Greased lighting : implications of circadian lipid metabolism for cardiometabolic health

Berg, R. van den; Berg R. van den

Chapter 5

Effects of mistimed light exposure on atherosclerosis development in APOE*3-Leiden.CETP mice

Rosa van den Berg, Maaike Schilperoort, Sander Kooijman, Ashna Ramkisoensing, Padmini P.S.J. Khedoe, Lianne Wee-Pals, Sanne Boekestijn, Karina Wagner, Johanna H. Meijer, Claudia P. Coomans, Jimmy F.P. Berbée, Patrick C.N. Rensen, Nienke R. Biermasz

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ABSTRACT

Shift work is a risk factor for atherosclerotic disease in humans. However, whether shift work is causally involved in atherosclerosis and which facet of shift work is responsible are unknown. Here, we investigated the hypothesis that mistimed light exposure may causally contribute to atherosclerosis by performing studies in APOE*3-Leiden.CETP mice, a well-established model for human-like lipoprotein metabolism and atherosclerosis. In two separate experiments, female mice were either subjected to constant light (LL) or to three different rotating light schedules, compared to control 12h light-dark (LD) schedule. While LL disturbed the circadian rhythm to some extent, it did not affect atherosclerotic lesion development. In contrast, a weekly 6 h advance light schedule increased plaque severity. Moreover, weekly reversal of LD schedule increased plaque severity as well as plaque size. Increased atherosclerotic development could not be attributed to higher plasma cholesterol levels. In conclusion, mistimed light exposure per se aggravates atherosclerotic development in mice, which may at least partly explain the association between shift work and cardiovascular disease in humans. Future studies should focus on underlying mechanisms.

INTRODUCTION

Cardiovascular diseases (CVD) are currently the leading cause of death world-wide [1]. The main cause of CVD is atherosclerosis, to which a pro-inflammatory state and dyslipidemia are key contributors [2]. Dyslipidemia is characterized by increased levels of plasma triglycerides (TG) and LDL-cholesterol and decreased HDL-cholesterol [3]. Plasma levels of lipids display day-night variations independent of food intake [4], suggesting that the biological clock is an important determinant of lipid levels. Consequently, disturbed biological clock function may contribute to dyslipidemia and thereby increase the risk for CVD.

Epidemiological studies have repeatedly shown associations between disturbance of biological clock function, responsible for generating circadian (i.e. ~24 h) rhythms, and metabolic disorders such as obesity, type 2 diabetes and CVD [5-7]. Already in 1949 a Scandinavian observational study among factory workers reported an association between shift work and cardiovascular mortality [8]. Longitudinal studies indicate that shift work is indeed a causal risk factor for cardiovascular events, including hard end-points like ischemic stroke and myocardial infarction [9, 10]. However, the underlying mechanisms remained elusive. Meanwhile, the behavioral patterns of human activity, especially in industrialized countries, have undergone dramatic changes with respect to adherence to day and night rhythms. The use of electrical light has uncoupled the behavioral active period from the natural occurring day, social activities are shifted and the 24-hour economy necessitates working at night. Of note, in Europe approximately 20% of the working population is involved in some form of shift work [11].

Shift work likely contributes to adverse health outcomes via multifactorial pathways, including psychosocial factors, sleep loss, a decrease in physical activity, altered food intake quantity (i.e. an increase in caloric intake) and quality (i.e. changes in timing and choice of food) and lack of sun light exposure. An undervalued aspect is the contributing factor of mistimed light exposure itself. Prolonged light exposure is associated with obesity in humans [12-16]. Recently, we were able to show in mice that prolonged light exposure increases adiposity by attenuation of brown adipose tissue (BAT) activity, independent of food intake [17]. Upon activation, BAT takes up fatty acids from TG-rich lipoproteins to burn into heat, resulting in accelerated hepatic clearance of TG-rich lipoprotein remnants [18]. In fact, pharmacological activation of BAT in mice decreases plasma TG and cholesterol, accompanied with attenuation of atherosclerosis formation [19]. It is thus conceivable to reason that mistimed light exposure could reduce BAT activity, thereby increasing plasma cholesterol and accelerating atherosclerosis.

