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ORIGINAL ARTICLE

Regulation of the clock gene expression in human adipose tissue by weight loss

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BACKGROUND: The circadian clock coordinates numerous metabolic processes to adapt physiological responses to light–dark and feeding regimens and is itself regulated by metabolic cues. The implication of the circadian clock in the regulation of energy balance and body weight is widely studied in rodents but not in humans. Here we investigated (1) whether the expression of clock genes in human adipose tissue is changed by weight loss and (2) whether these alterations are associated with metabolic parameters.

SUBJECTS/METHODS: Subcutaneous adipose tissue (SAT) samples were collected before and after 8 weeks of weight loss on an 800 kcal per day hypocaloric diet (plus 200 g per day vegetables) at the same time of the day. Fifty overweight subjects who lost at least 8% weight after 8 weeks were selected for the study. The expression of 10 clock genes and key metabolic and inflammatory genes in adipose tissue was determined by quantitative real-time PCR.

RESULTS: The expression of core clock genes *PER2* and *NR1D1* was increased after the weight loss. Correlations of *PERIOD* expression with body mass index (BMI) and serum total, high-density lipoprotein and low-density lipoprotein (LDL) cholesterol levels and of *NR1D1* expression with total and LDL cholesterol were found that became non-significant after correction for multiple testing. Clock gene expression levels and their weight loss-induced changes tightly correlated with each other and with genes involved in fat metabolism (*FASN*, *CPT1A*, *LPL*, *PPARG*, *PGC1A*, *ADIPOQ*), energy metabolism (*SIRT1*), autophagy (*LC3A*, *LC3B*) and inflammatory response (*NFKB1*, *NFKBIA*, *NLRP3*, *EMR1*).

CONCLUSION: Clock gene expression in human SAT is regulated by body weight changes and associated with BMI, serum cholesterol levels and the expression of metabolic and inflammatory genes. Our data confirm the tight crosstalk between molecular clock and metabolic and inflammatory pathways involved in adapting adipose tissue metabolism to changes of the energy intake in humans.

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INTRODUCTION

A wide range of physiological parameters depends on the daytime, including the sleep–wake cycle, thermogenesis, food intake and hormone secretion.[1] Circadian rhythms are controlled by molecular clocks that synchronize energy intake and expenditure with the day/night cycle. The master pacemaker in the suprachiasmatic nucleus of the hypothalamus adjusts subsidiary clocks in peripheral tissues via the humoral factors and autonomic innervation.[1,2]

The molecular clock comprises two interlocked feedback loops: (i) the major loop includes the *PER/CRY* genes regulated positively by transcription factors *CLOCK* and *BMAL1* and negatively by their own protein products; (ii) an additional loop established through the activators *RORα/RORγ* and the repressors *NR1D1/NR1D2* (*REV-ERBα/REV-ERBβ*) regulating *BMAL1* and possibly *CLOCK* transcription.[1] The circadian oscillator adjusts functions of numerous tissue-specific clock-controlled genes, including transcription factors.[1,2] Microarray studies have shown that about 5–25% of the transcriptome in the liver, heart, adipose and other tissues display circadian oscillations, including components of carbohydrate, cholesterol, lipid metabolism and detoxification pathways, as well as genes contributing to inflammation response.[3–5] Conversely, metabolic signals feedback on the circadian machinery, modulating circadian gene expression and behavior. Changes in food composition and feeding time lead to the activation of epigenetic and transcriptional regulatory mechanisms providing a fine tuning of circadian clock.[4,6–9]

White adipose tissue (WAT) also demonstrates strong circadian variations of metabolic function. During the active phase, nutrients are stored in WAT in form of triglycerides, and during the inactive fasting phase, the adipose tissue release triglycerides in form of free fatty acids, which serve as energy substrates for other organs.[10] Numerous key enzymes involved in both processes are regulated by circadian clock.[4,11] Furthermore, many adipokine hormones, that is, leptin, adiponectin and visfatin, are released in humans in a circadian manner.[12,13] The intimate interplay between components of circadian clocks and metabolic regulation in WAT was confirmed by a range of animal models where the knockouts of different clock components affect various aspects of glucose and lipid metabolism.[14–17] For example, an adipose-specific deletion of clock gene *Bmal1* in mice results in obesity and shifts the diurnal rhythm of food intake although the overall food

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intake stays normal.[17] In humans, a range of polymorphisms within core clock genes are associated with obesity and success of weight loss therapy.[18,19]

Alterations of clock oscillations in human peripheral tissues are shown in many metabolic diseases, such as obesity, type 2 diabetes and metabolic syndrome.[20,21] Clock gene expression in visceral adipose tissue of obese subjects was shown to be associated with metabolic syndrome parameters, such as waist circumference, total cholesterol and low-density lipoprotein (LDL) cholesterol.[22] Recently, the effect of food intake/fasting on the circadian gene expression was demonstrated in subcutaneous adipose tissue (SAT) in humans.[3] However, to our knowledge there are no publications about the effect of weight loss on circadian mechanisms in human adipose tissue and its role in the metabolic regulation.

Therefore, the aim of our study was to investigate the effects of weight loss on the clock gene expression in SAT in humans. Moreover, we studied association of clock gene expression with biochemical parameters of glucose and fat metabolism and with expression of metabolic and inflammatory genes in SAT.

MATERIALS AND METHODS

Subjects and design of the study

The study individuals were part of the Potsdam subcohort of the Diet, Obesity and Genes study described in detail previously.[23] Briefly, 50 overweight generally healthy persons (body mass index (BMI) $\geqslant 27$ kg m^{-2}, age <65 years) participated in the study. Exclusion criteria were BMI >45 kg m^{-2}, liver or kidney diseases, cardiovascular diseases, diabetes mellitus (type 1 or type 2), special diets/eating disorders, systemic infections/chronic diseases, cancer within the past 10 years, weight change >3 kg within the previous 3 months and other clinical disorders or use of prescription medication that might influence the outcome of the study.[23] The study was approved by the ethics committee of Potsdam University, Potsdam, Germany and registered at www.clinicaltrials.gov (NCT00390637). All subjects gave written informed consent before taking part in the study.

After the first clinical investigation day (CID1), appropriate subjects followed an 8-week low-calorie diet (Modifast, Nutrition et Sante, France) consisting of 800 kcal per day. In addition, 200 g of vegetables per day was allowed. Weight loss, compliance and adverse events were checked at regular intervals during the dietary intervention. Subjects who lost at least 8% weight after 8 weeks underwent the second clinical investigation day (CID2) as described previously.[23] In total, 50 subjects were selected for the study and participated at CID1 and CID2.

