the

circadian rhythm of each cell is generated by a set of interlocked autoregulatory transcriptional-translational feedback loops, which consist of activating and repressing elements (Bell et al., 2005).

The core components of the clockwork have been identified in mammals, including *Clock, Bmal1, Period* (*Per1, Per2, Per3*), *Cryptochrome* (*Cry1, Cry2*), and *Rev-erbα*. In brief, Clock, Bmal1 proteins and the clock paralog neuronal PAS-domain protein 2 (NPAS2) activate the transcriptional process of *Period* (*Per1, Per2, Per3*) and *Cryptochrome* (*Cry1, Cry2*) via so-called E-box in their promoter (He l̀e`ne Due et al., 2009). In turn, when the levels of *Per* and *Cry* mRNAs and their protein products reach the peak, the protein products, especially Cry proteins , start to inhibit Clock/Bmal1 transactivation activity, resulting in the decline of *Per* and *Cry* mRNA levels(Green et al., 2008). By degradation processes, the concentration of Per and Cry proteins decrease gradually and thus the inhibition is eventually reversed. A cycle of this autoregulatory feedback loop is approximately 24 hours. In addition, *Clock* and *Bmal1* are the members of basic-helix-loop-helix (bHLH)-Period-Arnt-Single-minded (PAS) family and have ability to bind to DNA promoter elements (e.g. E-box). E-boxes with the nucleotide sequence CACGTG, are frequently expressed in the regulatory region of clock-controlled genes (CCGs). Besides the core regulatory loop, *Per* and *Cry* are also modified by casein kinase Iε (CKIε) and F-box protein (FBXL3) (Busino et al., 2007; Godinho et al., 2007; Siepka et al., 2007). This may contribute to maintaining the transcriptional cycle of the Clock-Bmal1 activator (Eide et al., 2005).

The core negative feedback loop is modulated by other accessory pathways. These loops involve the predominant factors, namely retinoic acid receptor-related orphan receptor α (RORα) and reverse erythroblastosis virus α (Rev-erbα). *Rev-erbα* is directly activated by *Clock-Bmal1* complex, but in turn, this accessory pathway feeds back to *Bmal1* expression. Both of the transcription factors can bind to the ROREs region of *Bmal1* and subsequently, they compete with each other. *RORα* is an activator of *Bmal1*, while *Rev-erbα* has a repressor role in the transcription. Indeed, *RORα* and *Rev-erbα* act as integrators, dynamically driving rhythmic *Bmal1* expression.

Interaction of transcription factors is responsible not only for the regulation of the clock network, but also for nucleocytoplasmic transport and posttranslational regulation such as phosphorylation,

acetylation and ubiquitination influence the clock oscillator (Gallego et al. 2007). Different levels of phosphorylation of the transcription factors in the cytoplasm provide a time delay between formation and activity in the nucleus, which is necessary for oscillation. For phosphorylation of Per and Cry protein, CKIε is responsible for the further levels of regulation (Lowrey et al., 2000; Allada et al., 2005; Yagita et al., 2002; Kondratov et al., 2003).

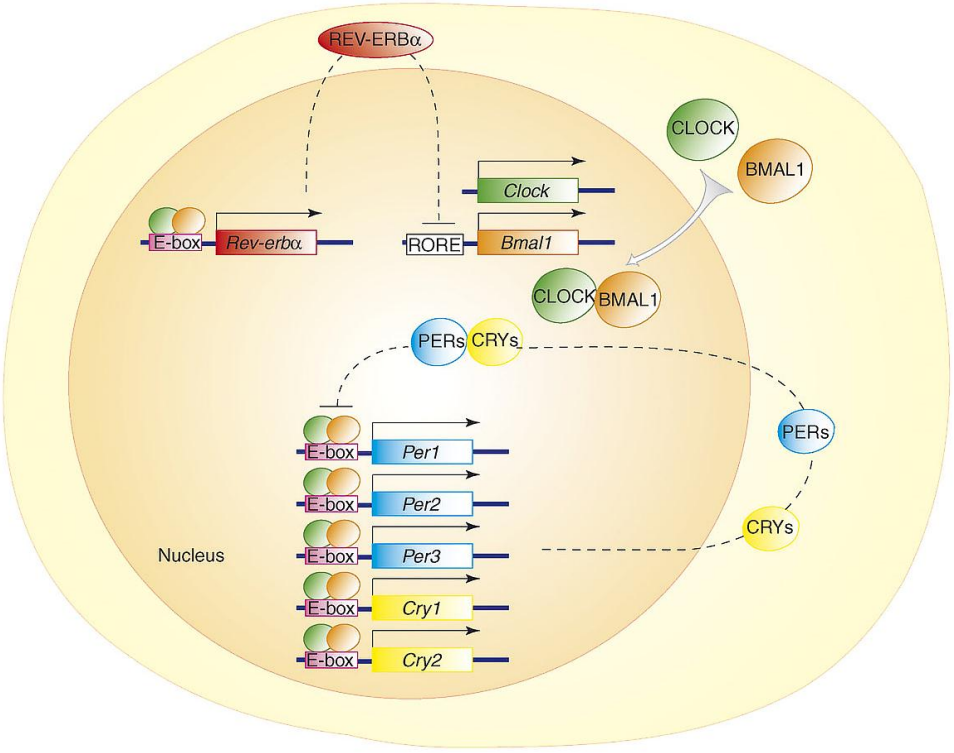


Figure 1.4: The transcriptional-translational feedback loop of the circadian timing system in mammals: The molecular mechanism of rhythm generation consists of two interlocked feedback loops: (1) the major loop includes the *Per/Cry* genes regulated positively by transcription factors *Clock* and *Bmal1* and negatively by their own protein products; (2) an accessory loop is established through the activators RORα and the repressors Rev-erbα, regulating *Bmal1* expression. The circadian oscillator adjusts the functions of numerous tissue-specific clock-controlled genes including transcription output regulators of metabolism (nuclear receptors, transcription factors *Tef*, *Dbp*, *HLF* and etc). Figure adapted from Akira et al. 2006.

1.3 Circadian system and health

1.3.1 Nutrient signaling and circadian clock system

Recent studies have demonstrated that many transcriptional factors serve an important role in modulating components of the circadian clocks as they are sensitive to food or metabolites. The core components related to this pathway are identified as NAD+, SIRT1, AMPK, NAMPT, Rev-erbα, and PPARγ (Aaron et al., 2007; Green et al., 2008). Ample evidence indicates that the NAD+-dependent histone deacetylase sirtuins mediate the caloric restriction or acute nutrient withdrawal response at the chromatin level of organisms ranging from yeast to mammals (Haigis et al., 2006; Longo et al., 2006).

SIRT1 is an orthology of yeast Sir2, a key factor which regulates Clock, Bmal1, Per2 and Cry1 in mammals (Nemoto et al., 2004; Cohen et al., 2004; Asher et al., 2008; Nakahata et al., 2008 2009; Ramsey et al., 2009). As shown in cultured cells and mouse livers, lack of *SIRT1* leads to a significant increase in the transcription of circadian clock genes (Nakahata et al., 2008). In turn, *Clock* and *Bmal1* directly control nicotinamide phospho-ribosyltransferase (NAMPT), the rate-limiting enzyme in NAD+ salvage pathway (Marina et al., 2010). Mouse experiments show that levels of *NAMPT* and *NAD* are reduced in $Clock^{-19/-19}$ and $Bmal1^{-/-}$ mice, whereas they increase in the *Cry1* and *Cry2* knock-out mice. Given that the levels of *SIRT1* and *Clock* expression remain relatively stable throughout the circadian cycle, and additionally, they closely interact with each other, therefore the *Clock-SIRT1* complex may be important in targeting other clock genes and metabolic genes (Nemoto et al., 2004; Cohen et al., 2004; Asher et al., 2008; Nakahata et al., 2008 2009; Ramsey et al., 2009). Insight into this hypothesis is demonstrated in *SIRT1*-knockout mice that acetylation of *Bmal1* and *Per2* are significantly increased. SIRT1's activity depends on the ratio of NAD+/NADH (Lin et al., 2004), NAD+ stimulates SIRT1 while NADH inhibits SIRT1 activity. Indeed, SIRT1 is considered as a sensor of metabolic changes, and its level is increased upon caloric restriction, enabling the cells to survive in the reduced nutrient condition. For example, $SIRT1^{-/-}$ mouse models exhibit defective metabolic processes (Chen et al., 2005; Boily et al., 2008), while *SIRT1* transgenic mice utilize energy more efficiently, which protects mice against high-fat-diet-induced metabolic disease (Bordone et al., 2007; Banks et al., 2008; Pfluger et al., 2008).

AMP-activated protein kinase (AMPK), another highly conserved nutrient sensor, negatively regulates the level of *Cry1* via phosphorylation (Lamia et al., 2009). On the other hand, AMPK displays in a circadian manner, and it is regulated by cellular nutrient status and hormone secretion (Green et al., 2008). It is activated by increased AMP:ATP ratio, thus, inhibition or consumption of ATP production can upregulate the signaling pathway. A glowing body of evidence shows that SIRT1 and AMPK often converge in the actions of metabolic processes. SIRT1 affects AMPK signaling via the deacetylation of kinase LKB1, while AMPK modulates SIRT1 activity through NADH:NAD+ ratio and NAMPT (Canto et al., 2009).

All these findings contribute to understanding the cross-talk between nutrient sensors and clock genes. In a word, levels of NAMPT, NAD+, and SIRT1 display circadian rhythm, the transcription of NAMPT is directly modulated by the clock, meanwhile NAMPT is the rate-limiting enzyme in the NAD+ salvage pathway and SIRT1 is NAD+-dependent. Therefore, SIRT1 is indirectly modulated by the circadian clock.

Many clock-controlled genes are also parts of cellular pathways, therefore, the connection between metabolism and clock genes is bidirectional, and disruption of the circadian timing track may induce metabolic disorders (Yang et al., 2006; Kohsaka et al., 2007).

The family of orphan nuclear receptors RORα and Rev-erbα are considered as a molecular link between the circadian clock and metabolism and they also contribute to the basis of a circadian clock gene interlocking feedback loop as described above. These ligand-activated transcription factors, containing binding sites in promoter regions for core clock genes, display circadian rhythm in peripheral tissues and also have important roles in lipid and carbohydrate metabolism (Yang et al., 2006). For example, Rev-erbα regulates hepatic gluconeogenesis, adipocyte differentiation, and triglyceride mobilization, and negatively regulates the activity of *Bmal1* (Yin et al., 2007; Duez et al., 2008). In Contrast, RORα is a positive driver for *Bmal1* expression, and it mediates lipid metabolism in skeletal muscle (Fontaine et al., 2007). The peroxisome proliferator activated receptor γ (PPARγ) induces *Bmal1* expression directly, for example, PPARγ knockout mice exhibit significant reduction in the circadian gene expression, heart rate and blood pressure (Wang et al., 2008). Bmal1 serves as a mediating factor in adipocyte differentiation, and decreases effects of PPARγ (Shimba et al., 2005; Fontaine et al., 2007). In addition, *PPARγ* co-activator 1α (*PGC-1α*) modulates *Bmal1* and *Rev-erbα* expression, and

PGC-1α displays in a circadian manner. Loss of *PGC-1α* results in abnormal metabolic rate and clock gene expressions (Liu et al., 2007).

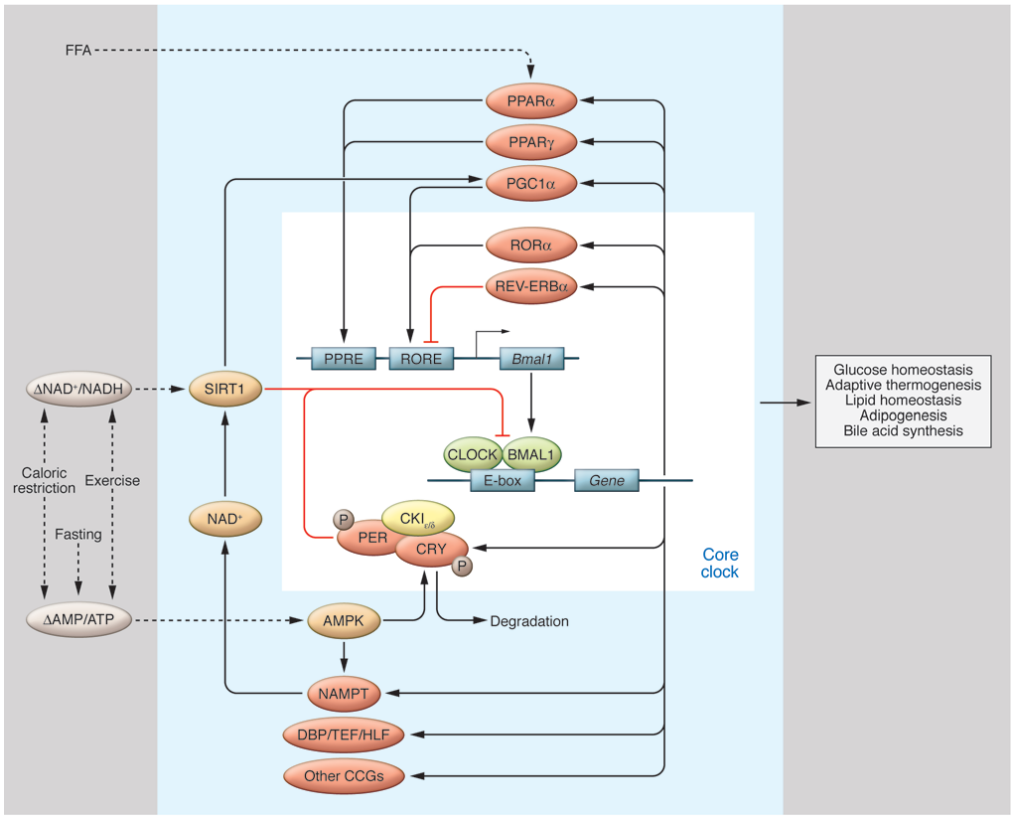


Figure 1.5: The connection of molecular clock and the nutrient sensors is a complex network. The molecular clock consists of series of transcriptional-translational feedback loops. The *Clock-Bmal1* heterodimer drives rhythmic expression of *Per, Cry* and other clock-controlled metabolic genes, which modulate physiological and metabolic processes, including glucose homeostasis, lipid metabolism, and adipogenesis. Most of these clock target genes negatively feed back to the clock due to changes in cell nutrient status via nutrient sensors. All these core components form a complex network between metabolism and clock genes to coordinate the dynamic balance of internal environment (Huang et al., 2011).