Although it has been established that mistimed light exposure by rotating light shifts increases breast cancer development in mice [20] and shift advance increases mortality [21], the effects of mistimed light exposure on lipid metabolism and atherosclerosis development are currently unknown. Therefore, the aim of the present study was to investigate the role of mistimed light exposure on atherosclerosis development. To investigate this, we subjected APOE*3-Leiden.CETP female mice, which have a humanized

lipoprotein metabolism and develop human-like atherosclerosis, to different light regimes and determined the effect on atherosclerosis development.

MATERIAL AND METHODS

Animal Husbandry

All animal experiments were approved by the Institutional Ethics Committee on Animal Care and Experimentation (Leiden University Medical Center). Mice heterozygous for the APOE*3-Leiden gene were crossbred at our own facility with mice expressing human cholesteryl ester transfer protein (CETP) to yield APOE*3-Leiden.CETP (E3L.CETP) transgenic mice [22], a mouse model with a human-like lipoprotein metabolism [23]. Female E3L.CETP mice of 8-12 weeks old were fed *ad libitum* with Western-type diet (WTD) containing 15% fat from cocoa butter, 1% fat from corn oil (diet T, HopeFarms, Aldrich, Zwijndrecht, the Netherlands), enriched with 0.1% cholesterol. During a run-in period of three weeks mice were housed under standard 12h:12h light:dark (LD) conditions. Afterwards, mice were randomized based on fasting plasma total cholesterol (TC), TG, body weight and age to experimental groups described below. Mice were group-housed (n=3/cage) in clear plastic cages, placed in light-tight cabinets fitted with diffuse white fluorescent light with an intensity of approximately 85 μW/cm^2. In the last two weeks of the experiment, behavioral activity patterns were assessed by housing mice individually in cages fitted with passive infrared detectors.

Experimental Design

In two separate atherosclerosis experiments, the effects of prolonged light exposure and of mistimed light exposure on atherosclerosis development were investigated. Blood drawing and final experiments started at ZT4 (ZT = Zeitgeber time in hours where ZT0 represents lights on) and groups were alternated. In the first experiment, mice were randomized to either a regular 12h:12h LD cycle or to constant light exposure (LL) (n=18/group) for the total duration of 14 weeks. In week 2, 4, 8 and 12 after start of light exposure intervention unfasted blood samples were obtained. At the same time, body weight was measured and food intake was determined by weighing food on the lid of the cage. After 14 weeks, body composition was assessed by EchoMRI-100 (EchoMRI, Houston, Texas), mice were anesthetized by intraperitoneal injection of acepromazin (6.25 mg/kg; Sanofi Sant Nutrition Animale, Libourne Cedex, France), midazolam (6.25 mg/kg; Roche, Mijdrecht, the Netherlands), and fentanyl (0.31 mg/kg; Janssen-Cilag, Tilburg, the Netherlands), and blood was drawn retro-orbitally into EDTA coated cups for immune cell analysis. Afterwards, mice were killed by cervical dislocation and organs were harvested for further analysis.

In the second experiment, mice were randomized to either LD, a 6-h phase advance every week (advance), a 6-h phase delay every week (delay) or a weekly reversal of the 12h light-dark cycle (LD-DL) (n=15/group) for the total duration of 15 weeks. In week 3, 7, 11 and 15 after start of the light regime, when light regimes were aligned among the groups,

unfasted blood was drawn at ZT4 on the 2nd day and also on the 6th day after a shift where indicated. At the same time, body weight and food intake were measured as described above. At week 11, blood was collected into EDTA-coated cups and used for immune cell analysis. After 15 weeks, mice were killed by CO_2 inhalation, blood was drawn into EDTA-coated cups and organs were harvested for further analysis.

