Rhythmic diurnal gene expression in human adipose tissue from individuals who are lean, overweight and have type 2 diabetes

Running Title: Rhythmic human adipose gene expression

Daniella T Otway,¹ Simone Mäntele,¹ Silvia Bretschneider,¹ John Wright,¹ Paul Trayhurn,² Debra J Skene,¹ M Denise Robertson,¹ Jonathan D Johnston¹

¹Faculty of Health and Medical Sciences, University of Surrey, UK
²School of Clinical Sciences, University of Liverpool, UK

Corresponding author: Dr Jonathan D Johnston
Faculty of Health and Medical Sciences
University of Surrey
Guildford
Surrey GU2 7XH
UK
Tel: +44 (0) 1483 686470
Fax: +44 (0) 1483 68
j.johnston@surrey.ac.uk


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OBJECTIVE – Previous animal studies suggest a functional relationship between metabolism, type 2 diabetes and the amplitude of daily rhythms in white adipose tissue (WAT). However, data interpretation is confounded by differences in genetic background and diet, or limited sampling points. We have taken the novel approach of analysing serial human WAT biopsies across a 24-hour cycle, in controlled laboratory conditions.

RESEARCH DESIGN AND METHODS – Lean (n = 8), overweight/obese (n = 11), or overweight/obese with type 2 diabetes (n = 8) volunteers followed a strict sleep-wake and dietary regime for 1 week prior to the laboratory study. They were then maintained in controlled light-dark conditions, semi-recumbent posture and fed hourly during wake periods. Subcutaneous WAT biopsies were collected 6-hourly over 24-hours and gene expression was measured by quantitative PCR.

RESULTS – Lean individuals exhibited significant (\(P < 0.05\)) temporal changes of core clock (\(PER1, PER2, PER3, CRY2, BMAL1, DBP\)) and metabolic (\(REVERB\alpha, RIP140, PGC1\alpha\)) genes. The \(BMAL1\) rhythm was in approximate antiphase with the other clock genes. Surprisingly, there was no significant effect (\(P > 0.05\)) of increased body weight or type 2 diabetes on rhythmic gene expression.

CONCLUSIONS – The robust nature of these rhythms and their relative phasing indicate that WAT can now be considered as a peripheral tissue suitable for the study of \textit{in vivo} human rhythms. Comparison of data between subject groups clearly
indicates that obesity and type 2 diabetes are not related to the amplitude of rhythmic WAT gene expression in humans maintained under controlled conditions.
INTRODUCTION

Circadian clocks regulate daily changes in a wide range of physiological and behavioural functions, including the timing of sleep. Disruption of circadian biology or sleep has profound metabolic consequences (1), which have lead to the development of a major research effort to understand the links between these processes. By understanding the temporal regulation of metabolic physiology, it is anticipated that common behaviours and clinical interventions can be optimised to reduce the impact of modern society on the incidence of metabolic diseases, such as diabetes.

There are clear links between circadian clocks and metabolism. In mouse models, transgenic disruption of key clock-related genes induces marked metabolic phenotypes (2-5). In humans, chronic desynchrony of internal circadian time with the external environment (e.g. during shift work) correlates with increased incidence of obesity, type 2 diabetes and cardio-metabolic diseases (6). Moreover, consistent with the transgenic mouse data, recent work has linked polymorphisms of human circadian clock genes with metabolic dysfunction (7-9).

The mammalian circadian timing system consists of a ‘master’ clock within the suprachiasmatic nuclei (SCN) of the hypothalamus and a series of ‘peripheral’ clocks, which are located in extra-SCN regions of the brain as well as in almost all other tissues (10). The environmental light-dark cycle synchronises the circadian system by acting through the SCN clock, which then maintains temporal order of the peripheral clocks via multiple rhythmic outputs (10). These SCN-driven pathways include
neuronal (e.g. sympathetic tone), endocrine (e.g. melatonin and cortisol secretion) and behavioural (e.g. feeding) activity.