Subjects were invited to the CIDs after at least 10-h overnight fasts and were asked to avoid alcohol consumption or exercise the day before the respective CID. SAT biopsies were taken at 0900 hours at each CID from contralateral sites at the level of the umbilicus. The skin was anesthetized with 1% lidocaine. A small incision was made and 1 g of adipose tissue was removed under sterile conditions by using a cutting needle biopsy handy (14-G, Somatex, Berlin, Germany). After removal, tissue samples were shock-frozen in liquid nitrogen and stored at -80 °C until RNA isolation.

Measurement of laboratory parameters

A detailed description of anthropometric and laboratory measurements has been published previously.[23] Body fat content was measured using the dual-energy X-ray absorptiometry. Blood samples obtained after the overnight fasting were analyzed for glucose, triglycerides, total cholesterol and high-density lipoprotein (HDL) cholesterol with standard methods, and LDL cholesterol was calculated from these data. Serum insulin was measured using an enzyme immunometric assay for the IMMULITE automated analyzer (Diagnostic Products Corporation, Los Angeles, CA, USA). C-reactive protein was measured using the COBAS Integra 400 analyzer (Roche Diagnostics, Mannheim, Germany).

Quantitative real-time PCR (qRT-PCR)

Total RNA from SAT biopsies was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). Synthesis of cDNA from total RNA (1 μg) was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). qRT-PCR was performed by ABI Prism 7700 sequence detection system using Power SYBR Green PCR Master Mix (Applied Biosystems). Each cDNA sample was measured as triplicates in optical 384-well plates. Quantification of mRNA levels was made by the standard curve method. Expression levels of all target genes were normalized to the expression of ribosomal protein large protein 0. Sequences of primers were designed with the Primer Express software (PE Applied Biosystems, Darmstadt, Germany) and are shown in Supplementary Table S1.

Statistical analysis

Power calculation was completed using the G*Power software version 3.1.9.2 (http://www.gpower.hhu.de/). For the sample size of 50 subjects, the probability is 80% (the power 0.80) that the study detects a treatment difference at a two-sided 0.05 significance level, if the effect size is 0.40.

SPSS 20.0 (SPSS, Chicago, IL, USA) was used for all statistical analyses. If not stated otherwise, data are given as means ± s.e.m. Weight loss-induced changes of clinical parameters and normalized gene expression were calculated as a ratio of the value after weight loss to that before weight loss. Presence or absence of normal distribution was verified by the Kolmogorov–Smirnov test. Not normally distributed parameters were logarithmically transformed. Pearson's simple coefficient was used for correlation analysis, and two-sided Student's *t*-test was applied to estimate differences between groups. *P*-value of <0.05 was considered significant. For multiple testing, the false-discovery rate (FDR) correction was applied if not stated otherwise. A gene-phenotype network was constructed for weight loss-induced changes of clinical parameters and gene expression using Cytoscape v.2.8.1 (http://cytoscape.org). Only correlation links significant after the FDR correction were used for the network construction.

RESULTS

Clinical characteristics of subjects

Fifty overweight subjects who achieved a weight loss of at least 8% after 8 weeks were selected for the study (age 40.8 ± 0.9 years, BMI 34.2 ± 0.6 kg m^{-2}; Table 1). The mean weight loss for the group was 10.8 ± 0.4% of initial weight (Table 1). The mean loss of total body fat was 16.2 ± 1.9%. As expected, triglycerides, total cholesterol and LDL cholesterol levels, C-reactive protein and systolic and diastolic blood pressure were decreased after 8 weeks of hypocaloric diet (Table 1).

Table 1. Clinical characteristics of subjects

	CID1	*CID2*
N (%, male)	50 (38)	
Age (years)	40.8 ± 0.9	
Weight (kg)	101.2 ± 2.2	90.3 ± 2.0**
Waist circumference (cm)	107.6 ± 1.6	97.9 ± 1.4**
BMI (kg m^{-2})	34.2 ± 0.6	30.6 ± 0.5**
Waist-to-hip ratio	0.91 ± 0.01	0.88 ± 0.01**
Total body fat (%)	38.3 ± 1.3	32.1 ± 1.2**
Fasting glucose (mmol l^{-1}l)	5.23 ± 0.05	4.89 ± 0.05**
Fasting insulin (pmol l^{-1})	58.5 ± 4.9	39.0 ± 3.1**
HOMA-IR (mmol mU l^{-2})	2.77 ± 0.24	1.54 ± 0.13**
Triglycerides (mmol l^{-1})	1.48 ± 0.09	1.18 ± 0.06**
Total cholesterol (mmol l^{-1})	5.34 ± 0.13	4.66 ± 0.11**
HDL cholesterol (mmol l^{-1})	1.24 ± 0.04	1.20 ± 0.03
LDL cholesterol (mmol l^{-1})	3.43 ± 0.12	2.92 ± 0.09**
C-reactive protein (mg l^{-1})	3.36 ± 0.32	2.51 ± 0.39**
Systolic pressure (mm Hg)	127.0 ± 2.0	116.7 ± 1.7**
Diastolic pressure (mm Hg)	83.9 ± 1.3	76.6 ± 1.1**

Abbreviations: BMI, body mass index; HDL, high-density lipoprotein; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; LDL, low-density lipoprotein. Data were collected before (CID1) and after 8 weeks of weight loss (CID2) on a 800-kcal hypocaloric diet. Data are presented as mean ± s.e.m. Statistical differences were determined by Student's *t*-test. *$P<0.05$, **$P<0.01$.

Regulation of the clock gene expression in SAT by weight loss

To determine whether weight loss affects the peripheral clock in SAT, we measured the expression levels of 10 clock genes in adipose tissue biopsies taken at the same daytime before and after 8 weeks of hypocaloric diet using qRT-PCR. These genes included most of core clock genes whose protein products are necessary for the generation and regulation of circadian rhythms (*PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *CLOCK*, *BMAL1*, *NR1D1*) and two clock-controlled transcription factors (*TEF* and *DBP*) and were selected from studies indicating their association with metabolic diseases and corresponding clinical markers.[3,20–22] Expression of core clock genes *PER2* and *NR1D1* was increased after weight loss ($P = 6.1 \times 10^{-6}$, and $P = 0.031$, respectively) (Figure 1a). Expression levels of other clock genes did not change. Clock gene expression levels tightly correlated with each other at both investigation days ($P < 0.01$ after FDR correction) (Supplementary Table S2).

Relationships between clock gene expression and biochemical parameters

Because the clock gene machinery coordinates numerous metabolic processes,[1,2] we further analyzed correlations between expression levels of clock genes in SAT, anthropometric parameters and metabolic blood markers. Notably, we observed only a small number of correlation links of clock gene expression with anthropometric and biochemical parameters (Table 2).