Biochemistry

Blood was drawn via the tail vein into paraoxon coated capillaries. Plasma was isolated by centrifugation, snap frozen into liquid nitrogen and stored at -80°C until further analyses. Plasma TC and TG were analysed by commercially available enzymatic kits according to manufacturer's protocols (Roche Molecular Biochemicals, IN, USA). HDL was isolated by precipitation of ApoB-containing lipoproteins. Hereto, 20% polyethylene glycol (Sigma Aldrich) in 200 mM glycine-buffered saline (pH 10) was added to plasma, centrifuged for 30 minutes at 6,000 rpm, and HDL-C was determined in the supernatant. Non-HDL-C was calculated by subtracting HDL-C from TC.

Immune cell composition

Immune cell composition of freshly isolated whole blood (100 µl) was determined using Sysmex using a XT-2000i hematology analyzer (Sysmex Corporation, Japan).

Histological analysis of the heart

Hearts were fixated in phosphate buffered 4% paraformaldehyde and embedded in paraffin and cross-sections of 5 µm were made in the aortic valve region. Sections were stained with haematoxylin-phloxine-saffron for histological analysis. Per mouse, lesion area was quantified in the aortic root starting from the appearance of open aortic valve leaflets in four subsequent sections with 50 µm intervals. Lesion area was determined by analysis using Image J software. Lesion severity was scored according to the guidelines of the American Heart Association adapted for mice [24].

Histological analysis of adipose tissue

Interscapular brown adipose tissue (iBAT) was fixated in phosphate buffered 4% paraformaldehyde, embedded in paraffin and cross-sections of 5 µm were made. Sections were stained with Mayer's haematoxylin and eosin. BAT lipid droplet content was quantified using ImageJ software.

Gene expression analysis

A part of iBAT was snap frozen and stored at -80°C for gene expression analysis and protein analysis (see below). Total RNA was isolated using TriPure (Roche) according to the manufacturer's instructions. 1 µg of total RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Real-time PCR was carried out on a CFX96 PCR machine (Bio-Rad) using IQ SYBR-Green Supermix (Bio-Rad). Expression levels were normalized to 36B4 as housekeeping gene.

Behavioral analysis

Behavioral patterns were analysed by plotting actograms using Clock lab (Actimetrics, Wilmette, IL, USA). F-periodogram analysis was performed on activity binned into 10 minutes interval of the last 10 consecutively recorded experimental days according to the algorithm of Dörrscheidt and Beck [25].

Statistical analysis

Comparisons between group averages were performed using Student's T-test or ANOVA with Fisher LSD post-hoc test (compared to LD light schedule), where appropriate (Graphpad Prism v.6). Correlations between two dependent variables were made using Pearson's correlation analysis. Data are presented as means ± SEM. Differences at P values < 0.05 were considered statistically significant.