1.3.2 Circadian clock affects metabolism

Microarray studies have shown that about 5-25% of the transcription in mammalian liver, heart, skeletal muscle, adipose and other tissues, and display circadian oscillations in mRNA expression levels including components of carbohydrate, cholesterol, lipid metabolism, glycolysis and gluconeogenesis, oxidative phosphorylation and detoxification pathways, and also

the rate-limiting enzymes in many of these pathways (Zvonic et al., 2006; Storch et al., 2002; Vollmers et al., 2009; Loboda et al., 2009; Panda et al., 2002).

The range of evidence presented in some recent excellent reviews confirmed the crosstalk between components of circadian and metabolic cycles (Asher et al., 2011; Maury et al., 2010; Green et al., 2008). Particularly, alterations of clock oscillations are observed in many metabolic diseases such as obesity, type 2 diabetes, metabolic syndrome and associated cardiovascular disease in animal models and humans (Ando et al., 2009; Ando et al., 2005; Maury et al., 2010).

The circadian system and the metabolic system are fundamentally linked in two ways. On one hand, the circadian clock regulates metabolic processes in mammals in preparation for temporal variations in the abundance of nutrients (Green et al., 2008). Analysis of animal models with genetic disruption of circadian rhythms has provided insight into the role of several core circadian clock genes in metabolism. Indeed, homozygous *Clock* mutant mice are hyperphagic and obese, and develop hyperleptinemia, hyperlipidemia, hepatic steatosis, hypoinsulinemia and hyperglycemia (Turek et al., 2005). Gene disruption in *Bmal1* induces an impaired gluconeogenesis, hyperleptinemia, glucose intolerance, and dyslipidemia (Rudic et al., 2004; Lamia et al., 2009). Dysregulation of circadian rhythms such as poor sleep patterns or circadian desynchronization can have a severe impact on energy homeostasis and contribute to metabolic disease and mental disorders (Van Cauter et al., 2007). Sleep restriction in humans leads to a profound decrease in the rate of glucose disposal, a reduction in insulin secretion and alterations in neuroendocrine control of appetite (Spiegel et al., 1999, 2004).

Animal studies also very clearly show that circadian mechanisms regulate essential physiological and biological processes, including tissue growth (Fu et al., 2005), blood pressure, heart rate (Curtis et al., 2007) and blood glucose levels (Rudic et al., 2004). Shift work is strongly associated with risk of metabolic syndrome, high body mass index, gastrointestinal disturbances and cardiovascular events (Li et al., 2011; Ellingsen et al., 2007; Knutsson et al., 2003; Oishi et al., 2005; Sookoian et al., 2007).

1.3.3 Metabolic processes feed back into circadian system

Conversely, metabolic signals also feed back into the circadian system, modulating circadian gene expression and behavior. Beside light, food availability and feeding regimens are confirmed

to be dominant external cues affecting the SCN clock and peripheral clocks (Green et al., 2008). Indeed, in rodents which normally feed at night, restriction of food availability to the daytime completely inverses the rhythmic expression of clock genes in peripheral tissues such as adipose tissue, liver, pancreas, heart, skeletal muscle, and kidney but not in the central SCN (Damiola et al., 2000) and/or Caloric restriction with or without restricted feeding times also shifts the central SCN clock gene expression pattern (Mendoza et al., 2007; Challet et al., 2010). In addition, human studies show that compared with normal people, non-breakfast-eating and night-eating syndrome patients easily gain weight, developing obesity and diabetes (Andersen et al., 2004; Greenwood et al., 2008). Moreover, animal studies demonstrated alterations of behavioral and molecular circadian rhythms induced by a calorically dense high-fat diet (Kohsaka et al. 2007). And another study also suggested that under the condition of high-fat diet, circadian behavior and circadian patterns of metabolic gene expression are disrupted (Froy et al., 2010). Mouse studies carried out by Arble and his colleagues demonstrated that under a high-fat diet, mice fed during the 12-h light phase gain more weight than those fed only in the night phase (Arble et al., 2009).

In summary, metabolic processes are under the control of internal organic clock machinery, meanwhile, mRNA expression of the core factors in metabolism varies in the 24h circadian cycle. Taken together, metabolism and diurnal timing system form a functional network that co-organizes the physiological and pathophysiological processes.

1.3.4 Circadian clock system and immune function

Besides effects on metabolic processes, the circadian clock system may also regulate the immune function as shown in recent studies. The mechanism of how circadian clock and immune system interact with each other is not fully understood. Experimental frameworks and data from clinic epidemiology strongly suggest a close link between circadian clock and immune system. For example, the circadian symptom of rheumatoid arthritis (RA) such as morning stiffness is well-known, and the proinflammatory cytokines such as IL-1β, IL-6 and TNF-α reach the peak in the morning (Danis et al., 1992; Dasgupta et al., 1992; Choy et al., 2001; Hashiramoto et al., 2010). However, until now only the innate immune system has been examined in a few studies. Circadian disruption alters the number of lymphocytes, natural killer (NK) cells and anti-body titers in mammals and increases inflammatory cytokines such as IL-6, TNF-α and C-reactive protein (Hui et al., 2007; Renegar et al., 1998; Everson et al., 2005; Palma et al., 2006;

Mullington et al., 2009; Vgontzas et al., 2004; Shearer et al., 2001; Meier-Ewert et al., 2004). In *Per2* mutants, IFN-γ rhythmicity is absent, and in *Cry1*$^{-/-}$ *Cry2*$^{-/-}$ mice, rhythm in free-running period seems to be abolished (Okamura et al., 1999; Arjona et al., 2006). Shifted mice have a manifestly higher mortality compared with unshifted control mice when endotoxemic shock is induced by LPS (Castanon-Cervantes et al., 2010).

One new study using cultured $CD4^+$ T cells revealed immune responses under the circadian clock control, providing insights into understanding the adaptive immune system. In turn, the immune system also influences the circadian timing system. TNF-α-induced locomotor activity reduction and clock-controlled genes changes were triggered through alterations of clock gene expressions (Sun et al., 2006; Oishi et al., 2006; Coogan et al., 2008; Kelley et al., 2003; Dantzer et al., 2008; Cavadini et al., 2007; Bollinger et al., 2011).

1.4 Aim of this study

In short, the molecular and cellular knowledge of endogenous clocks has brought circadian time to the forefront of science, and thereby shows how temporal disorganisation can cause metabolic disorders and immune system dysregulations. These discoveries also provide insight into the development of targeted therapies, which will utilize master and peripheral clock networks to manage and investigate circadian vulnerabilities in system disease.

Despite of numerous clock gene studies in rodents, there is little to no information about the effect of food intake or food composition on circadian mechanisms in humans. Particularly, effects of food intake/fasting on the circadian gene expression were recently demonstrated in subcutaneous adipose tissue in humans (Loboda et al., 2009). One of the main problems of human studies is the choice of target tissue allowed to monitor the functions of the clock machinery by the least invasive method available. Notably, mixed blood cell populations such as whole blood cells, peripheral leucocytes or peripheral blood mononuclear cells (PBMC) are mostly used in human studies (Ando et al., 2009; Fukuya et al., 2007; Takimoto et al., 2005; Kusanagi et al., 2004; Archer et al., 2008; Boivin et al., 2003). However, the study of isolated cell types such as neutrophils or monocytes might be advantageous and more selective for understanding the functions and regulation of the clock machinery.

In this study, we first performed the comparative analysis of daily expression profiles of ten clock genes in human PBMC and isolated monocytes in twelve non-obese individuals. We then continued with TLR4 signaling and metabolic-related genes. Moreover, we compared the response of the clock gene, TLR4 signaling and metabolic-related gene expression profile in relation to a high-fat isocaloric diet using real-time PCR with analysis of the interrelations of clock genes by correlation analysis.

2. Materials and methods

2.1. Abbreviations

ACOX3	Peroxisomal acyl-coenzyme A oxidase 3
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
B2M	Beta-2-microglobulin
bHLH	Basic-helix-loop-helix
BMAL1 (ARNTL)	Aryl hydrocarbon receptor nuclear translocator-like
BMI	Body mass index
BSA	Albumin from bovine serum
CCGs	Clock-controlled genes
CD14	Cluster of differentiation 14
CD180	Cluster of differentiation 180
cDNA	Complementary DNA
CID	Clinical investigation day
CKI	Casein kinase I
CLOCK	Clock homolog
CPT1A	Carnitine palmitoyltransferase 1A
CRY	Cryptochrome 1 (photolyase-like)
DBP	D site of albumin promoter (albumin D-box) binding protein
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
EDTA	Ethylendiamine tetra acetic acid
et al	et alii
FASN	Fatty acid synthase
FCS	Fetal calf serum
g	g-force of centrifuges
HADH	Hydroxyacyl-COA dehydrogenase
HFD	High-fat diet

HPRT1	Hypoxanthine phosphoribosyltransferase 1
IDH3A	Isocitrate dehydrogenase 3 (NAD^+) alpha
IL	Interleukin
IVGTT	Intravenous glucose tolerance test
JUN	Jun proto-oncogene
kcal	Kilocalories
LDL	Low-density Lipoprotein
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MAPK3	Mitogen-activated protein kinase 3
MCP-1 (CCL2)	Monocyte chemotactic protein-1
MIP-1α (CCL3)	Macrophage inflammatory protein-1α
ml	Milliliter
mM	Millimolar
mRNA	Messenger RNA
MTT	Meal tolerance test
MW	Average
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAMPT	Nicotinamide phospho-ribosyltransferase
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor (kappa-light-chain-enhancer) of activated B-cells
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-Cells inhibitor alpha
NK	Natural killer
NUGAT	Nutrigenomic Analysis in Twins
OGTT	Oral glucose tolerance test
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline

PER	Period homolog
PK2	Prokineticin-2
PPAR	Peroxisome proliferator-activated receptor
PPIB	Peptidylprolyl isomerase B
qRT-PCR	Quantitative real-time PCR
RA	Rheumatoid arthritis
Rev-erbα (NR1D1)	Nuclear receptor subfamily 1, group D, member 1
RHT	Retino-hypothalamic tract
RNA	Ribonucleic acid
ROR	Retinoic acid receptor-related orphan receptor
RORE	Retinoic acid-related orphan receptor response element
RORα	Retinoic acid receptor-related orphan receptorα
SCN	Suprachiasmatic nucleus
SD	Standard deviation
SE	Standard error
SIRT1	Sirtuin 1
SREBP	Sterol regulatory element-binding protein
TEF	Thyrotrophic embryonic factor
TGF-α	Transforming growth factor -α
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor α
vs	Versus
µl	Microliter
µM	Micromolar

2.2 Materials

Chemicals	Supplier
2-Mercaptoethanol	Sigma-Aldrich,Saint Louis, USA
Agarose	Sigma-Aldrich,Saint Louis, USA
BSA	Sigma-Aldrich,Saint Louis, USA
CD14 MicroBeads, human	Miltenyi Biotec, Bergisch Gladbach, Germany
DNAse & RNAse -free water	MP Biomedicals Inc. ,Solon, Ohio, USA
EDTA	Merck KgaA, Darmstadt, Germany
Ethanol, absolute	Carl Roth GmbH, Karlsruhe, Germany
FCS Hyclone	Thermo Fisher Scientific, Waltham, USA
Ficoll-Paque™ PREMIUM 1.073 g/ml	GE Healthcare, Freiburg, Germany
PBS 10 x (Ca2+ / Mg2+-free)	GIBCO® Invitrogen, Karlsruhe, Germany

Equipment	Supplier
ABI Prism 7700 sequence detection system	Applied Biosystems, Darmstadt, Germany
Centrifuge 5417 R	Eppendorf Research, Hamburg, Germany
Combitips® PLUS	Eppendorf Research, Hamburg, Germany
MACS® MultiStand	Miltenyi Biotec, Bergisch Gladbach, Germany
MiniMACS™ Separator	Miltenyi Biotec, Bergisch Gladbach, Germany
Multipette® plus	Eppendorf Research, Hamburg, Germany
Multifuge 3S/ 3S-R	Heraeus, Berlin, Germany
Neubauer improved counting chamber	Superior,Marienfeld, Germany
ND-1000 spectrophotometer	Nanodrop, PeqLab, Erlangen, Germany
Pipetboy	Integra Biosciences, Fernwald, Germany
Pipette Research®, 0,5 µl – 10 µl	Eppendorf Research, Hamburg, Germany
Pipette Reference®, 10 µl – 100 µl	Eppendorf Research, Hamburg, Germany
Pipette Reference®, 100 µl – 1000 µl	Eppendorf Research, Hamburg, Germany

Kits	Supplier
High Capacity cDNA Reverse Transkription Kit	Applied Biosystems, Darmstadt, Germany
NucleoSpin RNA II Kit	Macherei-Nagel, Düren, Germany
Power SYBR Green PCR Master Mix	Applied Biosystems, Darmstadt, Germany

Software	Supplier
Primer Express	PE Applied Biosystems, Darmstadt, Germany
SDS 2.3	ABI, Foster City, USA

Solution	Supplier
PBS – EDTA	1x PBS, 5 mM EDTA
PBS – FCS	1x PBS, 5 mM EDTA, 1% FCS
MACS-Buffer	1x PBS, 2 mM EDTA , 0,5 % BSA, pH 7,2

Supplies	Supplier
384 Well Multiply® -PCR Plate	Sarstedt, Nürnberg, Germany
50ml Falcon tube	Eppendorf Research, Hamburg, Germany
7.5-ml EDTA monovettes	Sarstedt, Nürnberg, Germany
MS Columns	Miltenyi Biotec, Bergisch Gladbach, Germany
Safe-lock Eppendorf	Eppendorf Research, Hamburg, Germany
Serological pipette (25 ml, 10 ml, 5 ml)	Sarstedt, Nürnberg, Germany
Transferpipette (2 ml)	Sarstedt, Nürnberg, Germany

2.3 Methods

2.3.1 Subjects and design of the study

Clock gene expression was analyzed in thirty non-obese individuals in terms of the NUtriGenomics Analysis in Twins (NUGAT) study. The baseline examination of participants recruited from the general population of the Berlin-Brandenburg area in Germany included anthropometric measurements, blood sampling, a 75-g oral glucose tolerance test (OGTT) for 120 min, and a personal interview on lifestyle habits and medical history. Subjects with metabolic disturbances, weight changes >3 kg during three months before investigation or BMI difference between twins ≤ 2 kg/m^2 were not included in the study. Subjects were not entrained by an extended sleep/wake schedule before or during the study phase. The study was approved by the ethics commission of Charité University Medicine, Berlin, Germany, and written informed consent was obtained from all participants of the study.