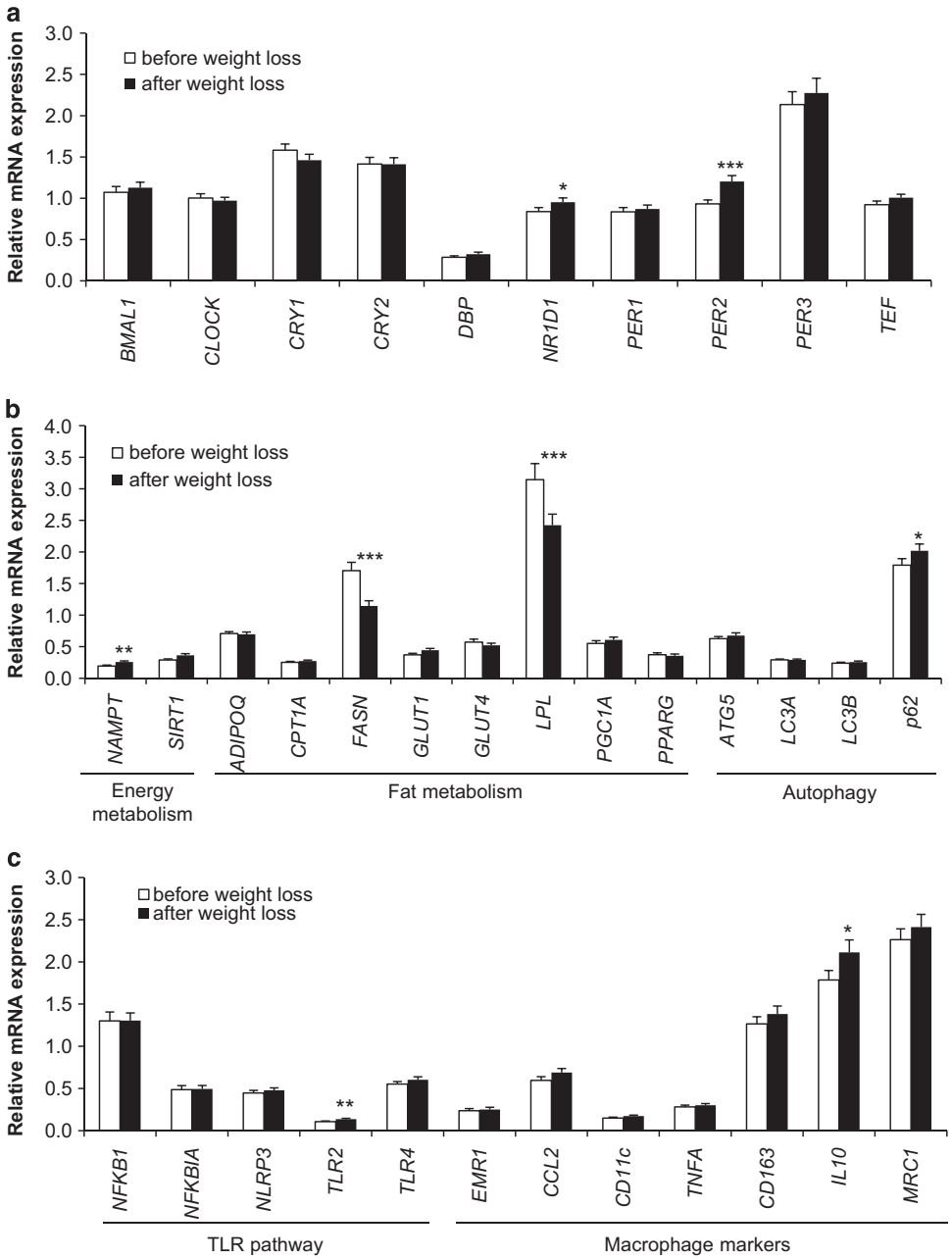

Figure 1. Gene expression in SAT before and after 8 weeks of weight loss. Expression levels of mRNA of clock genes (**a**), energy, fat metabolism and autophagy genes (**b**), as well as inflammatory genes (**c**) were measured by qRT-PCR and normalized to the expression levels of the housekeeping gene *RPLP0* ($n = 50$). *EMR1*—marker of macrophage accumulation in SAT; *CCL2*, *CD11c*, *TNFA*—M1 macrophage markers; *CD163*, *IL10*, *MRC1*—M2 macrophage markers. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by paired Student's *t*-test. Data are presented as mean ± s.e.m.

Table 2. Correlations of clock gene expression in SAT with anthropometric and biochemical parameters

		Weight	*Waist circumference*	*Body fat, %*	*WHR*	*BMI*	*Total cholesterol*	*HDL cholesterol*	*LDL cholesterol*	*Triglycerides*	*CRP*	*Fasting glucose*	*Fasting insulin*	*HOMA-IR*
BMAL1	CID1													
	CID2											−0.342*		
CLOCK	CID1													
	CID2													
CRY1	CID1													
	CID2													
CRY2	CID1							0.336*						
	CID2						0.388**		0.357*					
DBP	CID1							**0.517****						
	CID2				−0.295*		0.400**		0.367*					
NR1D1	CID1													
	CID2						0.331*		0.313*					
PER1	CID1	−0.332*	−0.381**			−0.363*		0.413**						
	CID2						0.289*		0.302*					
PER2	CID1					−0.308*								
	CID2			−0.288*		−0.297*	0.442**	0.309*	0.456**					
PER3	CID1		−0.293*			−0.322*	0.378**		0.366*					
	CID2						0.344*		0.342*					
TEF	CID1													
	CID2	−0.296*			−0.308*			0.326*				−0.322*		

Abbreviations: BMI, body mass index; CRP, C-reactive protein; HDL, high-density lipoprotein; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; LDL, low-density lipoprotein; SAT, subcutaneous adipose tissue; WHR, waist-to-hip ratio. Correlations of clock gene expression levels before (CID1) and after the weight loss (CID2) are presented. Only significant correlation links (*r*-values) are shown. *$P < 0.05$. **$P < 0.01$ without correction for multiple testing. Correlation significant after false-discovery rate correction is in bold.

We examined in detail associations of clock genes *PER2* and *NR1D1* whose expression was regulated by weight loss. Interestingly, the expression levels of *PER2* negatively correlated with BMI and body fat percentage ($P < 0.05$; Table 2). We also found several associations of *PER2* (as well as *PER1* and *PER3*) with serum total, HDL and LDL cholesterol levels and of *NR1D1* with total and LDL cholesterol ($P < 0.05$; Table 2). However, after correction for multiple testing, these correlation links became non-significant. After FDR correction, significant correlations were only detected for *DBP* expression with HDL cholesterol at CID1 ($r = 0.517$, $P = 0.033$; Table 2).