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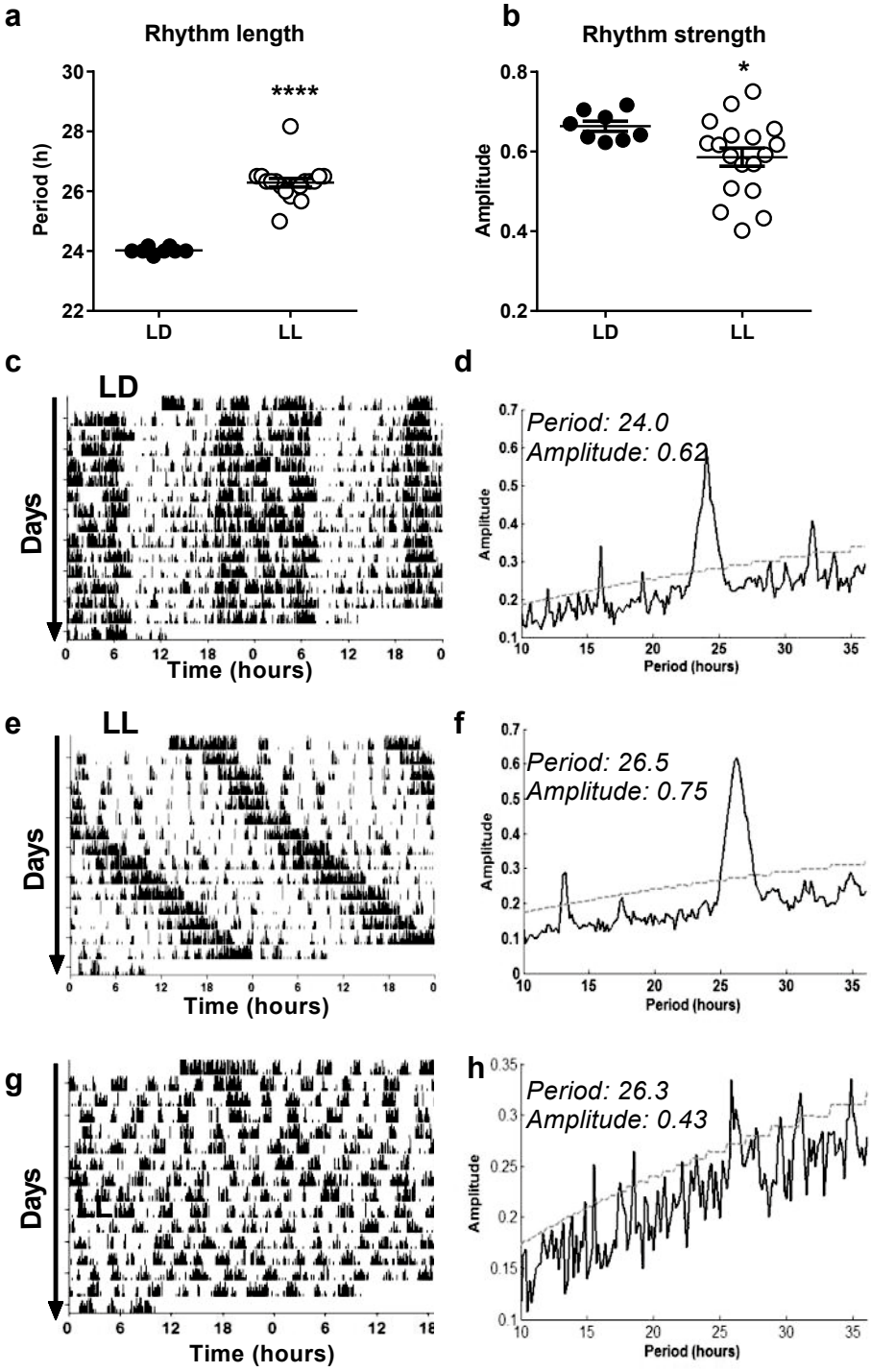
RESULTS

Constant light attenuates circadian rhythm strength in APOE*3-Leiden.CETP mice

Dyslipidemic APOE*3-Leiden.CETP female mice were subjected to 14 weeks of either constant light (LL) or normal light dark cycles (LD). The effect of light intervention on the circadian behavior was assessed by monitoring spontaneous physical activity rhythms via passive infrared detectors. Periodogram analysis demonstrated that LL lengthened the period of physical activity rhythmicity, compared to the LD mice (26.29 ± 0.14 *vs.* 24.02 ± 0.03 h; p<0.0001) (Fig. 1a). In addition, average rhythm strength was decreased in LL mice compared to LD mice (0.59 ± 0.02 *vs.* 0.66 ± 0.01; p=0.038) (Fig. 1b). LL mice showed considerably higher variation compared to LD mice (CV 16% *vs.* 5%) and between various LL mice we observed different physical activity patterns between high and low amplitude actograms (Fig. 1c-h). These data indicate that, while chronic LL exposure does not render all mice arrhythmic as determined by physical activity patterns, LL clearly attenuates the rhythmic circadian clock output.