During six weeks before high-fat intervention, participants consumed an isocaloric high-carbohydrate diet (55% carbohydrates, 15% proteins, 30 % fat) to ensure the homogenous nutrition conditions for all subjects (Fig.2.3.1). Then the subjects consumed an isocaloric high-fat diet (40% carbohydrates, 15% proteins, 45 % fat) for six weeks including standard high-fat food during the first and last week provided by our research center. The quality of the diet was controlled by nutrition consulting, weight gain monitoring and use of nutritional protocols.

Subjects participated in three clinical investigation days (CID): after the high-carbohydrate diet phase (CID1) and after one (CID2) and six weeks (CID3) of high-fat diet (Fig.2.3.1). During each investigation day, individuals underwent anthropometrical measurements as well as intravenous glucose tolerance tests (IVGTT) and meal tolerance tests (MTT). CIDs started in the morning after an overnight fast.

Blood samples were taken from the forearm vein at three time points during the CIDs: in the morning (8.00-10.00), in the middle of the day, 3h 15 min after the first sampling (11.30-13.00), and in the late afternoon, 7 h 30min after the first sampling (15.00-17.00).

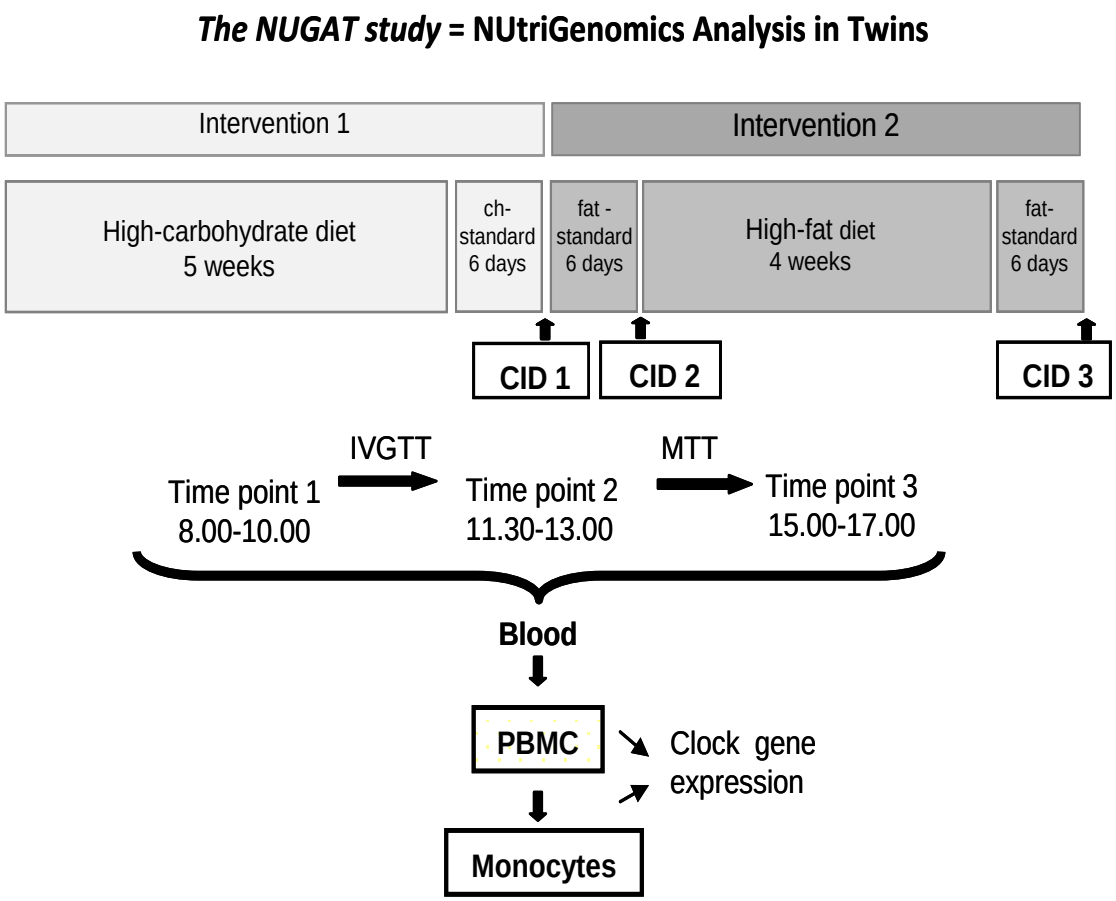


Fig.2.3.1 Design of the study. After six weeks of the isocaloric high-carbohydrate diet, participants consumed the isocaloric high-fat diet for six weeks. Subjects were examined on three clinical investigation days: after the high-carbohydrate diet phase (CID1), after one (CID2) and after six weeks (CID3) of high-fat diet. Blood samples were collected at three time points during each CID: in the morning (8:00 to 10:00), at noon (11.30-13.00), and in the late afternoon (15.00-17.00). From each subject, nine blood samples were taken over the study duration. (IVGTT - intravenous glucose tolerance test; MTT - meal tolerance test).

2.3.2 Measurement of laboratory parameters

Anthropometry was performed as described previously. Blood samples obtained after the overnight fasting were analyzed for glucose, cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol, and triglycerides using standard methods. Serum insulin was measured using a commercial enzyme-linked immunosorbent assay.

2.3.3 Isolation of human PBMC and monocytes

PBMC and monocytes were isolated immediately after the blood sampling. PBMC were isolated by gradient centrifugation using Ficoll-Paque™ PREMIUM 1.073 g/ml followed by two washing steps with PBS-EDTA (5mM) and PBS-EDTA-fetal bovine serum (1%) buffer to remove platelets.

The principle of density gradient centrifugation allows PBMC to be separated from other blood components. The PBMC fraction consists of lymphocytes, monocytes and macrophages. The medium Ficoll-Paque™ PREMIUM 1.073 g/ml was used for the separation. Blood fractions with higher density, such as erythrocytes and granulocytes were at the bottom of the tube after centrifugation, thereby enriching the lower density cells at the interface (e.g. PBMC and platelets). This principle is based on the method developed by BÖYUM.

From each twin pair, peripheral blood samples were collected at three time points (at 08:30, 11:45 and 16:00) during each investigation day in 7.5-ml EDTA monovettes. EDTA acts as an anticoagulant. The isolation procedure was on ice to avoid physiological processes and gene expression changes in the cells.

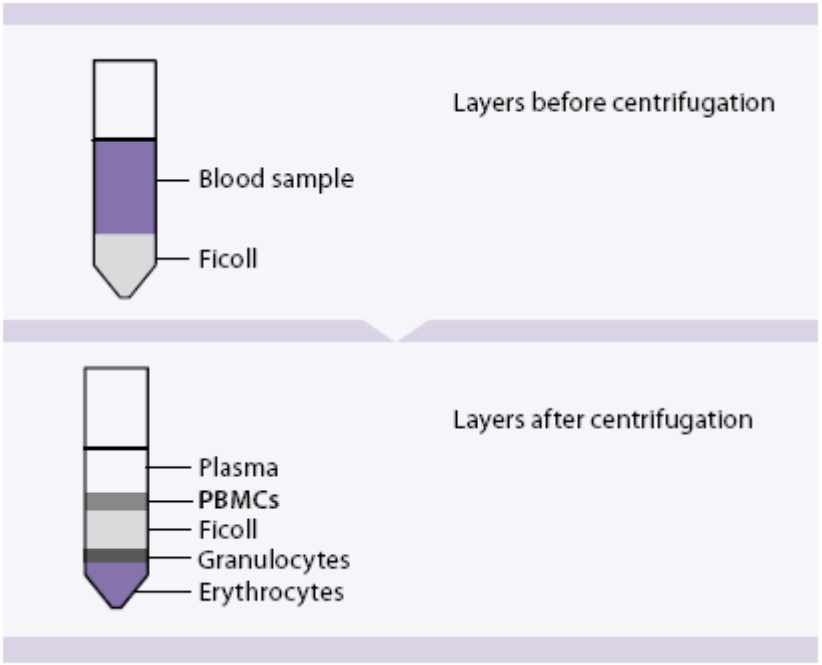


Figure 2.3.2 PBMC were isolated by gradient centrifugation. Ficoll-Paque™ PREMIUM 1.073 g/ml was used for the separation. Blood fractions with higher density, such as erythrocytes and granulocytes, were at the bottom of the tube after centrifugation, thereby enriching the lower density cells at the interface (e.g. PBMC and platelets).

The blood was diluted 3:5 with PBS-EDTA and laid over 15 ml Ficoll-Paque™ PREMIUM. The subsequent centrifugation (400 g, 40 min, 4 °C, minimum acceleration / brake) was used for the separation of blood components. The PBMC-rich interphase was harvested by using a transfer pipette and transferred into a new 50ml Falcon tube. This was followed by a washing step with PBS-EDTA and another with PBS-FCS. The cells were resuspended in 20 ml of appropriate buffer and centrifuged for 15 min at 200 g and 4 °C. The pellet was resuspended in 1 ml MACS buffer and followed by cell counting by using a Neubauer counting chamber. On average, 12-15 million PBMCs were obtained from 7.5 ml of whole blood sample. Approximately 4 million of these cells were directly used for RNA isolation of PBMC.

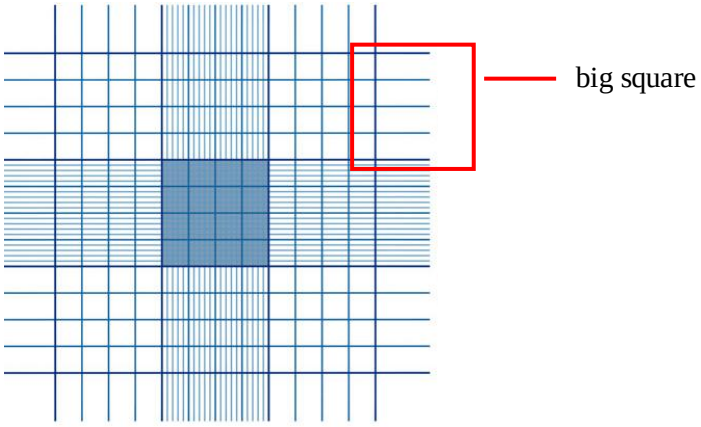


Figure 2.3.3 Cell counting by using a Neubauer counting chamber.

Calculate the volume (x) to remove 4 Million. PBMC

--> $\frac{\text{4 Million cells}}{\text{counted cells}} = \frac{\text{x (ml)}}{\text{1 ml}}$

Monocytes were isolated from the PBMC fraction to high purity (≥95%) by magnetic cell sorting using anti-CD14-coated beads according to manufacturer's recommendations, and their yield was 10-20% from PBMC.

Monocytes can be separated from other cell fractions by incubating with magnetic nanoparticles coated with antibodies against a particular surface antigen. According to the manufacturer's recommendations, cells were resuspended in MACS-buffer at a concentration of 80 µl per 10^7

cells. CD14 MicroBeads was added to the cell suspension at a concentration of 20 µl per 10^7 cells. Then the cell suspension was incubated for 15 min on ice.

After incubation, 1 ml MACS buffer was added to the suspension and centrifuged for 5 min at 500g, 4 ° C. The cell pellets were resuspended in 500 µl MACS-buffer. After washing the column with 500 µl MACS-buffer, the cell suspension was loaded into the column. In this step, the cells which were attached to the nanoparticles remained in the column, while other cells flowed through. After washing the column 3 times with 500 µl MACS buffer, it was removed from the magnet. To elute monocytes, the cells were rinsed in 1 ml MACS buffer by pushing it through the column. Extracted cells were immediately lysed in 350 µl NucleoSpin lysis buffer plus 3.5 µl ß-mercaptoethanol.

2.3.4 RNA extraction

Total RNA was extracted by NucleoSpin RNA II Kit according to the manufacturer's instructions.