Relationships between clock gene expression and the expression of inflammatory and metabolic genes

We further analyzed how the clock gene expression is associated with the expression of inflammatory and metabolic genes in SAT. For this, the expression of genes involved in energy metabolism, fat metabolism, autophagy and Toll-like receptor (TLR) pathway, as well as macrophage markers, cytokines and adipokines, were measured before and after 8 weeks of hypocaloric diet using qRT-PCR (Figures 1b and c). These genes were selected from previously published studies indicating their responsiveness to dietary weight loss programs and association with obesity and metabolic syndrome.[24–28] As expected, the expression of lipogenic genes *FASN* and *LPL* in SAT was decreased after weight loss ($P < 0.05$; Figure 1b). Expression levels of the energy metabolism gene *NAMPT* (coding visfatin) and autophagy marker *P62*, as well as TLR pathway gene *TLR2* and anti-inflammatory cytokine *IL10*, were increased after weight loss ($P < 0.05$) (Figures 1b and c).

Expression levels of clock genes demonstrated a large number of correlations with metabolic and autophagy genes in SAT, especially with fat metabolism genes *CPT1A*, *FASN*, *LPL*, *PGC1A* and *PPARG*, as well as with the adiponectin gene *ADIPOQ* ($P < 0.05$; Table 3). Furthermore, a range of correlation links between clock genes and genes of TLR pathway (especially *NFKB*, *NFKBIA*, *NLRP3*), as well as the macrophage accumulation marker *EMR1*, was found ($P < 0.05$; Table 4). Moreover, we investigated the association of clock gene expression with macrophage polarization markers and detected strong correlations with M2 macrophage markers *CD163* and *MRC1* at both CIDs ($P < 0.05$; Table 4). Notably, mRNA expression of cytokines (*CCL2*, *TNFA* and *IL10*) demonstrated only minor correlation links with clock genes (Table 4).

Relationships of weight loss-induced changes of clinical parameters and gene expression in SAT

Thereafter, we investigated correlations of weight loss-induced changes of clinical parameters and gene expression in SAT calculated as a CID2 to CID1 ratios. We first studied only relationships between clock genes and parameters significantly changed by weight loss (Figure 1a, Table 1). We found correlations of weight loss-induced changes of the clock gene *NR1D1* with serum triglyceride, *NAMPT* and *TLR2* alterations ($r = 0.528$, $P = 0.003$; $r = 0.439$, $P = 0.019$, and $r = 0.396$, $P = 0.042$ after the FDR correction, respectively), whereas no relationships of the clock gene *PER2* were detected. Notably, body weight and BMI alterations correlated only with the expression changes of lipogenic gene *LPL* ($r = 0.403$, $P = 0.038$ and $r = 0.403$, $P = 0.038$ after the FDR correction, respectively) but not with clock gene expression in SAT (Figure 2).

To provide an overview of interconnections between changes of all studied genes and parameters upon weight loss, we constructed a gene-phenotype network using the Cytoscape software (Figure 2). In this network, nodes with most connections were the inflammatory gene *NFKBIA*, fat metabolism genes *PPARG* and *CPT1A* and clock genes *PER3*, *CRY2* and *DBP*, as well as autophagy genes *LC3A* and *LC3B* (Figure 2). A much lower number of links was found for changes of biochemical and anthropometrical parameters. However, some of the clock genes studied (*NR1D1*, *BMAL1*, *PER1*, *PER3*) showed direct and indirect connections with serum lipid markers, that is, triglycerides, total and LDL cholesterol (Figure 2).

DISCUSSION

Previous studies showed that clock genes are expressed in both subcutaneous and visceral fat in humans and their expression is associated with the metabolic syndrome parameters.[22] Circadian rhythm of clock genes was detected in human adipose tissue

Table 3. Associations of clock gene expression with the expression of metabolic and autophagy genes in SAT

		Energy metabolism		*Fat metabolism*								*Autophagy*			
		NAMPT	*SIRT1*	*ADIPOQ*	*CPT1A*	*FASN*	*GLUT1*	*GLUT4*	*LPL*	*PGC1A*	*PPARG*	*ATG5*	*LC3A*	*LC3B*	*P62*
BMAL1	CID1	**0.356***		**0.345***	**0.458****	0.285*	**0.426****		**0.402****	**0.342***	**0.360***				
	CID2	**0.426****			**0.406****		**0.411****		**0.436****	**0.500****	0.294*			**0.347***	
CLOCK	CID1	**0.431****		0.300*	**0.540****		0.312*		**0.535****	**0.417****	**0.577****			**0.393****	0.316*
	CID2			**0.397****					**0.450****	0.319*	0.314*	**0.395****	0.318*		0.307*
CRY1	CID1			**0.381****	**0.448****	**0.338***	0.314*	**0.364***	**0.520****	**0.387****	**0.485****		0.314*	**0.359***	**0.390****
	CID2		**0.338***	**0.407****	**0.434****				**0.612****	0.310*	**0.553****	**0.484****	**0.395****		**0.321***
CRY2	CID1	**0.466****	**0.649****	**0.703****	**0.768****	**0.729****	**0.673****	**0.629****	**0.822****	**0.600****	**0.785****		**0.457****	**0.715****	
	CID2		**0.432****	**0.546****	**0.487****	**0.443****	**0.374***	**0.539****	**0.770****	**0.649****	**0.737****	**0.393****	**0.477****	**0.438****	
DBP	CID1	**0.442****	**0.500****	**0.580****	**0.517****	**0.711****	**0.478****	**0.630****	**0.654****	**0.457****	**0.641****		**0.339***	**0.549****	
	CID2		**0.793****	**0.504****	**0.673****	**0.384****	**0.507****	**0.505****	**0.525****	0.314*	**0.708****		**0.598****	**0.653****	
NR1D1	CID1			0.294*	**0.497****	**0.327***	0.320*	**0.323***	**0.477****		**0.546****			**0.325***	**0.367***
	CID2			**0.422****				**0.337***	**0.444****	**0.561****	**0.333***				
PER1	CID1			**0.344***	**0.344***	**0.478****		**0.418****	**0.320***		**0.363***				
	CID2			**0.403****				0.285*	**0.358***	**0.354***	0.302*		**0.320***	0.288*	
PER2	CID1		**0.342***	**0.402****	**0.488****	**0.367****		**0.344***	**0.613****	**0.437****	**0.643****			**0.340***	**0.371****
	CID2		**0.418****	**0.469****	**0.401****				**0.461****	**0.631****	**0.496****	**0.348***	0.312*	0.304*	
PER3	CID1	**0.359***	**0.463****	**0.356***	**0.659****	**0.466****	**0.411****	**0.322***	**0.740****	**0.736****	**0.695****			**0.436****	
	CID2		**0.698****	**0.546****	**0.749****		**0.451****	**0.352***	**0.673****	**0.729****	**0.841****	**0.515****	**0.483****	**0.590****	
TEF	CID1				0.316*	**0.356***		**0.448****	**0.332***		**0.392****			0.286*	
	CID2		**0.448****	**0.343***	**0.458****				0.311*		**0.443****	0.287*	**0.449****	**0.432****	