Murine white adipose tissue (WAT), as with many other tissues, contains endogenous circadian timing properties (11,12) and approximately 10-20% of the murine WAT transcriptome is estimated to exhibit 24-hour variation (13,14). Importantly, mice that are genetically susceptible to obesity and type 2 diabetes (15) or fed a high fat diet (16) exhibit reduced amplitude rhythms of gene expression in WAT, leading to the hypothesis that the robustness of WAT clocks is functionally associated with metabolic phenotype.

This hypothesis has not been rigorously tested in humans. The principal reason for this important gap in our knowledge stems from the difficulty in obtaining multiple serial biopsies of tissue from human volunteers. To date, the literature on rhythmic gene expression in human WAT has primarily relied on either analysis of single time point biopsies maintained in culture (17), or the relative expression of genes at a single time point (18,19). Clearly neither of these methods is ideal for the assessment of in vivo 24-hour rhythms.

In this study, serial human biopsies across a 24-hour cycle have been analysed using a carefully controlled protocol designed to minimise the effect of confounding factors on rhythmic physiology. We have studied 3 defined groups of human volunteers to investigate daily WAT rhythms; lean individuals have been compared with subjects who are overweight/obese or overweight/obese with type 2 diabetes.
RESEARCH DESIGN AND METHODS

All aspects of the study were conducted in accordance with the Declaration of Helsinki and received a favourable ethical opinion from the Surrey Research Ethics Committee and the University of Surrey Ethics Committee.

Participants. Volunteers completed questionnaires to assess general health and ensure normal sleep and diurnal preference. They then attended a pre-screening session at the University of Surrey, typically within 1 month of the laboratory session and during the early afternoon. Body mass index (BMI, m²/kg), waist circumference were measured; fasting blood samples were provided for measurement of plasma HbA1c, insulin and glucose concentration, and homeostatic model assessment of insulin resistance (HOMA-IR; calculated using HOMA Calculator v2.2 software, Diabetes Trial Unit, University of Oxford, UK).

27 male volunteers were recruited; 8 lean, 11 overweight and 8 overweight with type 2 diabetes. Of the patients with type 2 diabetes, average time since diagnosis was 6.9 ± 2.3 years; 3 were controlled by diet and exercise; the other 5 patients were treated with combinations of metformin, statins, ramipril and lisinopril. One participant within the lean group was a smoker, and was required to refrain from smoking for one week prior to the study. None of the participants had undertaken shift work within 5 years of the study, nor crossed any time zones within one month of the study.

For 1 week prior to the laboratory study, volunteers were required to maintain prescribed daily feeding times and sleep-activity (sleep 22:30-06:30), which were
monitored using wrist actigraphy (AWL, Cambridge Neurotechnology, Cambridge UK), sleep diaries, food diaries and recorded messages on a laboratory time-stamped answer phone. Participants were also required to refrain from eating fatty or sugary foods and drinking alcohol or caffeine throughout the week. For the final three days of this baseline week, food was provided by the research team, to enable control of timed behaviour, together with both the quantity and quality of nutrient intake. During these three days, the daily caloric content of the supplied food was basal metabolic rate x 1.5 with approximately 35% of energy from fat.

**Laboratory study.** All experimental procedures were carried out at the Surrey Clinical Research Centre. Volunteers arrived in the afternoon of day 0 for a night of adaptation. Throughout the 2-day study, they were required to maintain a semi-recumbent posture. They were required to remain awake with lights-on between 06:30-22:30 (440-825 lux in direction of gaze) and allowed to sleep with lights-off between 22:30-06:30 (0 lux). During the waking period, participants were fed with hourly nutritional drinks (Fortisip; Nutricia, Schiphol, Netherlands) and were allowed to drink water *ad libitum*. Daily energy intake was basal metabolic rate x 1.1, divided equally over the waking hours.