The isolated cells were lysed already in 350 µl NucleoSpin lysis buffer plus 3.5 µl ß-mercaptoethanol. The mixture was shaken and applied into NucleoSpin®Filter and then centrifuged for 1 min at 11,000 x g. 350 µL 70% ethanol was added to the homogenized lysate and mixed by pipetting up and down for 5 times in order to adjust RNA binding conditions. After another centrifugation for 1 min at 11,000 x g, the eluate was discarded and the column was washed with 350 µl membrane desalting buffer. After another centrifugation (1 min, 11,000 g), the DNA-digesting enzyme deoxyribonuclease was used to remove DNA from the silica membrane of the column. 10 µl DNAase was diluted in 90 ul DNAase reaction buffer to make DNAase solution. 95 µl DNase solution was applied to the column and incubated for 15 min at room temperature. Silica membrane was washed and dried for 3 times. The sample was washed in buffer RA2 once, then washed twice in buffer RA3 and finally eluted with 60 µl RNase-free water.

The RNA concentration measurement and quality control was performed by using an ND-1000 spectrophotometer.

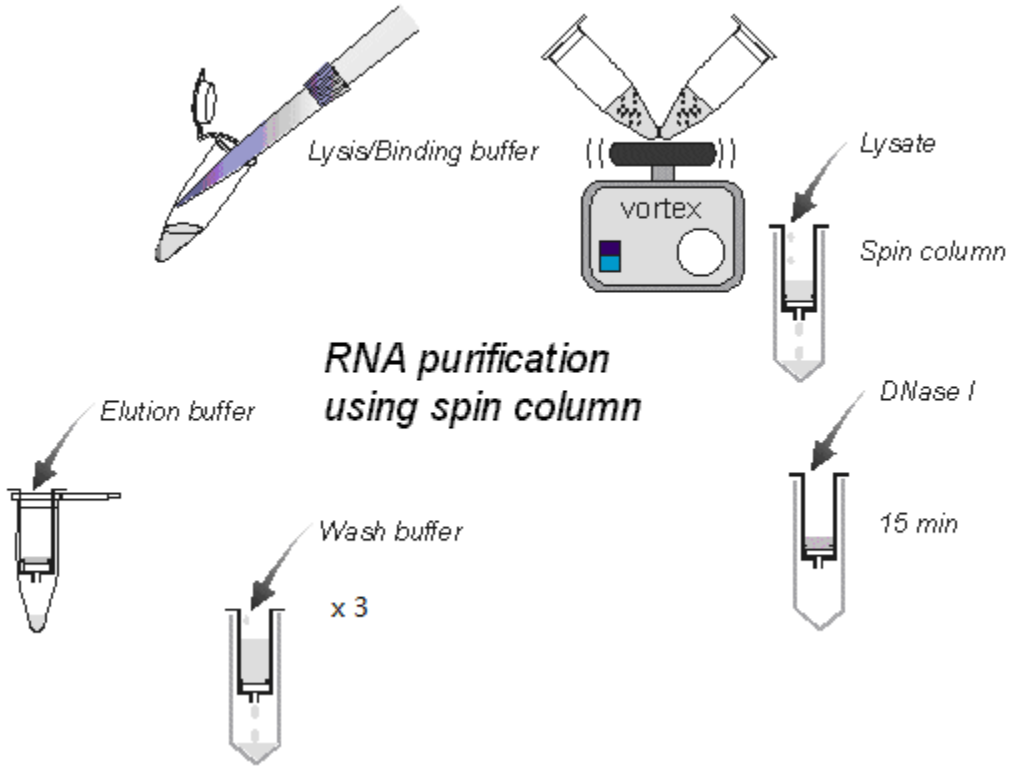


Figure 2.3.4 The protocol of RNA extraction. Total RNA was extracted by NucleoSpin RNA II Kit according to the manufacturer's instructions

2.3.5 cDNA synthesis

Single-stranded cDNA was synthesized with High-Capacity cDNA Reverse Transcription Kit™ using random hexamers as described in the manufacturer's instructions (Table 2.3.1).

Component	Volume/Reaction (ul)
RNA	10.0
10× RT Buffer	2.0
25×dNTP Mix (100 mM)	0.8
10× RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free H_2O	3.2
Total per reaction	20.0

Table 2.3.1 Reaction mixture (20 uL) for reverse transcription.

Then, optimal thermal cycler conditions were chosen for use with the High Capacity cDNA Reverse Transcription Kits.

The thermal cycling conditions were as follows: an annealing at 25 ℃ for 10 minutes, followed by reverse transcription at 37 ℃ for 120 minutes, enzyme inactivation for 5 min at 85 ℃, and cooling down. Then cDNA was diluted 1:30 and stored at - 80 ℃.

2.3.6 Primer design

mRNA sequences were available in the GenBank database of International Center for Biotechnology Information (NCBI). The corresponding sequence of the gene was determined and the primers were designed by using the Primer Express software. The distribution of the four bases in the primer was randomised, importantly, the 3'-end of the primer contained less than three consecutive hydrogen bonds (G or C). Tm values of the upstream primer were consistent with the downstream primer.

Moreover, the primers were intron-spanning designed to eliminate unwanted amplification of genomic DNA. The base sequences were aligned against the sequences of the human genome in the database of the NCBI to ensure specific hybridization of the primer with the sequence of the target gene. The primer sequences are listed in Table 2.3.2.

Gene symbol	Gene ID	Primer sequence	
		forward	reverse
Clock genes			
CLOCK	9575	ATTCCACAAGGCATGTCCCA	TTTGCTTCTATCATGCGTGTCC
BMAL1	406	CATTAAGAGGTGCCACCAATCC	CAAAAATCCATCTGCTGCCC
PER1	5187	ATTCCGCCTAACCCCGTATGT	CCGCGTAGTGAAAATCCTCTTG
PER2	8864	AGCAGGTGAAAGCCAATGAAG	AGGTAACGCTCTCCATCTCCTC
PER3	8863	GTCCAAGCCTTACAAGCTGGTTT	GACCGTCCATTTGTTGGCAT
NR1D1	9572	TGACCTTTCTCAGCATGACCAA	CAAAGCGCACCATCAGCAC
CRY1	1407	GGGACCTGTGGATTAGTTGGG	GCTCCAATCTGCATCAAGCAA
CRY2	1408	TGCATCTGTTGACACTCATGATTC	GGTACTCCCCCAGCCCAG
DBP	1628	GAAAAATCCAGGTGCCGGA	CGTTGTTCTTGTACCGCCG
TEF	7008	AACCGTGTCCAGCACAGAATCT	GGTCCGGATTGAAGTTCACATC
TLR4 signaling			
CD14	929	TCTCAACCTAGAGCCGTTTCT	CCTTGACCGTGTCAGCATAC
CD180	4064	TCCCTGACACTCTACCAAAC	TAATCTGGCACCTAGTTAAATC
NFKBIA / IKBA	4792	CTGGGCCAGCTGACACTAG	CATCATAGGGCAGCTCGTC
MAPK3	5595	CCACATTCTGGGCATCCTG	CACCTTGGTCTTGGAGGGC
JUN	3725	CAGCCCACTGAGAAGTCAAA	CACCAATTCCTGCTTTGAGA
IL1β	3553	GCAATGAGGATGACTTGTTCTTTG	CAGAGGTCCAGGTCCTGGAA
IL6	3569	AGCCCTGAGAAAGGAGACATGTA	TCTGCCAGTGCCTCTTTGCT
IL8	3576	CTGGCCGTGGCTCTCTTG	CCTTGGCAAAACTGCACCTT
IL10	3586	ACGGCGCTGTCATCGATT	GGCATTCTTCACCTGCTCCA
TNFα	7124	GGACCTCTCTCTAATCAGCCCTC	TCGAGAAGATGATCTGACTGCC
CCL2	6347	CATAGCAGCCACCTTCATTCC	TCTGCACTGAGATCTTCCTATTGG
CCL3	6348	GTTCTCTGCATCACTTGCTG	GGCTGCTCGTCTCAAAGTAG

Gene symbol	Gene ID	Primer sequence	
		forward	reverse
Fat metabolism			
LPL	4023	TGCAGGAAGTCTGACCAATAAG	CCCTCTGGTGAATGTGTGTAAG
FASN	2194	AGACACTCGTGGGCTACAGCAT	ATGGCCTGGTAGGCGTTCT
CPT1A	1374	ATTATGCCATGGATCTGCTG	AGCGGAGCAGAGTGGAATC
ACOX3	8310	ACGGTCTGGATAATGGTTTC	GCTGCCTGACGTCCTTAAAG
IDH3A	3419	CAGGGAAGTTGCAGAAAGCT	TGCACACAAGTCACTAAGGATG
HADH	3033	GAAGACCCTGAGCACCATAG	AAAGATTGTATGTTCAGCAGCA
NAMPT	10135	GCCAGCAGGGAATTTTGTTA	TGATGTGCTGCTTCCAGTTC
Housekeeping genes			
HPRT1	3251	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
B2M	567	CTATCCAGCGTACTCCAAAG	AAACCCAGACACATAGCAAT
PPIB	5479	ACAGGAGAGAAAGGATTTGG	CTCACCGTAGATGCTCTTTC

Table 2.3.2 Primers used for real-time PCR.

2.3.7 Quantitative real-time PCR

Expression of eight core clock genes (*PER1-3, CRY1-2, BMAL1, CLOCK, REV-ERBα*), and two clock-controlled transcription factors (*DBP, TEF*) was measured by quantitative real-time PCR (qRT-PCR). QRT-PCR was performed in a 384-well plate in ABI Prism 7700 sequence detection system. QRT-PCR was carried out on equal amounts of cDNA in triplicate for each sample using Power SYBR Green PCR Master Mix. The thermal cycling conditions of qRT-PCR were as follows: an initial denaturation at 95 ℃ for 10 minutes, followed by 45-50 cycles of denaturing at 95 ℃ for 15 sec, and annealing/extension for 1 min at 60 ℃. Quantification of mRNA levels was performed by the standard curve method. Clock gene expression was normalized to the geometric mean of three reference genes *PPIB* (peptidylprolyl isomerase B, cyclophilin B), *B2M* (beta-2-microglobulin) and *HPRT1* (hypoxanthine phosphoribosyltransferase 1) widely used as a constitutively expressed internal control in mononuclear cells.

All oligonucleotides for the qRT-PCR were designed with Primer Express software (Table 2.3.2). PCR primer pairs for qRT-PCR were located in different exons to avoid possible amplification

of genomic DNA. Amplified PCR products were confirmed by electrophoresis in 1.5% agarose gel.

2.3.8 Statistical analysis

All values were expressed as the means ± SE if not stated otherwise. Differences between means of two groups were analyzed using the Wilcoxon test. For multiple comparisons between CIDs or time points, the Friedman test was used. Correlation analyses were performed by using the Pearson test. A p-value<0.05 was considered statistically significant. Statistical analyses were performed with R software and SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

3. Results

This section is divided in two parts. In the first part, I will focus on the comparative analysis of clock gene expression in human PBMCs and monocytes under the influence of a high-fat diet. In the second part, I will present results concerning the interference of a high-fat diet in clock gene, fat metabolic gene and cytokine expression.

3.1 Clinical characteristics of subjects

Thirty non obese individuals (age 37.47 ± 17.22, BMI 23.23 ± 2.58) with normal glucose tolerance measured in OGTT were involved in this study. Baseline characteristics of population studied are presented in Table 3.1.

After six weeks of high-fat diet, no changes of anthropometrical characteristics, such as BMI, waist-to-hip ratio, total body fat or leukocyte number were found. However, alterations of biochemical parameters were detected. Indeed, the total cholesterol level was increased already after one week, and LDL cholesterol level after six weeks of diet intervention. Interestingly, the level of triglyceride was significantly decreased after six weeks of HFD.

	CID1	CID2	CID3
Anthropometry			
N	30	30	30
Sex (m/f)	13/17	13/17	13/17
Age (years)	37.47 ±17.22	37.53 ±17.16	37.53 ±17.16
BMI (kg/m^2)	23.23 ±2.58	23.23 ±2.58	23.23 ±2.56
Waist-to-hip ratio	0.83 ±0.09	0.83 ±0.09	0.83 ±0.09
Total body fat (%)	24.39 ±7.74	•	24.24 ±7.22
Blood parameters			
Fasting glucose cap. (mmol/l)	5.47 ±0.92	5.09 ±0.60	5.62±0.65
Fasting insulin (pmol/l)	47.27 ±33.87	50.59 ±41.64	41.20 ±33.61
Triglycerides (mmol/l)	1.05 ±0.43	1.05±0.39	1.00 ±0.40 *
Total cholesterol (mmol/l)	4.45 ±0.84	4.67 ±0.75 *	4.78 ±0.88 *
HDL cholesterol (mmol/l)	1.31 ±0.40	1.37 ±0.39 *	1.40 ±0.41
LDL cholesterol (mmol/l)	2.67 ±0.70	2.83 ±0.63	2.93 ±0.75 **
Leukocyte (gpt/l)	5.47 ±1.89	5.51 ±1.71	5.49 ±2.25

Table 3.1 Clinical characteristics of subjects. Data were collected at the beginning of the high-fat isocaloric diet (CID 1), after one week (CID 2) and six weeks of intervention (CID 3). • Data were not collected. Data are presented as mean±SD. * $p<0.05$, ** $p<0.01$

3.2 Comparative analysis of clock gene expression in human PBMC and monocytes (twelve non-obese individuals)

Because expression of clock genes in human monocytes was poorly studied until now, we first analyzed the daily variation of mRNA expressions of the clock genes in isolated monocytes and compared it with their expression in PBMCs in twelve non obese individuals. Expression of mRNA of ten clock genes was measured by real-time PCR at three time points of three CIDs during the day time (totally nine samples from each subject) and normalized to the geometric mean of *HPRT, B2M* and *PPIB* expression.