Abbreviation: SAT, subcutaneous adipose tissue. Correlations of gene expression levels before (CID1) and after the weight loss (CID2) are presented. Only significant correlation links (*r*-values) are shown. *$P < 0.05$. **$P < 0.01$ without correction for multiple testing. Correlations significant after false-discovery rate correction are in bold.

Table 4. Associations of clock gene expression with the expression of inflammatory genes in SAT

		TLR pathway					*Total macrophage marker*	*M1 macrophage markers*			*M2 macrophage markers*		
		NFKB1	*NFKBIA*	*NLRP3*	*TLR2*	*TLR4*	*EMR1*	*CCL2*	*CD11c*	*TNFA*	*CD163*	*IL10*	*MRC1*
BMAL1	CID1	**0.362****	**0.371****	**0.345***		**0.355***	0.312*						**0.352***
	CID2	0.318*		0.305*	**0.511****		**0.530****		0.290*		**0.362***		**0.426****
CLOCK	CID1	**0.596****	**0.548****	**0.412****	0.309*		**0.381****		**0.459****	**0.360***	**0.466****		**0.484****
	CID2	**0.347***	**0.462****		**0.378****	**0.451****					**0.496****		**0.447****
CRY1	CID1	**0.573****	**0.517****	**0.416****	0.293*	0.316*	0.310*	0.296*	0.313*		**0.528****		**0.588****
	CID2	**0.649****	**0.576****	**0.502****	**0.441****	**0.474****		**0.428****			**0.669****	**0.420****	**0.697****
CRY2	CID1	**0.547****	**0.751****	**0.535****		**0.495****	**0.537****		**0.345***	**0.344***	**0.425****		**0.535****
	CID2	**0.657****	**0.624****	**0.414****	**0.444****	**0.331***	**0.422****				**0.615****		**0.639****
DBP	CID1		**0.543****			**0.331***	**0.403****	−0.282*					
	CID2	0.319*	**0.685****			**0.441****	0.290*		0.300*				
NR1D1	CID1	**0.489****	**0.514****	**0.438****									**0.369****
	CID2	**0.447****	**0.443****	**0.372****	**0.498****	**0.418****	**0.342***				**0.455****		**0.416****
PER1	CID1						**0.385****						
	CID2		**0.398****										
PER2	CID1	**0.568****	**0.607****	**0.498****			**0.437****				**0.370****		**0.449****
	CID2	**0.562****	**0.579****	**0.345***	0.318*	**0.454****					**0.340***		**0.353***
PER3	CID1	**0.578****	**0.777****	**0.640****	0.301*		**0.491****		**0.528****	**0.491****	**0.668****		**0.690****
	CID2	**0.700****	**0.782****	**0.364***	**0.401****	**0.555****	**0.370***				**0.534****	0.290*	**0.652****
TEF	CID1												
	CID2		**0.437****			**0.460****							**0.378****

Abbreviations: SAT, subcutaneous adipose tissue; TLR, Toll-like receptor. Correlations of gene expression levels before (CID1) and after the weight loss (CID2) are presented. Only significant correlation links (*r*-values) are shown. *$P < 0.05$, **$P < 0.01$ without correction for multiple testing. Correlations significant after false-discovery rate correction are in bold.

explants[29] and SAT biopsies[3] which confirms that human adipose tissue possesses own peripheral circadian oscillators independent of the central circadian control mechanism. In our study, we demonstrated for the first time that clock gene expression in human SAT is altered upon body weight changes and associated with BMI, serum cholesterol levels and the expression of metabolic and inflammatory genes.

A temporal component is obviously having an important role in the regulation of adipose tissue functions and body weight. Indeed, changes in meal timing influence obesity and success of