Four subcutaneous WAT biopsies were taken under local anaesthetic (lidocaine) from four different sites of the upper buttock region of each participant at 6-hourly intervals for 24-hours, beginning at 10:30 on day 1. The sampling order of the four biopsy sites was randomised between groups and subjects. This WAT depot is known to exhibit metabolic activity (20).
**Analysis of gene expression.** WAT biopsies were washed with saline and snap-frozen in liquid nitrogen before storage at -80°C. Total RNA was extracted using TRIZOL, according to the manufacturer’s instructions. cDNA was synthesised and expression was measured for genes integral to the circadian clock (*PER1, PER2, PER3, CRY2, BMAL1, DBP*) and involved in metabolic activity (*REVERBα, RIP140, PGC1α*) by quantitative Taqman real-time PCR as described previously (21). Primer-probe sets were designed in-house using Primer Express software (Table 1). Standard curves ($R^2 > 0.99$) were generated using human genomic DNA (Promega Corporation, Southampton, UK) and expression of all genes was normalised to *GAPDH*, as in previous studies of adipose clock gene expression (15,16).

**Statistical analysis.** Pre-screen data were analysed using 1-way ANOVA with Tukey post-hoc test. Correlation analyses were conducted using linear regression, and the gene expression time course from biopsies was performed using 1-way or 2-way repeated measures ANOVA (factors time and group) with Tukey post-hoc test.
RESULTS

Pre-screen data are shown in Table 2. Despite efforts to age-match the participants, there was a significant \( P < 0.05 \) difference in age, with post-hoc analysis revealing a significant difference only between the overweight and type 2 diabetes groups. However, subsequent analysis using age as a co-variant revealed that age per se did not alter gene expression (data not shown). There were significant \( P < 0.05 \) differences in BMI, waist circumference, fasting plasma glucose and insulin concentrations, HbA1c levels and HOMA-IR, with highest values occurring in participants with type 2 diabetes. There was no significant difference in plasma insulin, glucose, HbA1c or HOMA-IR between lean and overweight/obese groups, indicating that our overweight/obese participants were insulin-sensitive.

In lean individuals, there was a significant \( P < 0.05 \) effect of time on the expression of multiple genes involved in circadian \( (\text{PER}1, \text{PER}2, \text{PER}3, \text{CRY}2, \text{BMAL}1, \text{DBP}) \) and metabolic functions \( (\text{REVERB} \alpha, \text{RIP}140, \text{PGC}1 \alpha) \) (Fig. 1). Minimum expression of \text{REVERB} \alpha and \text{DBP} occurred around the middle to end of the afternoon. In keeping with circadian gene expression in other tissues (10), the phase of \text{PER}1, \text{PER}2, \text{PER}3 and \text{CRY}2 was slightly delayed compared to \text{REVERB} \alpha and \text{DBP}, with minimum expression occurring around the evening light-dark transition. The \text{BMAL}1 rhythm was in approximate antiphase to the other circadian genes, with maximal expression around the evening light-dark transition.

Analysis by 2-way repeated measures ANOVA of the data from all of the experimental groups confirmed a significant \( P < 0.05 \) effect of time on gene
expression (Fig. 2). However, surprisingly, we did not observe a significant effect of experimental group on the expression of any gene. The only gene to exhibit a significant ($P < 0.01$) time x group interaction was *BMAL1*. Tukey post-hoc analysis revealed that *BMAL1* expression 16.5 hours after lights-on (equivalent to clock time 23:00) was significantly ($P < 0.05$) lower in the overweight/obese participants than in the lean or type 2 diabetic groups.
DISCUSSION

Consistent with a previous study (22), our data reveal robust diurnal rhythmicity within human WAT in vivo. To date, limited data have been published concerning clock gene expression in this tissue. Due to difficulties in acquiring human tissue, some studies have measured clock gene expression in WAT collected at a single time point (18,19), although it is clearly difficult to infer rhythmical changes from such a temporally limited data set. An elegant compromise has been to culture explants of tissue taken at a single time point and then measure gene expression in fragments of the biopsies over a 24-hour time course (17). Although such studies highlight endogenous WAT rhythms, they do not reflect gene expression in vivo. We therefore measured gene expression in human tissue explants harvested across a whole 24-hour cycle to provide in vivo human WAT rhythms.