3.2.1 Daily variation of clock gene expression in human PBMC and monocytes

The mRNA expression of *PER1-3*, *BMAL1*, *DBP*, *TEF* and *NR1D1* exhibited significant daily variation ($p<0.01$ in Friedman test). Despite the intersubject variability, distinct temporal patterns were detected (Fig. 3.1). *PER1-3*, *DBP*, *TEF* and *NR1D1* showed the highest levels in the morning and dropped to trough levels in the afternoon (Fig. 3.1 A, B). Notably, the change in *PER1* had the largest daily variance and significance among the genes studied, with up to 10-fold change in some individuals. In contrast to these genes, *BMAL1* demonstrated an upward trend and reached maximal expression in the afternoon ($p=0.007$ in Friedman test) (Fig. 3.1 C). The mRNA levels of *CLOCK* and *CRY1-2* (Fig. 3.1 D) showed no variation throughout the day. In PBMCs, similar temporal expression profiles were detected (Fig. 3.1). During all three CIDs, the clock genes studied showed the same daily variation.

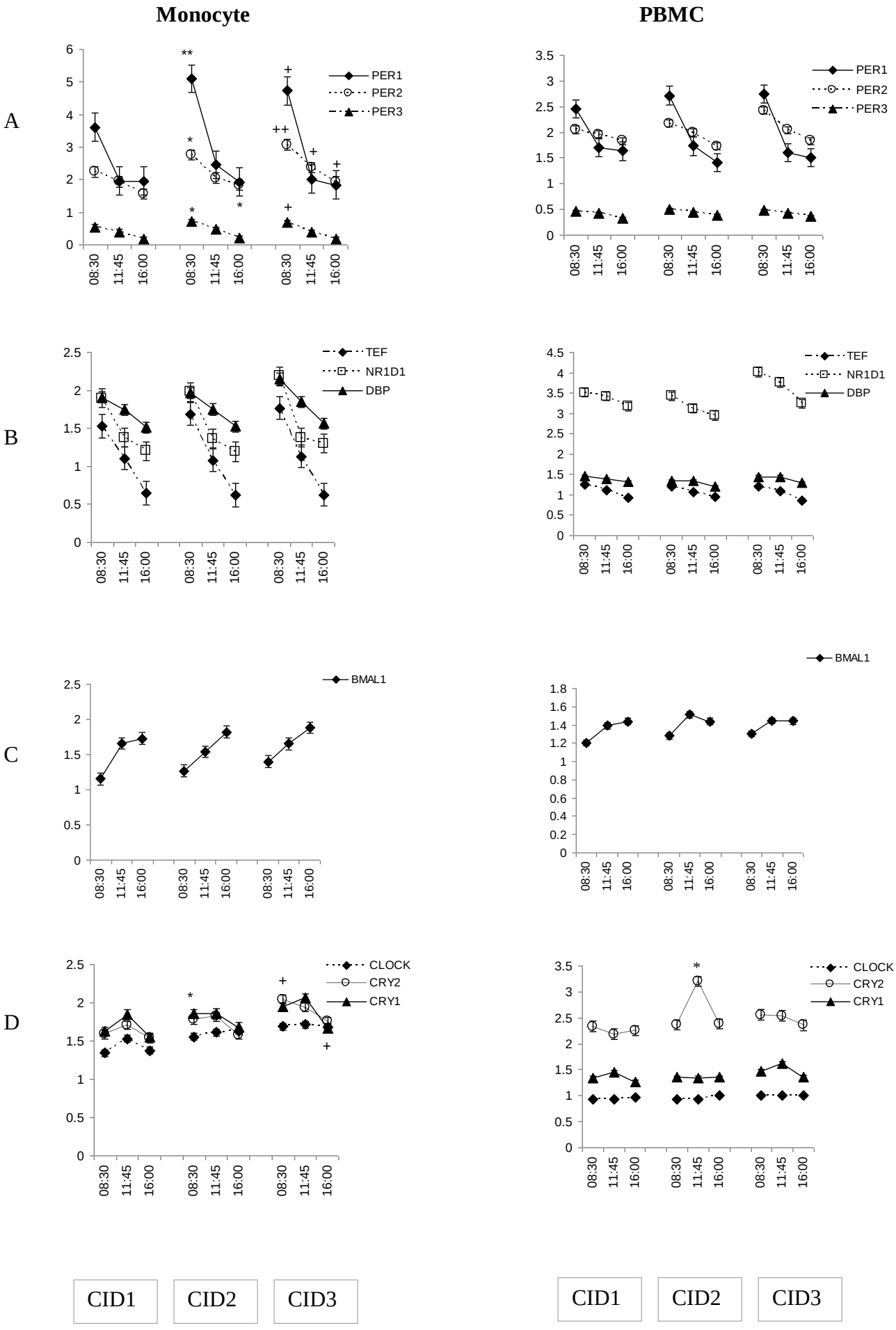

Monocyte
PBMC
A
B
C
D
PER1
PER2
PER3
TEF
NR1D1
DBP
BMAL1
CLOCK
CRY2
CRY1
08:30
11:45
16:00
CID1
CID2
CID3
CID1
CID2
CID3

Figure 3.1 Temporal profiles of clock gene expression in human PBMC and monocytes. Expression of ten clock genes was measured at three time points of three CIDs by qRT-PCR and normalized to the geometric mean of three reference genes *PPIB, B2M* and the *HPRT* expression. The genes were grouped into panels by their temporal profiles: A, B – genes that showed high levels in the morning and low levels in the afternoon (downward trend); C – genes that showed lower expression in the morning and increased in the afternoon (upward trend); D - genes that showed relatively small daily changes. The p-value for time-dependent change was determined by two-way ANOVA for time and patient. Data were presented as mean±SE. *P<0.05, ** P<0.01 vs. CID 1; + p< 0.05, ++ P<0.01 vs. CID 1.

Remarkably, amplitudes of daily changes of *PER1, PER3, DBP, TEF* and *BMAL1* were significantly higher in monocytes compared with the mixed PBMC population. Expression levels of some of the clock genes studied were also different between these two cell populations.

Interestingly, some twin pairs demonstrated very similar expression levels and daily changes of clock gene expression, whereas in other pairs these patterns were absolutely different.

3.2.2 HFD effects on the clock gene expression levels

To investigate the effects of HFD on the clock machinery, we compared the clock gene expression at the same time points before and after the diet interventions. In response to HFD, in monocytes, the morning expression of the *Period* genes *PER1, PER2* and *PER3* increased after one week of HFD (P=0.005, P=0.041and p=0.015 for *PER1, PER2* and *PER3*, respectively) (Fig. 3.1 A). The amplitude of daily expression changes of the aforementioned genes was accordingly increased (41.27%, 23.21% and 29.09% for *PER1, PER2* and *PER3*, respectively). The morning expression of *PER1* and *PER3* was upregulated after six weeks of HFD (p=0.049 and p=0.019 for *PER1* and *PER3*, respectively). Additionally, *PER2* expression increased in the afternoon after one week (p=0.034) and throughout the investigation day after 6 weeks (P=0.006, p=0.02 and p=0.034 for three time points, respectively). Moreover, expression of *CLOCK, Cry1* and *Cry2* was also significantly higher after diet intervention (P=0.12, P=0.034 and P=0.019). However, in PBMCs, only *Cry2* increased after one week of HFD intervention. No Influence of HFD on the expression of other clock genes was found.

3.2.3 Correlation analysis of clock gene expression in PBMC and monocytes

To better define the relationship among circadian clock gene expression under the diet interventions, we analysed correlation of all ten clock genes after one week and six weeks using the Pearson test.

Correlation analysis in PBMCs revealed that there were close links among clock genes. However, such relationship was disrupted after the diet intervention (Table 3.2).

	CID1			CID2			CID3		
Clock genes	TP1	TP2	TP3	TP1	TP2	TP3	TP1	TP2	TP3
BMAL1	CRY1 0.672	CRY1 0.580	CRY1 0.584		PER1 0.751	CRY1 0.682			
CLOCK	CRY2 0.634	CRY2 0.749	**CRY2 0.825**	CRY2 0.580	CRY1 0.643	CRY2 0.581	PER2 0.738	PER2 0.662	
	PER2 0.774	PER1 0.612	PER1 0.586	PER2 0.669	CRY2 0.815	PER1 0.713		PER3 0.598	
		PER2 0.668	PER2 0.745		PER3 0.640	PER2 0.674			
			TEF 0.665			PER3 0.655			
CRY1	PER3 0.654			CRY2 0.805	CRY2 0.739		PER3 0.688		CRY2 0.580
				PER2 0.631	PER2 0.782				PER1 0.659
									PER3 0.701
CRY2			PER2 0.771	PER2 0.818			PER2 0.778	PER1 0.724	
								PER2 0.862	
PER1	PER2 0.702	PER3 0.611	PER2 0.662			PER2 0.595		PER2 0.810	PER2 0.740
	PER3 0.735	TEF 0.668				PER3 0.636			PER3 0.629
PER2			TEF 0.700			**PER3 0.880**			PER3 0.633
PER3			TEF 0.799						
Other core components									
DBP	CLOCK 0.703		CLOCK 0.642	CRY2 0.599	CRY2 0.645	NR1D1 0.629	CLOCK 0.727	CLOCK 0.712	**NR1D1 0.851**
	CRY2 0.607		CRY2 0.643	NR1D1 0.761			CRY2 0.742	CRY2 0.755	PER1 0.759
	NR1D1 0.692		**NR1D1 0.870**				PER2 0.749	NR1D1 0.822	PER2 0.706
	PER2 0.659		PER1 0.701					PER1 0.774	PER3 0.709
			PER2 0.843					***PER2 0.917***	
			PER3 0.668						
			TEF 0.742						
NR1D1	CLOCK 0.712	CLOCK 0.616	CLOCK 0.709	CLOCK 0.630	CRY1 0.655	CLOCK 0.608	**CLOCK 0.826**	CLOCK 0.755	CLOCK 0.578
	PER1 0.710	PER1 0.705	CRY2 0.708	CRY1 0.606	PER2 0.646	CRY1 0.606	CRY2 0.600	***CRY2 0.908***	CRY2 0.597
	PER2 0.922	PER2 0.590	**PER1 0.842**	CRY2 0.777			PER2 0.816	PER1 0.697	PER3 0.594
			PER2 0.908	PER2 0.712				**PER2 0.872**	
			TEF 0.594						

Table 3.2 Correlation analysis of clock genes in PBMCs after diet intervention (6 twin pairs). (1) CID1: at the beginning, CID2: after one week, CID3: after six weeks of diet intervention. (2) TP: time point, TP1: 08:30, TP2: 11:45, TP3: 16: 00. Data were presented as $\gamma_{Pearson.}$ 0.01<p<0.05, 0.001<p<0.01, **0.0001<p<0.001**, ***p<0.0001.***

Correlation analysis in monocytes revealed that there were close links among clock genes. However, such relationship was disrupted after the diet intervention (Table 3.3)

	CID1			CID2			CID3		
Clock genes	TP1	TP2	TP3	TP1	TP2	TP3	TP1	TP2	TP3
BMAL1	CRY1 0.593		CLOCK 0.593	***PER2 0.915***					PER1 0.586
			CRY1 0.674						
CLOCK	CRY2 0.696	**CRY1 0.871**		CRY1 0.698	CRY1 0.656	CRY2 0.801	CRY1 0.787	**CRY1 0.871**	CRY1 0.619
	PER1 0.704	CRY2 0.679		CRY2 0.579	***CRY2 0.904***	PER3 0.736	CRY2 0.741	**CRY2 0.899**	CRY2 0.643
	PER3 0.652	PER1 0.684					PER2 0.822	**PER2 0.849**	***PER2 0.920***
		PER2 0.920						TEF 0.579	
		PER3 0.752							
CRY1		CRY2 0.739	CRY2 0.604	CRY2 0.694	PER2 0.581	CRY2 0.601	***CRY2 0.911***	**CRY2 0.829**	**CRY2 0.828**
		PER2 0.894	PER1 0.757		PER3 0.787	PER1 0.597	PER2 0.795	PER1 0.653	
		PER3 0.682			TEF 0.773	PER2 0.720		**PER2 0.861**	
CRY2		PER2 0.740				PER3 0.598	PER2 0.772	PER1 0.668	TEF 0.670
								PER2 0.776	
								TEF 0.753	
PER1	PER3 0.637	PER1 0.778	PER3 0.690		PER3 0.787	**PER2 0.867**	PER3 0.701	PER2 0.585	TEF 0.646
		PER3 0.750			TEF 0.773			TEF 0.610	
		TEF 0.741							
PER2	PER3 0.658	PER3 0.739			TEF 0.626	PER3 0.601			PER3 0.597
PER3		**TEF 0.890**			TEF 0.631		TEF 0.581		
Other core components									
DBP	PER3 0.618	**NR1D1 0.859**	PER1 0.663	NR1D1 0.796	CRY1 0.607	NR1D1 0.795	CLOCK 0.628	**CLOCK 0.856**	**CLOCK 0.836**
			PER3 0.689		CRY2 0.588	PER1 0.803	CRY1 0.581	CRY1 0.808	CRY2 0.788
					NR1D1 0.703	PER2 0.578	CRY2 0.676	CRY2 0.723	NR1D1 0.792
							NR1D1 0.853	**NR1D1 0.834**	PER1 0.640
							PER2 0.730	**PER2 0.886**	PER2 0.803
								TEF 0.590	PER3 0.639
									TEF 0.758
NR1D1	CLOCK 0.637	CLOCK 0.702	CRY2 0.657	PER1 0.703	PER3 0.669	PER1 0.696	PER2 0.585	CLOCK 0.642	**CLOCK 0.874**
	PER1 0.732	CRY2 0.689						CRY2 0.719	CRY1 0.597
	PER2 0.704	PER2 0.580						**PER2 0.863**	CRY2 0.651
	PER3 0.925								PER1 0.646
									PER2 0.809
									PER3 0.673