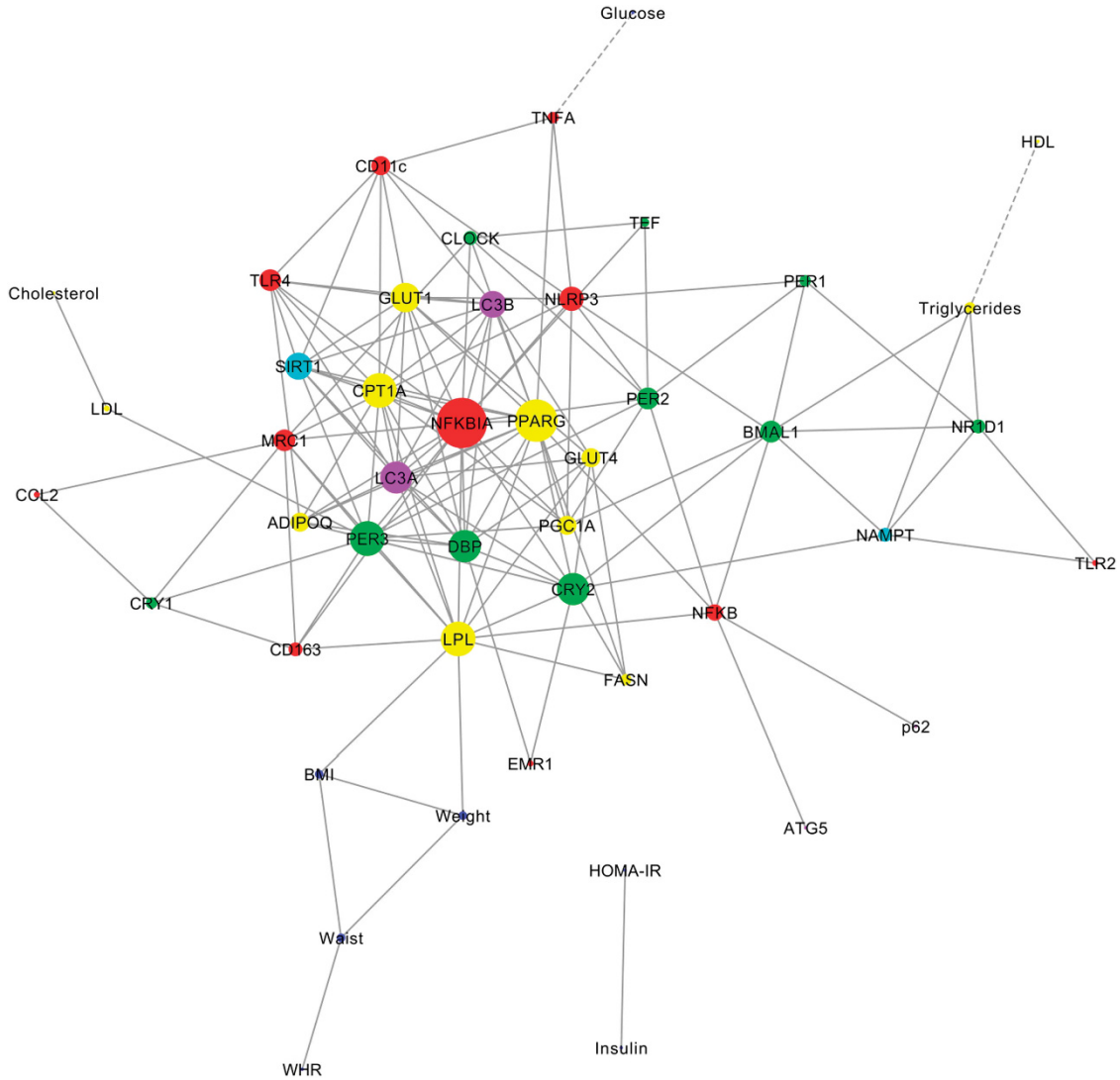

Figure 2. Involvement of clock genes in the regulation of inflammation and fat metabolism by the weight loss. A gene-phenotype network was constructed for weight loss-induced changes of clinical parameters and gene expression calculated as CID2/CID1 ratios as described in Materials and methods section. Only correlation links significant after the FDR correction were used for the network construction. Node size reflects the number of direct connections a parameter has within network. Clock genes are shown in green, energy metabolism genes in light blue, fat metabolism genes in yellow, inflammation genes in red, autophagy genes in purple and clinical parameters in dark blue. Positive correlations are shown with continued lines; negative correlations are shown with broken lines.

weight loss therapy. The timing of the main meal predicted weight loss during a 20-week dietary intervention independent from total 24-h caloric intake.[30] Interestingly, *SIRT1* and *CLOCK* combined genotype is associated with evening preference and weight loss resistance in a obesity treatment.[19] The importance of caloric distribution across the day on weight loss therapy was also supported by a study showing that eating two large meals a day (breakfast and lunch) yielded more weight loss than consuming six mini-meals with the same number of calories.[31] In shift workers, unusual feeding time induces a disruption of the circadian system and impairment of metabolic functions.[32]

In our study, we demonstrated that changes of energy homeostasis, in turn, affect the circadian system modulating circadian gene expression. We observed an increase of *PER2* and *NR1D1* expression in SAT after weight loss and correlations of *PERIOD* genes and *NR1D1* with serum cholesterol levels. Our observations confirmed mouse data showing that both PERIOD and NR1D1 are crucially involved in the regulation of metabolic functions. *Nr1d1*−/− mice increase body weight on both regular chow and high-fat diets (HFDs) and utilize more free fatty acids as energy substrate.[15] NR1D1 regulates the activity of sterol regulatory element-binding protein (SREBP) in the circadian manner and thereby changes the daily expression of SREBP target genes involved in cholesterol and lipid metabolism.[33] *Per2*$^{-/-}$ animals loose body weight and decrease body fat content on a regular chow diet owing to an overactivation of peroxisome proliferator-activated receptor gamma (PPARγ),[34] whereas on a HFD *Per2*-deficient animals develop significant obesity.[16] In humans, *PER2* expression in adipose tissue correlated with waist circumference and serum cholesterol levels,[22] and *PERIOD* gene expression in blood monocytes was associated with the expression of numerous metabolic and inflammatory genes.[3] Moreover, diurnal oscillations of *PERIOD* genes in blood monocytes were rapidly altered by the isocaloric change of food composition in humans.[9]

Generally, clock gene expression levels and their weight loss-induced changes in SAT strongly correlated with each other and with the expression of metabolic and inflammatory genes. Interestingly, in the weight loss network, nodes with most

connections were fat metabolism genes *PPARG* and *CPT1A* and inflammatory gene *NFKBIA*, whereas lipogenic genes *FASN* and *LPL* greatly downregulated by the weight loss are not included in the network. Our data confirmed, that carnitine palmitoyl transferase 1 (CPT1A), the rate-limiting enzyme of mitochondrial fat oxidation, and PPARG, the transcription factor regulating of adipocyte differentiation, are deeply involved in the regulation of fat metabolism by weight loss in humans. Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA) is involved in the regulation of the NFKB pathway activity. Interestingly, all three genes were previously shown to demonstrate circadian oscillations,[4,9] suggesting the involvement of clock genes in their regulation. Moreover, we showed here for the first time the strong correlations of clock genes with a marker of macrophage accumulation in adipose tissue *EMR1* and with M2 macrophage markers *CD163* and *MRC1*. Obesity is associated with the phenotypic switch of adipose tissue macrophages from an alternatively activated (M2) to a classically activated (M1) phenotype.[28] Interestingly, in our study we found the mRNA increase of the anti-inflammatory cytokine *IL10* after the weight loss which may suggest the shift of macrophage polarization toward M2 macrophages.

We also found that autophagy markers *LC3A* and *LC3B* are tightly interconnected with clock genes and metabolic and inflammatory genes in SAT. Moreover, another autophagy marker *P62* was upregulated by weight loss. Autophagy, a major degradative pathway eliminating unnecessary, damaged or potentially harmful cellular components, was shown to be involved in the regulation of lipid droplet biogenesis and breakdown and adipose tissue phenotype.[26] Some studies demonstrated enhanced autophagy in adipocytes from obese patients,[26] whereas other authors showed severe reduction in autophagic flux, which was partially recovered after bariatric surgery.[35] Notably, recent work has demonstrated that autophagy is rhythmically activated in a circadian manner in the mouse liver.[36]