Figure 1. (right page) Constant light attenuates circadian rhythm strength in APOE*3-Leiden.CETP mice. *APOE*3-Leiden.CETP mice fed WTD were exposed to LD or LL (n=18/group) for 14 weeks. During the last 10 days, mice were individually housed and behavioral activity was monitored by passive infrared monitors (n=8 LD, n=18 LL). Rhythm length (a) and rhythm strength (b) was calculated using F periodogram analysis. Representative double-plotted actogram (c) and periodogram (d) is shown for 1 LD mouse. Double-plotted actograms (e, g) and periodograms (f, h) are shown for 2 LL mice. Gray dotted line represents significance threshold. * = p<0.05; **** = p<0.0001 (unpaired T-test). Data is presented as individual data points including means ± SEM.*


a
Rhythm length
Period (h)
30
28
26
24
22
****
LD
LL
b
Rhythm strength
Amplitude
0.8
0.6
0.4
0.2
*
LD
LL
c
LD
Days
0 6 12 18 0 6 12 18 0
Time (hours)
d
Period: 24.0
Amplitude: 0.62
Amplitude
Period (hours)
e
LL
Days
Time (hours)
f
Period: 26.5
Amplitude: 0.75
Amplitude
Period (hours)
g
Days
Time (hours)
h
Period: 26.3
Amplitude: 0.43
Amplitude
Period (hours)

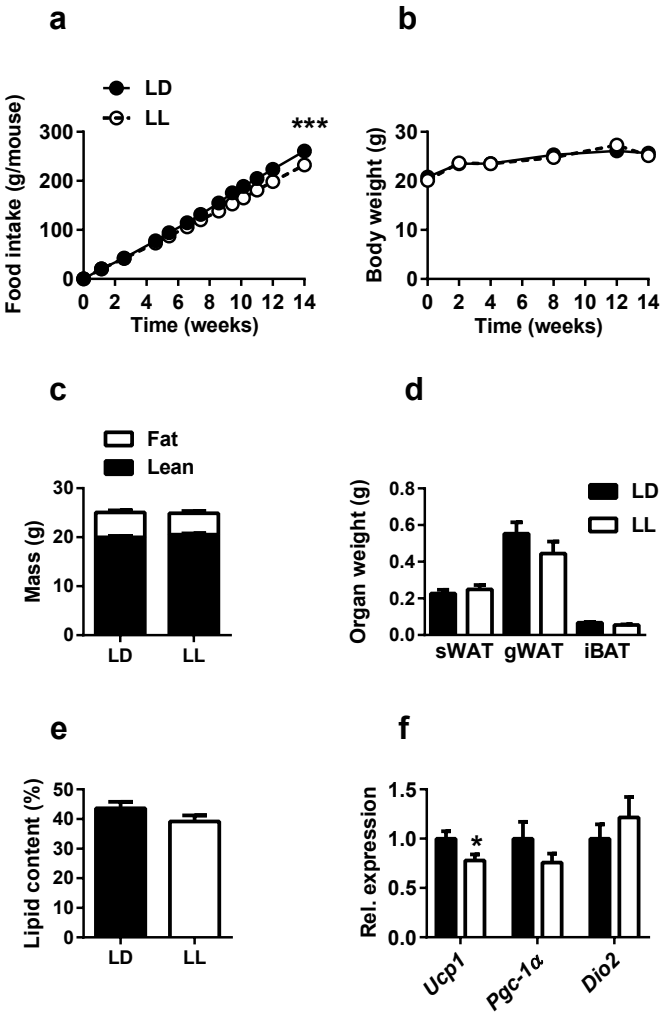


Figure 2. Constant light decreases food intake without increasing body weight or adiposity in APOE*3-Leiden.CETP-mice. *APOE*3-Leiden.CETP mice fed WTD were exposed to LD or LL (n=18/group) for 14 weeks. Food intake (a) and body weight (b) were determined at regular intervals. After 14 weeks, body composition (fat mass versus lean mass) was determined by EchoMRI (c) and subcutaneous white adipose tissue (sWAT), gonadal WAT (gWAT) and interscapular brown adipose tissue (iBAT) were weighed (d). Part of iBAT was stained with H&E for lipid content quantification (e) and in part used for gene expression analysis by qPCR (f). Expression is normalized for 36b4 and relative to mean LD expression. * = p<0.05; *** = p<0.001 (unpaired T-test). Data is presented as means ± SEM.*

Constant light decreases food intake without altering body weight or adiposity in APOE*3-Leiden.CETP mice