Table 3.3 Correlation analysis of clock genes in monocytes after diet intervention (6 twin pairs). (1) CID1: at the beginning, CID2: after one week, CID3: after six weeks of diet intervention. (2) TP: time point, TP1: 08:30, TP2: 11:45, TP3: 16: 00. Data were presented as $\gamma_{Pearson.}$ $0.01<p<0.05$, $0.001<p<0.01$, **$0.0001<p<0.001$**, ***$p<0.0001$*.**

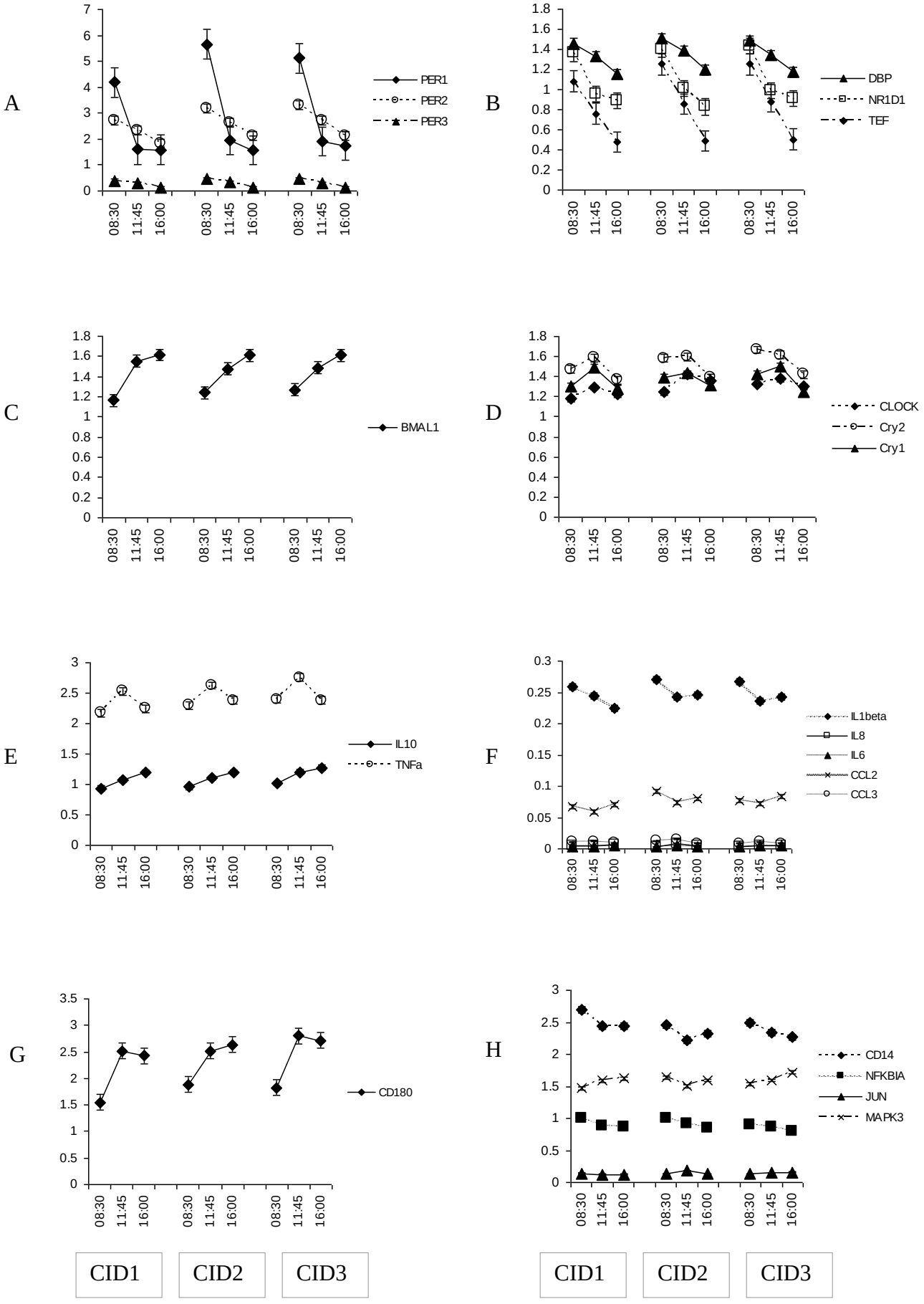
3.3 Analysis of clock gene expression, cytokine and metabolic gene expression in human monocytes (thirty non-obese individuals)

PBMCs are widely used in human studies, however, they are a population of several cell types including monocytes, lymphocytes and macrophages. Each cell subpopulation could display different circadian rhythm and responses to the diet intervention, so this may contribute to the different results from PBMCs and monocytes.

3.3.1 Daily variation of clock gene, cytokine and metabolic gene expression in human monocytes

Data from thirty non-obese individuals show that rhythmic expression of ten clock genes in monocytes was consistent with the results described above (Fig. 3.2 A, B, C, D). In addition, the mRNA expression of *CD14*, *CD180* and *NFKBIA* as well as *NAMPT*, *FASN* and *CPT1A* displayed significant daily variation. *CD14*, *CPT1A* and *NAMPT* showed the highest levels in the morning and dropped to trough levels in the afternoon (Fig. 3.2 H, K). In contrast to these genes, *NFKBIA* showed the lowest level in the morning and reached the peak in the afternoon (Fig. 3.2 H). *CD180* demonstrated an upward trend and reached maximal expression in the middle of the day, while *FASN* showed the highest level in the afternoon (Fig. 3.2 G, J). The mRNA levels of *IL1beta* (*ILβ*), *IL8*, *IL10*, *TNFα*, *IL6*, *CCL2*, *CCL3*, *JUN*, *MAPK3*, *ACOX3* as well as *HADH*, *IDH3A* and *LPL* (Fig. 3.2 E, F, I) showed no variation throughout the day. During all three CIDs, the clock genes studied showed the same daily variation.

Monocytes


A
PER1
PER2
PER3
B
DBP
NR1D1
TEF
C
BMAL1
D
CLOCK
Cry2
Cry1
E
IL10
TNFa
F
IL1beta
IL8
IL6
CCL2
CCL3
G
CD180
H
CD14
NFKBIA
JUN
MAPK3
08:30
11:45
16:00
CID1
CID2
CID3

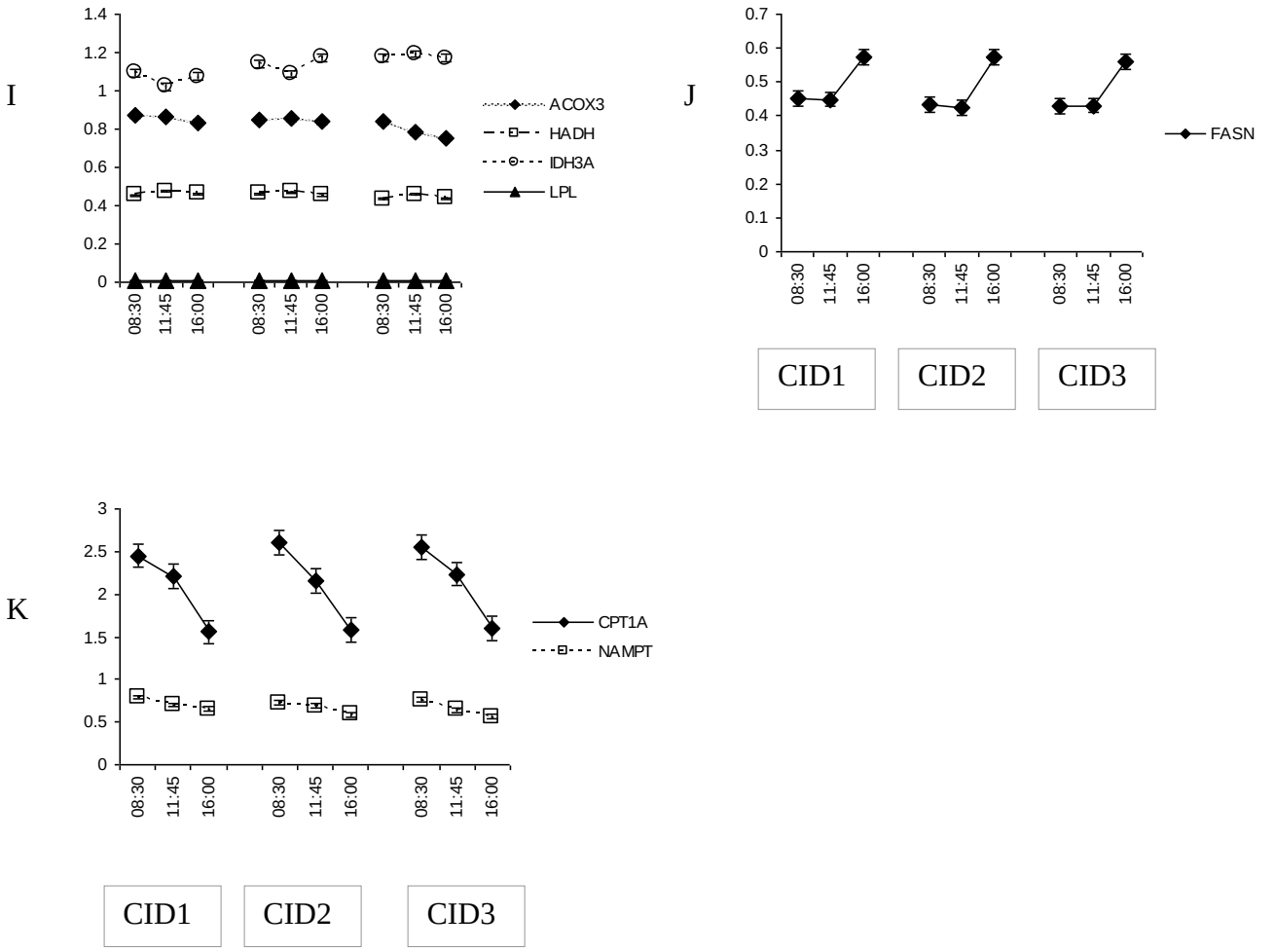


Figure 3.2 Temporal profiles of clock gene, cytokine, and fat metabolic gene expression in human monocytes. Expression of ten clock genes as well as cytokine and fat metabolic gene was measured at three time points of three CIDs by qRT-PCR and normalized to the geometric mean of three reference genes PPIB, B2M and the HPRT expression. The genes were grouped into panels by their temporal profiles: A, B, K – genes that showed high levels in the morning and low levels in the afternoon (downward trend); C, J – genes that showed lower expression in the morning and increased in the afternoon (upward trend); D, E, F, H, I - genes that showed relatively small daily changes. G - genes that showed an upward trend and reached maximal expression in the middle of the day . The p-value for time-dependent change was determined by two-way ANOVA for time and patient. Data were presented as mean±SE. *P<0.05, ** P<0.01 vs. CID 1; + p< 0.05, ++ P<0.01 vs. CID 1.

3.3.2 HFD effects on the clock gene, cytokine and fat metabolic gene expression levels

We also compared the clock gene, cytokine and metabolic gene expression at the same time points before and after the diet interventions. In response to HFD, the morning expression of the *Period* genes *PER1, PER2* and *PER3* increased after one week of HFD (P=0.014, P=0.017and p=0.048 for *PER1, PER2* and *PER3,* respectively) (Fig.3.2. A), and *PER2* expression was upregulated in the afternoon after one week of HFD (p=0.034). Intriguingly, *PER2* expression increased during the day after six weeks of HFD (p=0.006, p=0.017 and p=0.025, respectively), and *PER1* expression was upregulated in the middle of the day after six weeks of HFD (p=0.033). Moreover, the morning expression of *CD14* decreased after one and/or six weeks of interventions (P=0.042 and P=0.048), and *CD14* expression decreased in the middle of the day after one week of diet (p=0.019) (Fig.3.2. H). In addition, the morning expression of *NFKBIA* and the second time point of *ACOX3* expression diminished after six weeks (p=0.021 and p=0.042) (Fig.3.2. H,I). No Influence of HFD on the expression of other genes detected was found.