However, it is not known whether weight change primarily induces clock alterations and in this way affects the expression of metabolic and inflammatory genes in SAT, or vice versa. These mechanisms are obviously complex and pleiotropic and are intensively studied in mice fed with HFD. In this model, mechanisms of the circadian cycle disruption involve the impairment of CLOCK:BMAL1 chromatin recruitment and cyclic activation of surrogate pathways through transcription factors PPARγ, SREBP-1, CREB1 and SRF, as well as regulation of redox state and 5′-adenosine monophosphate-activated protein kinase activity.[4,37,38] However, the weight loss-induced changes of the circadian oscillations in WAT are poorly studied. Interestingly, in mice, HFD-induced changes of peripheral circadian rhythms do not require the onset of obesity and were reversible when obese HFD-fed mice were switched to the normal chow diet for 2 weeks.[4] At least in mice, the nutritional challenge, and not the development of obesity, seems to cause the reprogramming of the clock.[4] Furthermore, it might be expected that the aforementioned molecular pathways are involved in the 'normalization' of clock functions by the weight loss. Additionally, two key enzymes of energy metabolism, NAMPT, an enzyme that provides a rate-limiting step in the NAD+ synthesis, and SIRT1, NAD+ dependent histone deacetylase, might be involved in this regulation. Indeed, activity of these enzymes is regulated by the energy intake and they can interact with and affect the core clock mechanism.[37,39] In our study, the increase of the *NAMPT* expression in SAT was observed by weight loss, and this correlated with changes of *NR1D1* mRNA expression. This finding is in agreement with mouse data indicating that obese HFD-fed mice demonstrated a loss of NAD+ oscillation accompanied by a dampening of *Nampt* cyclic transcription.[4] Moreover, we found that *SIRT1* expression in human SAT was associated with both clock and metabolic genes by weight loss.

In contrast to tight gene–gene correlations, we found a much lower number of links between clock gene expression and biochemical and anthropometrical parameters similarly to our previously published data on blood monocytes.[9] Nevertheless, *PERIOD* and *NR1D1* expression in SAT was associated with serum cholesterol levels. In the gene-phenotype network constructed for weight loss-induced changes, some clock genes (*NR1D1*, *BMAL1*, *PER1*, *PER3*) also showed connections with serum lipid markers, that is, triglycerides, total and LDL cholesterol. Gomez-Abellan *et al.*[22] demonstrated previously that clock gene expression in visceral adipose tissue of obese subjects was associated with total cholesterol and LDL cholesterol and waist circumference. Interestingly, in our study, we observed association of *PER2* expression levels with BMI only before and after weight loss but not for weight loss-induced changes. In the weight loss network, no correlations of obesity indices (BMI, waist-to-hip ratio, waist circumference, body weight) with clock gene expression in SAT were found, pointing out rather to the indirect regulation of clock genes by weight changes in humans. It should also be kept in mind that mRNA levels not necessarily mirror the level of protein expression in cells and tissues that complicates interpretation of the gene expression data obtained in human samples and the investigation of underlying molecular mechanisms.

Further limitation of our study is that the expression of clock genes was assessed only at one time point and we were therefore not able to investigate the change of whole 24-h circadian rhythms. We standardized experimental conditions as much as possible to diminish the influence of nutritional and behavioral factors on the levels of gene expression in SAT. Subjects were invited to the CIDs after at least 10-h overnight fasts and were asked to avoid alcohol consumption or exercise the day before the respective CID. All subjects consumed similar diet before CID2 (Modifast drinks+200 g of vegetables per day). Nevertheless, we cannot exclude the influence of seasonal variation, changes of regular physical activity, food composition before CID1 and other factors on the gene expression in SAT. Further studies are needed to study the 24-h oscillations in SAT, although their implementation in humans is difficult owing to the high invasiveness.

In conclusion, we demonstrated that clock gene expression in human SAT is regulated by body weight changes and is associated with BMI and serum cholesterol levels. Our data confirm the tight crosstalk between molecular clock and metabolic and inflammatory pathways involved in adapting adipose tissue metabolism to the changes of energy intake. Further studies are needed to determine the role of clock genes in metabolic and immune changes induced by weight loss in humans and the exact mechanisms of this regulation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

AFHP, OG and OP designed the research; OP, SS, JG, VM and KK conducted the research; OP, MO and NR analyzed data and performed the statistical analysis; and OP, AK and AFHP wrote and edited the manuscript; OP had the primary responsibility for the final content. All authors read and approved the final manuscript.