Body weight and food intake were measured throughout the study and fat mass was determined after 14 weeks of light intervention. We observed a small but consistent decrease in food intake in mice subjected to LL (Fig. 2a), which resulted in a lower cumulative food intake in LL mice compared to LD mice over 14 days (232 ± 4 *vs*. 260 ± 5 g/mouse; p<0.0001). This difference in food intake did not result in differences in body weight of the mice over time (at 14 weeks 25.2 ± 0.6 *vs*. 25.7 ± 0.7 g; p=0.608) (Fig. 2b), nor in fat mass as measured by EchoMRI (4.3 ± 0.5 *vs.* 5.1 ± 0.5 g; p=0.301) (Fig. 2c). Also, wet tissue weights of subcutaneous and gonadal white adipose tissue (sWAT; gWAT) depots were similar in LL mice compared to LD mice after 14 weeks (sWAT 0.25 ± 0.02 *vs.* 0.23 ± 0.02 g; p=0.460) (gWAT 0.45 ± 0.07 *vs.* 0.55 ± 0.06 g; p=0.242) (Fig. 2d). BAT activation leads to increased delipidation of BAT, which is reflected by the weight and lipid content [26]. BAT weight did not decrease due to LL (iBAT 0.054 ± 0.004 *vs.* 0.067 ± 0.005 g; p=0.064) (Fig. 2d), which was in line with the lipid content, which was equal between mice exposed to LL and LD (39 ± 2 *vs.* 44 ± 2%; p=0.149) (Fig. 2e). Thermogenic gene *Ucp1* showed a decreased relative expression in LL compared to LD (0.78 ± 0.06 *vs.* 1.00 ± 0.07 fold change; p=0.028) (Fig. 2f), however this was not accompanied by a lower expression of thermogenic genes *Pgc1α* (p=0.221) and *Dio2* (p=0.400) (Fig. 2f).

Constant light increases total cholesterol exposure without increasing atherosclerosis

Compared to LD, LL exposure raised TC plasma levels after 4 weeks (+3.6 ± 1.5 mM; p=0.025), 8 weeks (+3.0 ± 1.5 mM; p=0.056) and after 12 weeks (+4.5 ± 1.9 mM; p=0.025) of light intervention (Fig. 3a). This increase in TC was accompanied by a slightly lower TG level after 4 weeks (-1.1 ± 0.5 mM; p=0.013) and 14 weeks (-1.1 ± 0.5 mM; p=0.044) (Fig. 3b), but not at the other time points. Determination of cholesterol in lipoprotein subfractions showed that TC levels were increased due to a selective increase in non-HDL-cholesterol levels, i.e. (V)LDL-cholesterol (Fig. 3c-d). We therefore calculated TC exposure for the duration of light intervention, which was elevated in LL mice compared to LD mice (288 ± 12 *vs.* 251 ± 11 mM*week; p=0.038) (Fig. 3e). Since atherosclerosis development depends on both dyslipidemia and inflammation, we explored whether light interventions may have altered immune cell composition by measuring white blood count in peripheral blood. Interestingly, we observed a decrease in white blood cell (2.8 ± 0.2 $*10^9$ *vs.* 4.7 ± 0.4$*10^9$ cells/l; p<0.001) and platelet counts (1.8 ± 0.2 $*10^{12}$ *vs.* 1.4 ± 0.1$*10^{12}$ cells/l; p=0.013) due to LL exposure (Fig. 3f).

We next determined atherosclerotic lesion formation by assessing atherosclerosis plaque size and severity in the aortic root. Despite the raised TC exposure in LL mice, we observed no difference in atherosclerotic lesion size between LL and LD exposed mice (11.3 ± 1.0$*10^4$ *vs.* 12.0 ± 1.2$*10^4$ mm^2; p=0.540) (Fig. 3g). Likewise, lesion severity was not different (66 ± 5 *vs*. 62 ± 4% mild lesions; p=0.579) (37 ± 4 *vs*. 34 ± 5 % severe lesions; p=0.580) (Fig. 3h). Since rhythm strength varied in the LL group, we explored whether