3.3.3 Correlation analysis of clock gene, cytokine and fat metabolic gene expression in monocytes

Correlation analysis in monocytes revealed that there were close links among clock genes. However, such relationship was disrupted after the diet intervention (data not shown)

Correlation analysis in monocytes revealed that there were close links among clock genes and genes related to inflammation, however, such relationship was disrupted after the diet intervention (Table 3.4)

	CID1			CID2			CID3		
Clock genes	TP1	TP2	TP3	TP1	TP2	TP3	TP1	TP2	TP3
BMAL1	IL8 0.542	JUN 0.499	IL10 0.370	IL10 0.380	CD180 0.392	CD180 0.499	CD180 0.553	CCL2 0.514	CD180 0.469
	NFKBIA 0.565	NFKBIA 0.378		NFKBIA 0.381	NFKBIA 0.607	TNFa 0.498	IL1beta 0.367	CD180 0.438	IL8 0.428
							IL10 0.434	IL10 0.581	JUN 0.386
								NFKBIA 0.464	
								TNFa 0.383	
CLOCK	IL1beta 0.375	IL8 0.464	CCL3 0.395	CD180 0.424	CD180 0.641	CD180 0.551	CCL2 0.421	CD180 0.687	CD180 0.704
	IL10 0.384	JUN 0.386	CD180 0.465	IL10 0.470	JUN 0.568	IL1beta 0.412	CD180 0.668	IL8 0.454	IL1beta 0.442
	JUN 0.558	MAPK3 0.672	IL1beta 0.422	JUN 0.467	NFKBIA 0.491	IL8 0.427	IL1beta 0.370	IL10 0.663	IL8 0.517
	MAPK3 0.558		IL10 0.623	MAPK3 0.446		IL10 0.593	IL8 0.424	JUN 0.672	IL10 0.565
	TNFa 0.364		NFKBIA 0.607	TNFa 0.491		JUN 0.624	IL10 0.692	TNFa 0.536	JUN 0.523
						TNFa 0.428	JUN 0.537		NFKBIA 0.361
							TNFa 0.710		TNFa 0.615
CRY1	IL10 0.377	IL8 0.615	IL10 0.478	CD180 0.422	JUN 0.539	CD180 0.523	CCL3 0.453	CD180 0.617	CCL3 0.594
	JUN 0.418	JUN 0.576	TNFa 0.443	IL8 0.437	TNFa 0.370	IL1beta 0.385	CD14 0.525	IL8 0.632	CD180 0.639
	MAPK3 0.529	MAPK3 0.515		IL10 0.482		IL8 0.492	CD180 0.487	IL10 0.587	IL1beta 0.390
	TNFa 0.440	TNFa 0.386		JUN 0.692		IL10 0.562	IL1beta 0.466	TNFa 0.560	IL8 0.530
				TNFa 0.608		JUN 0.592	IL8 0.516		IL10 0.549
						TNFa 0.580	IL10 0.553		JUN 0.703
							JUN 0.742		TNFa 0.560
							TNFa 0.713		
CRY2	JUN 0.491		IL1beta 0.377	**JUN 0.830**	CD180 0.500	CD180 0.368	CD14 0.550		CCL3 0.430
	TNFa 0.390		JUN 0.465		***JUN 0.914***	IL8 0.462	IL1beta 0.424	CD180 0.697	CD180 0.587
			TNFa 0.609			IL10 0.417	IL8 0.365	IL10 0.533	JUN 0.741
						JUN 0.741	JUN 0.795	**JUN 0.846**	TNFa 0.484
						TNFa 0.473	TNFa 0.596	TNFa 0.561	
NR1D1	IL1beta 0.458	IL8 0.444	JUN 0.531	IL8 0.411	IL8 0.369	CCL3 0.537	CD14 0.373	CCL3 0.420	CCL3 0.666
	JUN 0.484	TNFa 0.509	TNFa 0.668	JUN 0.507	JUN 0.520	CD180 0.378	CD180 0.377	CD180 0.624	CD180 0.646
	MAPK3 0.382			TNFa 0.453		IL8 0.529	JUN 0.491	IL8 0.505	JUN 0.512
	TNFa 0.543					IL10 0.390	TNFa 0.563	IL10 0.464	TNFa 0.581
						JUN 0.667		JUN 0.761	
						TNFa 0.450		TNFa 0.594	
PER1	IL1beta 0.515	IL8 0.390	TNFa 0.401	CD180 0.475	NFKBIA 0.462	CD180 0.414			
	IL8 0.468	MAPK3 0.477				IL8 0.671			
	NFKBIA 0.540					IL10 0.402			
						JUN 0.552			
PER2	IL6 0.364	CD180 0.367			IL6 0.539	CD14 0.453	IL6 0.473	IL6 0.425	MAPK3 0.373
	IL8 0.440	JUN 0.543			MAPK3 0.410	IL6 0.499		MAPK3 0.451	NFKBIA 0.386
	JUN 0.415				NFKBIA 0.593	MAPK3 0.487			
	NFKBIA 0.450					NFKBIA 0.405			
						TNFa 0.382			
PER3	IL1beta 0.541	IL1beta 0.500	CD14 0.366	IL6 0.412		CCL3 0.386	CD180 0.463	IL8 0.428	CCL2 0.398
	JUN 0.376	IL8 0.379	TNFa 0.470	IL8 0.412		IL1beta 0.571	JUN 0.639	JUN 0.452	CCL3 0.535
	MAPK3 0.498			JUN 0.402		IL8 0.431	TNFa 0.476		CD180 0.657
	TNFa 0.546			TNFa 0.452		JUN 0.648			IL1beta 0.404
						TNFa 0.439			IL10 0.380
									JUN 0.519
									TNFa 0.614

Table 3.4 Correlation analysis of clock genes and genes related to inflammation in monocytes after diet intervention (15 twin pairs). (1) CID1: at the beginning, CID2: after one week, CID3: after six weeks of diet

intervention. (2) TP: time point, TP1: 08:30, TP2: 11:45, TP3: 16: 00. Data were presented as $\gamma_{Pearson.}$ 0.01<p<0.05, 0.001<p<0.01, **0.0001<p<0.001**, ***p<0.0001.***

Correlation analysis in monocytes revealed that there were close links among clock genes and genes related to fat metabolism, however, such relationship was disrupted after the diet intervention (Table 3.5).

	CID1			CID2			CID3		
Clock genes	TP1	TP2	TP3	TP1	TP2	TP3	TP1	TP2	TP3
BMAL1	CPT1A 0.415		IDH3A 0.375		FASN 0.368	FASN 0.362	IDH3A 0.414	IDH3A 0.563	CPT1A 0.652
	IDH3A 0.512				HADH 0.632	HADH 0.481			IDH3A 0.542
					IDH3A 0.498	IDH3A 0.709			
CLOCK	CPT1A 0.578	CPT1A 0.584	CPT1A 0.541	CPT1A 0.435	CPT1A 0.473	CPT1A 0.670	CPT1A 0.710	CPT1A 0.748	ACOX3 0.498
	IDH3A 0.556	IDH3A 0.489	HADH 0.547	IDH3A 0.540	FASN 0.461	HADH 0.469	IDH3A 0.600	IDH3A 0.742	**CPT1A 0.828**
			IDH3A 0.570		HADH 0.597	NAMPT 0.373			IDH3A 0.815
			NAMPT 0.525						
CRY1	ACOX3 0.446	ACOX3 0.444	ACOX3 0.376	ACOX3 0.460	ACOX3 0.420	ACOX3 0.468	**CPT1A 0.828**	ACOX3 0.411	ACOX3 0.379
	CPT1A 0.805	CPT1A 0.699	CPT1A 0.642	CPT1A 0.769	CPT1A 0.775	**CPT1A 0.832**	IDH3A 0.613	***CPT1A 0.918***	CPT1A 0.788
	IDH3A 0.652	IDH3A 0.584	HADH 0.412	IDH3A 0.714	FASN 0.455	FASN 0.368			IDH3A 0.811
			IDH3A 0.500			HADH 0.544			
						IDH3A 0.768			
CRY2	ACOX3 0.371	CPT1A 0.390	ACOX3 0.394	ACOX3 0.453		ACOX3 0.364	CPT1A 0.697	CPT1A 0.744	CPT1A 0.650
	CPT1A 0.419	HADH 0.362	CPT1A 0.412	CPT1A 0.444		CPT1A 0.649		IDH3A 0.575	
		LPL 0.426	IDH3A 0.395			HADH 0.376			
		NAMPT 0.394	LPL 0.393			IDH3A 0.566			
NR1D1	ACOX3 0.376	ACOX3 0.437	ACOX3 0.436	ACOX3 0.566	ACOX3 0.362	ACOX3 0.488	ACOX3 0.364	CPT1A 0.652	ACOX3 0.463
	CPT1A 0.471	CPT1A 0.584	CPT1A 0.630	CPT1A 0.668	CPT1A 0.386	CPT1A 0.728	CPT1A 0.643	IDH3A 0.598	CPT1A 0.647
	IDH3A 0.434	IDH3A 0.491	HADH 0.416	IDH3A 0.579	IDH3A 0.417	IDH3A 0.588	IDH3A 0.475		IDH3A 0.719
	LPL 0.438		IDH3A 0.539		LPL 0.564				LPL 0.367
	NAMPT 0.477								
PER1	LPL 0.463	CPT1A 0.446	CPT1A 0.444		CPT1A 0.446	ACOX3 0.582		CPT1A 0.382	
		IDH3A 0.436	LPL 0.517		HADH 0.435	CPT1A 0.723			
					IDH3A 0.405	FASN 0.522			
						HADH 0.481			
						IDH3A 0.594			
PER2	CPT1A 0.384	CPT1A 0.527	CPT1A 0.410	CPT1A 0.528	CPT1A 0.421				
	FASN 0.462	IDH3A 0.459		IDH3A 0.507	HADH 0.373				
	LPL 0.501	LPL 0.542							
		NAMPT 0.405							
PER3	CPT1A 0.544	IDH3A 0.451		CPT1A 0.650	ACOX3 0.366	CPT1A 0.670	CPT1A 0.570	CPT1A 0.558	CPT1A 0.749
	IDH3A 0.565			FASN 0.434	CPT1A 0.451	IDH3A 0.608	IDH3A 0.632	IDH3A 0.565	IDH3A 0.702
	LPL 0.394			HADH 0.379	HADH 0.394		NAMPT 0.389		
				IDH3A 0.582	IDH3A 0.561				

Table 3.5 Correlation analysis of clock genes and fat metabolic genes in monocytes after diet intervention (15 twin pairs). (1) CID1: at the beginning, CID2: after one week, CID3: after six weeks of diet intervention. (2) TP: time point, TP1: 08:30, TP2: 11:45, TP3: 16: 00. Data were presented as γ_{Pearson}. $0.01<p<0.05$, $0.001<p<0.01$, **$0.0001<p<0.001$**, ***$p<0.0001$.***

4. Discussion

In this study, we first described in detail the daily expression profiles of clock genes in human monocytes and compared them with the PBMC fractions. Moreover, in the analysis of effects of an isocaloric HFD on circadian clock genes in humans, we demonstrated for the first time that clock oscillations in monocytes are affected by a HFD. Significant changes of *Period* gene expression were found in monocytes. However, in PBMCs, these alterations were not detected. Disruption of the circadian clock and its relationship with genes related to inflammation and fat metabolism after the diet were found in both PBMCs and monocytes.

We demonstrated that *PER1, PER2, PER3, BMAL1, REV-ERBα, DBP* and *TEF* were rhythmically expressed in human monocytes, whereas *CLOCK*, *CRY1* and *CRY2* displayed no significant daily expression changes. In PBMCs, similar temporal expression profiles were detected, confirming the results of previous studies in human PBMCs, peripheral leucocytes and blood cells (Fukuya et al., 2007; Takimoto et al., 2005; Kusanagi et al., 2004; Archer et al., 2008; Boivin et al., 2003) and other tissues (Bjarnason et al., 2001; Ripperger et al., 2006; Loboda et al., 2009). Remarkably, *PER1* demonstrated a most robust daily change in accordance with literature data (Fukuya et al., 2007; Loboda et al., 2009). In our study, *PER1-3, DBP, TEF* and *NR1D1* peaked in the early hours of the day, whereas *BMAL1* peaked in the afternoon. However, we didn't detect an atypical pattern of clock gene expression in PBMCs, which was described as similar circadian patterns of *BMAL1* and *PER2*. (Teboul et al., 2005).

Interestingly, the circadian patterns were more pronounced in isolated monocytes compared with PBMCs. PBMCs are a heterogeneous population of white blood cells consisting of monocytes (≈10 %) and predominat lymphocytes including T cells (~75%), B cells and NK cells (~25% combined). The relative contributions of these populations were shown to display circadian changes (Abo et al., 1981; Levi et al., 1988). Each of these cell types could exhibit different circadian rhythms and different responses to the diet interventions, so that the whole mixed population demonstrates the average rhythm obviously reflecting more or less that of the prevalent cell subpopulation. Such average rhythm could explain the relatively lower amplitude of circadian rhythms detected in human mixed blood cell populations such as peripheral leucocytes (Ando et al., 2009; Haimovich et al., 2010) in comparison with that in the liver and adipose tissue (Loboda et al., 2009; Oike et al., 2011). In our study, the amplitude of circadian rhythms in PBMCs was also lower compared with monocytes.

Moreover, in our study, we also found significant changes of clock gene expression after HFD intervention in isolated monocytes, which were undetectable in PBMC. Notably, the changes of clock gene expression observed in a mixed leucocyte population in response to endotoxin administration were reported to reflect changes occurring in the predominantly neutrophil subpopulation but not in monocytes (Haimovich et al., 2010). Although mixed blood cell populations are widely used in clinical studies (Ando et al., 2009), because such practical approach minimizes manipulation time, the aforementioned evidence confirms the limitations of the analysis of such populations.

However, the extraction of human monocytes from the blood samples is also a relatively simple and little-invasive method of monitoring of human circadian oscillator activity compared with repeated biopsies of subcutaneous adipose tissue or bone marrow which are too invasive to be useful (Loboda et al., 2009; Tsinkalovsky et al., 2007). Therefore, the investigation of the isolated monocytes or other single populations of blood cells could be the most suitable method for the study of the clock gene regulation in humans.

Monocytes are a part of mammalian innate immune system. They play multiple roles in immunological function including cytokine secretion and differentiation into macrophages and dendritic cells. Surprisingly, circadian rhythm and immune response are closely interrelated. Both immune cell number and function such as cytokine secretion, phagocytosis, lytic activity and antibody titers are subject to circadian regulation (Born et al., 1997; Lange et al., 2010; Boivin et al., 2003; Arjona et al., 2006; Keller et al., 2009; Hayashi et al., 2007, Mazzoccoli et al., 2010). In turn, proinflammatory cytokines produced by activated immune cells can reset the circadian clock in peripheral tissues during inflammation (Cavadini et al., 2007). In humans, acute systemic inflammation suppresses clock gene expression in peripheral blood leucocytes, monocytes and neutrophils and possibly uncouples it from the activity of the central clock (Haimovich et al., 2010). Moreover, circulating monocytes react directly to the alterations of metabolism as well as infections and are also involved in metabolic processes such as cholesterol homeostasis (Cuchel et al., 2006). Moreover, macrophages are closely linked to insulin sensitivity via regulation of the inflammatory subtype of macrophages in adipose tissue (Odegaard et al., 2007). Thus, the HFD-induced alterations of circadian clocks in monocytes might result in modulation of immune functions observed in obesity and the metabolic syndrome.