REFERENCES

1 Asher G, Sassone-Corsi P. Time for food: the intimate interplay between nutrition, metabolism, and the circadian clock. *Cell* 2015; **161**: 84–92.
2 Huang W, Ramsey KM, Marcheva B, Bass J. Circadian rhythms, sleep, and metabolism. *J Clin Invest* 2011; **121**: 2133–2141.
3 Loboda A, Kraft WK, Fine B, Joseph J, Nebozhyn M, Zhang C *et al.* Diurnal variation of the human adipose transcriptome and the link to metabolic disease. *BMC Med Genomics* 2009; **2**: 7.
4 Eckel-Mahan KL, Patel VR, de Mateo S, Orozco-Solis R, Ceglia NJ, Sahar S *et al.* Reprogramming of the circadian clock by nutritional challenge. *Cell* 2013; **155**: 1464–1478.
5 Keller M, Mazuch J, Abraham U, Eom GD, Herzog ED, Volk HD *et al.* A circadian clock in macrophages controls inflammatory immune responses. *Proc Natl Acad Sci USA* 2009; **106**: 21407–21412.
6 Damiola F. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev* 2000; **14**: 2950–2961.
7 Hatori M, Vollmers C, Zarrinpar A, DiTacchio L, Bushong EA, Gill S *et al.* Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet. *Cell Metab* 2012; **15**: 848–860.
8 Kohsaka A, Laposky AD, Ramsey KM, Estrada C, Joshu C, Kobayashi Y *et al.* High-fat diet disrupts behavioral and molecular circadian rhythms in mice. *Cell Metab* 2007; **6**: 414–421.
9 Pivovarova O, Jurchott K, Rudovich N, Hornemann S, Lu Y, Mockel S *et al.* Changes of dietary fat and carbohydrate content alter central and peripheral clock in humans. *J Clin Endocrinol Metab* 2015; **100**: 2291–2302.
10 Kumar Jha P, Challet E, Kalsbeek A. Circadian rhythms in glucose and lipid metabolism in nocturnal and diurnal mammals. *Mol Cell Endocrinol* 2015; **418**: 74–88.
11 Adamovich Y, Rousso-Noori L, Zwighaft Z, Neufeld-Cohen A, Golik M, Kraut-Cohen J *et al.* Circadian clocks and feeding time regulate the oscillations and levels of hepatic triglycerides. *Cell Metab* 2014; **19**: 319–330.
12 Benedict C, Shostak A, Lange T, Brooks SJ, Schioth HB, Schultes B *et al.* Diurnal rhythm of circulating nicotinamide phosphoribosyltransferase (Nampt/visfatin/PBEF): impact of sleep loss and relation to glucose metabolism. *J Clin Endocrinol Metab* 2012; **97**: E218–E222.
13 Gavrila A. Diurnal and ultradian dynamics of serum adiponectin in healthy men: comparison with leptin, circulating soluble leptin receptor, and cortisol patterns. *J Clin Endocrinol Metab* 2003; **88**: 2838–2843.
14 Turek FW. Obesity and metabolic syndrome in circadian clock mutant mice. *Science* 2005; **308**: 1043–1045.
15 Delezie J, Dumont S, Dardente H, Oudart H, Grechez-Cassiau A, Klosen P *et al.* The nuclear receptor REV-ERB alpha is required for the daily balance of carbohydrate and lipid metabolism. *FASEB J* 2012; **26**: 3321–3335.
16 Yang S, Liu A, Weidenhammer A, Cooksey RC, McClain D, Kim MK *et al.* The role of mPer2 clock gene in glucocorticoid and feeding rhythms. *Endocrinology* 2009; **150**: 2153–2160.
17 Paschos GK, Ibrahim S, Song WL, Kunieda T, Grant G, Reyes TM *et al.* Obesity in mice with adipocyte-specific deletion of clock component Arntl. *Nat Med* 2012; **18**: 1768–1777.
18 Garaulet M. PERIOD2 variants are associated with abdominal obesity, psycho-behavioral factors, and attrition in the dietary treatment of obesity. *J Am Diet Assoc* 2011; **110**: 917–921.
19 Garaulet M, Tardido AE, Lee YC, Smith CE, Parnell LD, Ordovas JM. SIRT1 and CLOCK 3111 T>C combined genotype is associated with evening preference and weight loss resistance in a behavioral therapy treatment for obesity. *Int J Obesity* 2012; **36**: 1436–1441.
20 Maury E, Ramsey KM, Bass J. Circadian rhythms and metabolic syndrome: from experimental genetics to human disease. *Circ Res* 2010; **106**: 447–462.
21 Ando H, Takamura T, Matsuzawa-Nagata N, Shima KR, Eto T, Misu H *et al.* Clock gene expression in peripheral leucocytes of patients with type 2 diabetes. *Diabetologia* 2009; **52**: 329–335.
22 Gomez-Abellan P, Hernandez-Morante JJ, Lujan JA, Madrid JA, Garaulet M. Clock genes are implicated in the human metabolic syndrome. *Int J Obes (Lond)* 2008; **32**: 121–128.
23 Gogebakan O, Kohl A, Osterhoff MA, van Baak MA, Jebb SA, Papadaki A *et al.* Effects of weight loss and long-term weight maintenance with diets varying in protein and glycemic index on cardiovascular risk factors: the diet, obesity, and genes (DiOGenes) study: a randomized, controlled trial. *Circulation* 2011; **124**: 2829–2838.
24 Rossmeislova L, Malisova L, Kracmerova J, Stich V. Adaptation of human adipose tissue to hypocaloric diet. *Int J Obes (Lond)* 2013; **37**: 640–650.
25 de Kreutzenberg SV, Ceolotto G, Papparella I, Bortoluzzi A, Semplicini A, Dalla Man C *et al.* Downregulation of the longevity-associated protein sirtuin 1 in insulin resistance and metabolic syndrome: potential biochemical mechanisms. *Diabetes* 2010; **59**: 1006–1015.
26 Kovsan J, Bluher M, Tarnovscki T, Kloting N, Kirshtein B, Madar L *et al.* Altered autophagy in human adipose tissues in obesity. *J Clin Endocrinol Metab* 2011; **96**: E268–E277.
27 Dasu MR, Devaraj S, Park S, Jialal I. Increased Toll-like receptor (TLR) activation and TLR ligands in recently diagnosed type 2 diabetic subjects. *Diabetes Care* 2010; **33**: 861–868.
28 Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* 2007; **117**: 175–184.
29 Gomez-Santos C, Gomez-Abellan P, Madrid JA, Hernandez-Morante JJ, Lujan JA, Ordovas JM *et al.* Circadian rhythm of clock genes in human adipose explants. *Obesity* 2009; **17**: 1481–1485.
30 Garaulet M, Gomez-Abellan P, Alburquerque-Bejar JJ, Lee YC, Ordovas JM, Scheer FA. Timing of food intake predicts weight loss effectiveness. *Int J Obes (Lond)* 2013; **37**: 604–611.
31 Kahleova H, Belinova L, Malinska H, Oliyarnyk O, Trnovska J, Skop V *et al.* Eating two larger meals a day (breakfast and lunch) is more effective than six smaller meals in a reduced-energy regimen for patients with type 2 diabetes: a randomised crossover study. *Diabetologia* 2014; **57**: 1552–1560.
32 Li Y, Sato Y, Yamaguchi N. Shift work and the risk of metabolic syndrome: a nested case-control study. *Int J Occup Environ Health* 2011; **17**: 154–160.
33 Le Martelot G, Claudel T, Gatfield D, Schaad O, Kornmann B, Lo Sasso G *et al.* REV-ERBalpha participates in circadian SREBP signaling and bile acid homeostasis. *PLoS Biol* 2009; **7**: e1000181.
34 Grimaldi B, Bellet MM, Katada S, Astarita G, Hirayama J, Amin RH *et al.* PER2 controls lipid metabolism by direct regulation of PPARgamma. *Cell Metab* 2010; **12**: 509–520.
35 Soussi H, Reggio S, Alili R, Prado C, Mutel S, Pini M *et al.* DAPK2 down-regulation associates with attenuated adipocyte autophagic clearance in human obesity. *Diabetes* 2015; **64**: 3452–3463.
36 Ma D, Panda S, Lin JD. Temporal orchestration of circadian autophagy rhythm by C/EBPbeta. *EMBO J* 2011; **30**: 4642–4651.
37 Nakahata Y, Sahar S, Astarita G, Kaluzova M, Sassone-Corsi P. Circadian control of the NAD+ salvage pathway by CLOCK-SIRT1. *Science* 2009; **324**: 654–657.
38 Lamia KA, Sachdeva UM, DiTacchio L, Williams EC, Alvarez JG, Egan DF *et al.* AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation. *Science* 2009; **326**: 437–440.
39 Ramsey KM, Yoshino J, Brace CS, Abrassart D, Kobayashi Y, Marcheva B *et al.* Circadian clock feedback cycle through NAMPT-mediated NAD+ biosynthesis. *Science* 2009; **324**: 651–654.

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