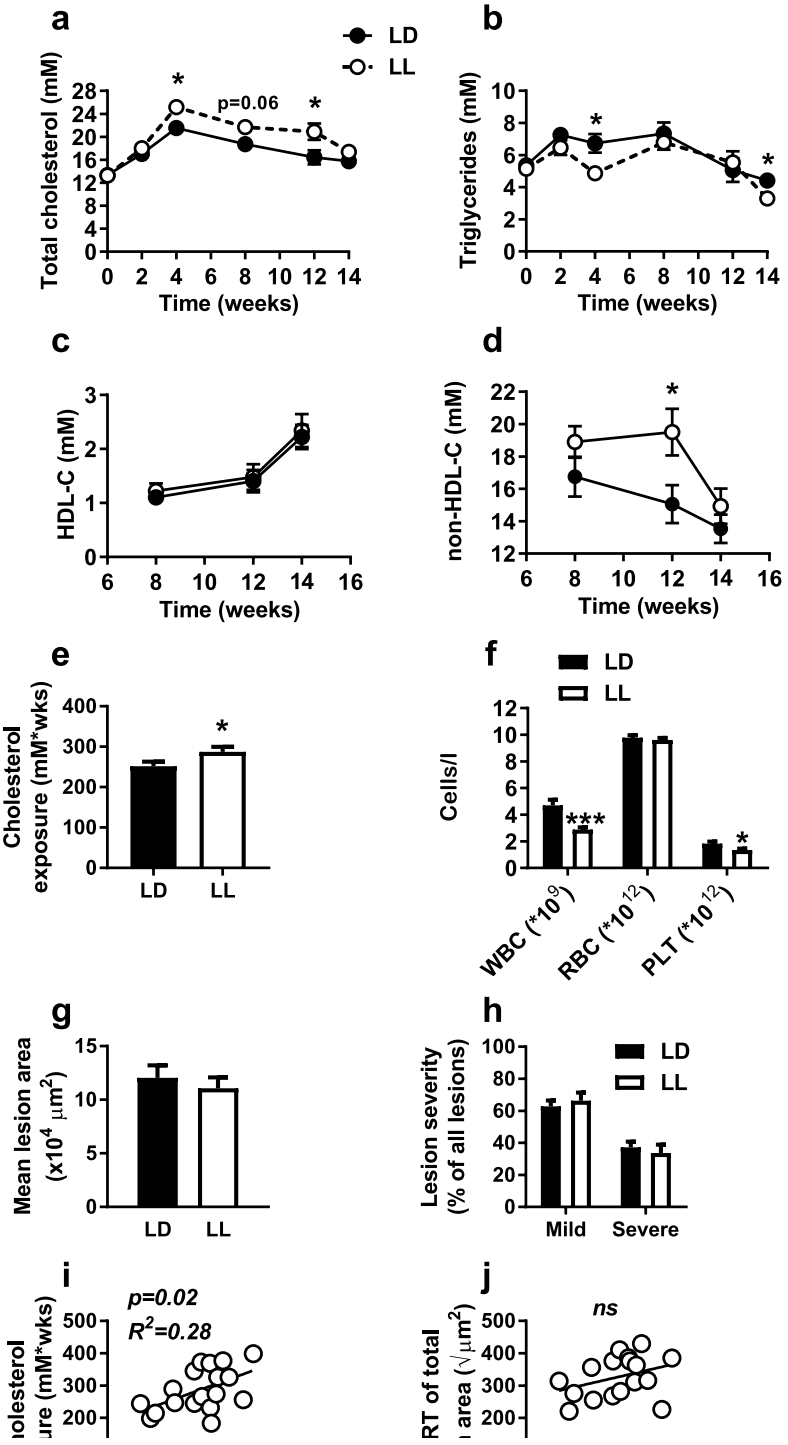

a
LD
LL
Total cholesterol (mM)
p=0.06
Time (weeks)
b
Triglycerides (mM)
Time (weeks)
c
HDL-C (mM)
Time (weeks)
d
non-HDL-C (mM)
Time (weeks)
e
Cholesterol exposure (mM*wks)
LD
LL
f
LD
LL
Cells/l
WBC (*10^9)
RBC (*10^12)
PLT (*10^12)
g
Mean lesion area (x10^4 µm^2)
LD
LL
h
Lesion severity (% of all lesions)
LD
LL
Mild
Severe
i
p=0.02
R^2=0.28
j
ns