Animal models suggest that obesity and type 2 diabetes reduce rhythm amplitude in murine WAT, as lean C57BL/6J mice exhibit higher amplitude WAT rhythms than obese/diabetic KK and KK-Ay mice (15). Other data revealed reduced amplitude rhythms in mice that have become obese due to a high-fat diet (16). However, Ando et al (15) compared mice with differing genetic backgrounds, which complicate interpretation of their data, and it is possible that dietary intervention could directly regulate gene expression rather than obesity per se.

In contrast to the above studies, we observed minimal differences between clock gene rhythms in human WAT. Interpretation of the minor difference in BMAL1 expression is not clear; although mouse Bmal1 has been implicated in the control of adipogenesis
in vitro (23), juvenile Bmal1−/− mice develop adipose depots comparably to their wild type litter mates (24). Furthermore, the similarity between gene expression profiles in lean individuals and those overweight with type 2 diabetes suggests that BMI is not the cause of the time x group interaction for BMAL1 in the overweight, non-diabetic individuals.

The similarity of gene expression profiles between our experimental groups may partly result from a lack of extreme phenotypic differences. Although there was not a large disparity in BMI between our groups, all participants fell into the current clinical guidelines for lean/healthy, obese and type 2 diabetic individuals. There was therefore no effect of obesity or type 2 diabetes per se on human WAT rhythmicity. It remains possible that severely obese individuals may exhibit reduced amplitude WAT rhythmicity; however, interpretation of data from such individuals is complicated by various confounding factors, such as co-morbidities associated with type 2 diabetes.

Another difference between our study and previous work (25) is the high level of glycaemic control in our participants. We specifically aimed to investigate the relationship between WAT rhythmicity, body weight and presence of type 2 diabetes. We therefore eliminated as many additional factors as possible. Diurnal adipose gene expression may be modulated by the level of glycaemic control and/or drugs taken by diabetic individuals. Alternatively, the association between metabolic state and WAT rhythmicity might vary between adipose depots. However, due to limitations of sampling and experimental group size, we could not directly address these possibilities in the current study.
The persistence of 24-hour rhythms in WAT from patients with type 2 diabetes now suggests that the link between human circadian and metabolic physiology occurs outside of WAT, at least in the earlier stages of the disease.
AUTHOR CONTRIBUTIONS

D.T.O. researched data and reviewed/edited the manuscript. S.M., S.B. and J.W. researched data. P.T., D.J.S. and M.D.R. reviewed/edited the manuscript. J.D.J. wrote the manuscript.
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REFERENCES (max 25)


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Data are presented as mean ± SEM. * P < 0.05 vs Lean group; # P < 0.05 vs Overweight/Obese group. HOMA-IR, homeostatic model assessment of insulin resistance.
FIGURE LEGENDS

FIG. 1. Robust 24-hour changes of gene expression in human WAT from lean individuals. Data are presented as mean ± SEM of n = 8-11 values, normalised to GAPDH. There was a significant effect of time (\(P < 0.05\), 1-way repeated measures ANOVA) on the expression of each gene. Horizontal bars represent wake (white) and sleep (black) periods during the laboratory study. Zeitgeber time 0 represents the time of lights-on (equivalent to clock time 06:30).

FIG. 2. Comparison of 24-hour gene expression profiles in human WAT from individuals who are lean (solid line, diamonds), overweight/obese (dashed line, squares) or overweight/obese with type 2 diabetes (dotted line, triangles). Data are presented as mean ± SEM of n = 8-11 values, normalised to GAPDH. For all genes, there was a significant (\(P < 0.05\)) effect of time but no subject group effect on gene expression (2-way repeated measures ANOVA). With the exception of \(BMAL1\) (\(P < 0.01\)), there was no significant interaction between time and subject group on gene expression. Horizontal bars represent wake (white) and sleep (black) periods during the laboratory study. Zeitgeber time 0 represents the time of lights-on (equivalent to clock time 06:30).