It is interesting that *CD14* expression significantly decreased after one and/or six weeks of interventions, *NFKBIA* expression reduced after six weeks, whereas *CD180, MAPK3, JUN, IL1β, IL6, IL8, TNFα, CCl2* and *CCl3* showed no significant changes. Together with the Toll-like receptor 4 (TLR 4) and lymphocyte antigen 96 (MD-2), CD14 acts as a co-factor for the recognition of pathogen-associated molecular patterns, especially lipopolysaccharide (LPS) (Kitchens et al., 2007; Tapping et al., 2000). NFKBIA is an inhibitor of NF-κB factors. Moreover, disruption of NF-κB signaling is related to inflammatory and autoimmune diseases, septic shock, viral infection, cancer and dysfunctional immune development. Thus, we supposed that high-fat diet might affect immune function through Toll-like signaling pathways. Moreover, correlation analysis in monocytes revealed disruption of correlation links among clock genes and cytokines after diet. Therefore, this indicated that diet intervention could affect the timing network and immune system, leading to various abnormalities in the organisms.

In this study, we demonstrated for the first time that clock oscillations in monocytes could be affected by diet interventions, confirming data obtained in other peripheral tissues in humans and animal studies. Kohsaka and his colleagues (Kohsaka et al., 2007) reported that a high-fat diet reduced the rhythmic mRNA expression of *Clock, Bmal1* and *Per2* in the liver and adipose tissues of mice. However, another mouse study could not confirm an impairment of the circadian clock in the liver and adipose tissues even though the mice developed a metabolic syndrome characterised by obesity, hyperlipidaemia and hyperglycaemia (Yanagihara et al., 2006).

In response to high-fat diet, in monocytes, the expression and amplitude of *PER1, PER2* and *PER3* increased after one and six weeks of intervention. *Period* genes, core component of the mammalian circadian clock, are implicated in cell cycle events, growth and metabolic regulation (Ko et al., 2006). *Per2*$^{-/-}$ mice showed an absence of diurnal feeding rhythms and developed significant obesity in response to a high-fat diet (HFD) (Yang et al., 2009). Indeed, *PER1* expression in human adipose tissue correlates with numerous genes involved in the metabolic regulation as well as cytokine expression (Loboda et al. 2009). Interestingly, *PER1* and *PER2* regulate the cell cycle and DNA damage repair, controlling tumor growth (Gery et al., 2010). Therefore, circadian dysfunction might be a link between metabolic diseases and cancer.

Clocks in peripheral tissues are well-known to be entrained by feeding (Green et al., 2008), and feeding patterns may play an important role in the synchronization of SCN-controlled and food entrainable oscillations. In this study, we firstly demonstrated that diet composition in humans

can also trigger a change of the synchronization state of the clock system. Indeed, the correlation analysis in monocytes and PBMCs reveals disruption of relationship among clock genes after the HFD intervention. The synchronization level of the circadian system obviously plays an important role in the organism, to adapt to metabolic and environmental challenges. Disruption of clock gene interactions is speculated to disturb the clock-mediated regulation of metabolic functions and may contribute to the pathophysiology of metabolic diseases (Gomez-Abellan et al., 2008). Particularly, the increase of the synchronization of clock expression was shown in human leucocytes during the acute period of systemic inflammation (Haimovich et al., 2010). Indeed, in animal models of shift work, the experimentally induced circadian disruption altered innate immune responses (Gomez-Abellan et al., 2008).

Thus, our results suggest that consumption of an isocaloric HFD can influence the circadian mechanism in humans already after a short time of intervention and emphasize the role of nutrition-clock interaction in the regulation of human metabolism. The daily changes of clock gene expression observed by us were similar to those found in adipose tissue (Loboda et al., 2009). However, further studies are required to investigate whether HFD effects on the clock gene expression in monocytes also mirrors changes of circadian rhythm in other tissues.

We demonstrated that *ACOX3* expression decreased after 6 weeks of intervention. It is well-known that *ACOX3* is related to the desaturation of 2-methyl branched fatty acids in peroxisomes. In humans, it is expressed at an extremely low level in tissues, thus it is undetectable by routine Northern-blot or immunoblot analysis or enzyme activity measurements. Despite the low amount, it plays an important role in peroxisomal fatty acid β-oxidation and the development of prostate cancer (Vanhooren et al., 1997; Zha et al., 2005; Westin et al., 2007). Therefore, effects of a high-fat diet on clockwork may affect lipid metabolism via *ACOX3*. Interestingly, we also found the correlation links of clock genes and fat metabolic genes were significantly altered. This contributed to the better understanding that diet influences metabolic processes and clock machinery, finally causing series of dysfunctions.

Indeed, the total cholesterol level was increased already after one week, and LDL cholesterol after six weeks of diet intervention. Low-density lipoproteins (LDL) carry cholesterol and other lipophilic substances in extrahepatic tissues and are often oxidized to permanently elevated concentrations in the blood. This situation may increase the risk of atherogenic diseases (Masson et al., 2009). Interestingly, the triglyceride level was significantly decreased after six weeks of

HFD. The triglyceride-lowering effect may indicate an increased mitochondrial fatty acid oxidation, caused by a reduced availability of other substrates (Schrauwen et al., 2000).

Mechanisms by which HFD influences circadian rhythmicity are not fully understood. The molecular mechanisms coupling the cell metabolic state to the core clock circuitry are the subject of intensive study. NAD^+-dependent enzymes such as sirtuins, poly[ADP-ribose] polymerases, redox- and/or temperature-dependent transcription factors (CLOCK, NPAS2, HSF1), nutrient-sensing transcriptional regulatory proteins (CREB-CBPCRCT2, FOXO-p300, nuclear receptors, PGC-1, and SP1 family members) and protein kinases (e.g., AMPK), are plausible candidates for conveying a cell's metabolic state to the core clock circuitry (Asher et al., 2011). At the cell level, fatty acids might regulate PPAR-α and PPAR-gamma controlling *BMAL1* and *NR1D1* expression (Teboul et al., 2009), as well as redox status and AMPK activity (Nakahata et al. 2009). Kohsaka (Kohsaka et al., 2007) demonstrated the phase shift of clock-associated nuclear receptors and regulators RORα, PPARα and PPARγ as well as SREBP-1c after HFD in mice.

In conclusion, our results suggest that a high-fat isocaloric diet can influence the circadian mechanism in humans already after 6 days of intervention and emphasize the role of nutrition-clock interaction in the regulation of human metabolism. High-fat diet induces alterations of clock gene expression in human monocytes which are undetectable in heterogenic PBMC populations. In addition, high-fat diet alters fat metabolic gene and cytokine expression in human monocytes after a short term. Moreover, isolated monocytes demonstrated more pronounced daily variability compared with PBMC. Therefore, investigation of isolated monocytes could be a new perspective and little-invasive method for the monitoring of the clock gene regulation and its molecular mechanisms in humans.

5. Summary

Background and aims: The circadian system regulates daily rhythms in various aspects of behavior and physiology including locomotor activity and energy homeostasis. On the other hand, metabolic processes feed back into the circadian clock as shown in human studies of obesity and type 2 diabetes and animal studies of high-fat diet interventions. However, little is known about the effect of nutrition on circadian mechanisms in humans. Another important question for human studies is the choice of target tissue investigated to monitor the functions of the clock machinery by a realizable and little-invasive method allowing repeated sampling over the day. To address this, we provided a comparative analysis of clock gene expression in human peripheral blood mononuclear cells (PBMC) and isolated monocytes before and after a high-fat diet intervention.

Materials and methods: Daily expression profiles of ten clock genes, fat metabolic genes and cytokines were determined by real-time PCR in thirty non obese healthy individuals as part of the NUtriGenomics Analysis in Twins (NUGAT) study. Gene expression was measured at three time points (in the morning, at noon and in the afternoon) during three investigation days. The blood sampling was carried out after 6 weeks of a low fat, high-carbohydrate diet before the beginning of a high-fat low carbohydrate isocaloric diet (45 % kcal from fat) and after one and six weeks of this intervention.

Results: (1) We demonstrated that *PER1, PER2, PER3, BMAL1, REV-ERBα, DBP* and *TEF* were rhythmically expressed in human monocytes (twelve non-obese subjects). The clock genes *CLOCK*, *CRY1* and *CRY2* displayed no significant daily expression changes. In PBMC, similar temporal expression profiles were detected. In response to high-fat diet, in monocytes, the expression and amplitude of *PER1, PER2* and *PER3* increased after one and six weeks of intervention, and with a relatively small increase in *CLOCK, CRY1* and *CRY2* after one or six weeks. In contrast, in PBMCs, we only found *CRY2* increased after one week of the high-fat diet. Correlation analysis revealed disruption of the relationship among clock genes after a high-fat diet in monocytes and PBMCs. (2) Similar rhythmic expression of ten clock genes in monocytse was detected in thirty non-obese individuals. In addition, mRNA expression of *CD14, CD180* and *NFKBIA* as well as *NAMPT, FASN* and *CPT1A* displayed significant daily variation, whereas other cytokines and fat metabolic genes studied showed no significant daily expression changes. Moreover, correlation analysis in monocytes revealed disruption of the relationship

among clock genes and its relationship with genes related to inflammation and fat metabolism after a high-fat diet.

Conclusion: Our results suggest that the consumption of a high-fat isocaloric diet can influence the circadian mechanism in humans already after 6 days of intervention and emphasize the role of nutrition-clock interaction in the regulation of human metabolism. High-fat diet induces alterations of clock gene expression in human monocytes which are undetectable in heterogenic PBMC population. Therefore, the investigation of the isolated monocytes could be a new perspective and a less-invasive method of the monitoring of the clock gene regulation and its molecular mechanisms in humans.

Keywords Circadian clock, peripheral blood mononuclear cells, monocytes, high-fat isocaloric diet

Einleitung:

Das zirkadiane System regelt verschiedene Aspekte des Verhaltens und physiologische Prozesse, wie die Ernährung und den Energiestoffwechsel. Andererseits, es ist aus humanen Adipositas- und Diabetes Typ 2-Studien sowie aus den Tierversuchen mit Hochfettdiäten bekannt, das metabolische Prozesse den zirkadianen Rhythmus beeinflussen. Über den Einfluss der Ernährung auf zirkadiane Mechanismen beim Menschen ist jedoch noch sehr wenig bekannt. Eine der wichtigen Fragen für humane Studien ist die richtige Auswahl des Zielgewebes für Untersuchung der Clock-Gene. Die Eingriffe müssen minimal invasiv sein mit der Möglichkeit wiederholter Probenentnahmen während eines Tages. Um dieses Problem anzugehen, haben wir eine Vergleichsanalyse der Effekte einer fettreichen Diät auf die Expression der Clock-Gene in humanen mononukleären Zellen des peripheren Blutes (PBMC) und isolierten Monozyten durchgeführt.

Materialien und Methoden

Im Rahmen der NUGAT-Zwillingsstudie (NUtriGenomics Analysis in Twins) wurde das tägliche Expressionsprofile von 10 Clock-Genen, Fettstoffwechsel-Genen und Zytokinen in 30 nicht übergewichtigen, gesunden Probanden mittels Real-Time PCR gemessen.

Die Genexpression wurde zu drei Zeitpunkten (morgens, mittags und nachmittags) während drei Untersuchungstagen gemessen. Die Blutentnahme erfolgte nach 6 Wochen einer fettarmen, kohlenhydratreichen Diät vor Beginn der Intervention sowie nach einer und sechs Wochen der fettreichen, kohlenhydratarmen und isokalorischen Diät (45% Kcal aus Fett).

Ergebnisse:

Wir haben zum ersten Mal gezeigt, dass die Expression der *PER1, PER2, PER3, BMAL1, REV-ERBα, DBP* und *TEF* Gene in humanen Monozyten (bei zwölf nicht übergewichtigen Probanden) einem Rhythmus unterliegt. Für die Gene *Clock, Cry1* und *Cry2* wurde keine tageszeitliche Veränderungen nachgewiesen. In PBMC wurde ein ähnliches zeitliches Expressionsmuster beobachtet. Die fettreiche Ernährung hat die morgendliche Expression und die Amplitude der drei *Period* Gene *Per1, Per2* und *Per3* in Monozyten nach einer und/oder sechs Wochen der Intervention signifikant erhöht. Im Gegensatz dazu in der PBMC-Fraktion, fanden wir keine

signifikanten Veränderungen der Expression der Clock-Gene. (2) Ähnliche rhythmische Expressionen wurden für zehn Clock-Gene in Monozyten in 30 nicht übergewichtigen Personen gemessen. Darüber hinaus wurden signifikante tägliche Veränderungen in mRNA-Expression von *CD14, CD180* und *NFKBIA* sowie *NAMPT, FASN* und *CPT1A* beobachtet, während die untersuchten Zytokin- und Fettmetabolismus-Gene keine signifikanten Schwankungen in täglichen Expression zeigten.

Diskussion:

Unsere Ergebnisse legen nahe, dass der Verzehr einer fettreichen und isokalorischen Diät den zirkadianen Mechanismus bei Menschen bereits nach 6 Tagen Intervention beeinflussen kann, und betonen die Rolle der Ernährung-zirkadiane Rhythmus Interaktionen bei der Regulation des humanen Stoffwechsels. Fettreiche Diät induziert Veränderungen in Clock-Genexpression in humanen Monozyten, die nicht in heterogener PBMC-Population nachweisbar sind. Daher könnte die Untersuchung der isolierten Monozyten eine Perspektive und wenig invasive Methode der Analyse der zirkadianen Genregulation und ihrer molekularen Mechanismen beim Menschen darstellen.

6. Reference

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Erklärung

Ich, Lu Ye, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: Influence of a high-fat diet on the expression of clock genes and genes related to the fat metabolism and LPS response in human monocytes selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.

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