Figure 3. (left page) Constant light increases total cholesterol exposure without increasing atherosclerosis. *APOE*3-Leiden.CETP mice fed WTD were exposed to LD or LL (n=18/group) for 14 weeks. Plasma total cholesterol (a) and triglycerides (b) was determined at regular intervals, HDL-cholesterol (c) and non-HDL-cholesterol (d) was determined after 8, 12 and 14 weeks. Total cholesterol exposure was calculated (e). After 14 weeks, mice were sacrificed and whole blood was collected and pooled (n=4-5/group) for cell count using Sysmex (f). Hearts were isolated and, at the aortic root, atherosclerotic lesion size (g) and severity (h) was determined. Rhythm strength was correlated to total cholesterol exposure (i) and atherosclerotic plaque area (j) for mice exposed to LL (Pearson correlation). * = p<0.05 (unpaired T-test). Data is presented as means ± SEM. WBC= white blood cells, RBC=red blood cells, PLT=platelets.*

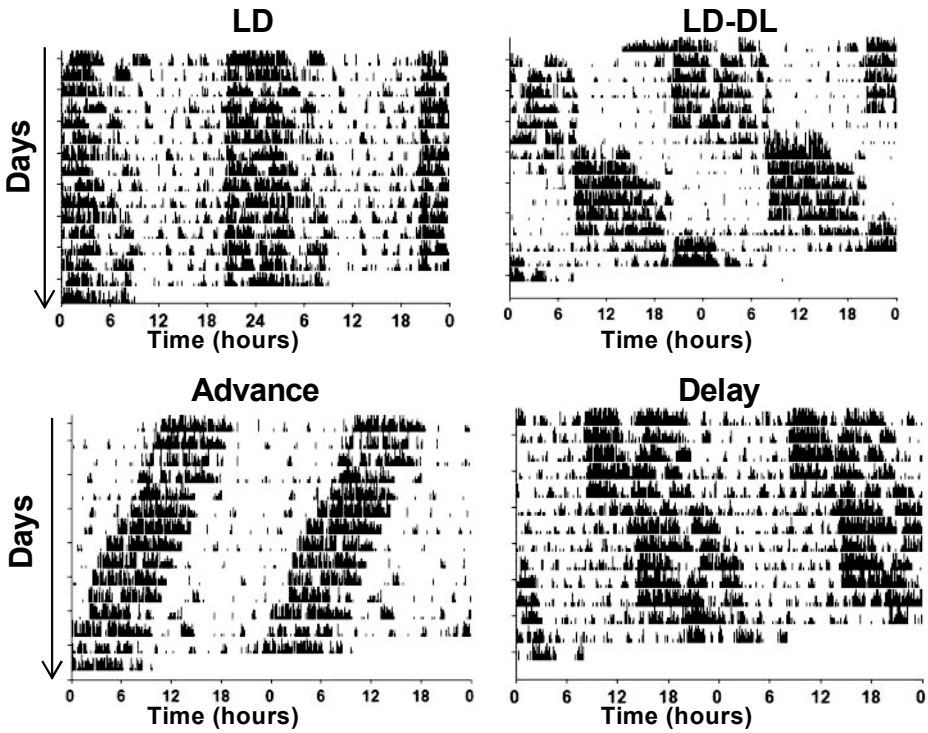


Figure 4. Representative actograms of mistimed light exposure. *APOE*3-Leiden.CETP mice fed WTD were exposed to LD, weekly 12 h reversal (LD-DL), weekly 6h advance or weekly 6h delay. During the last 10 days, mice were individually housed and behavioral activity was monitored by passive infrared monitors. Representative double-plotted actograms for 1 mouse per group are shown. Gray shading indicates dark period.*

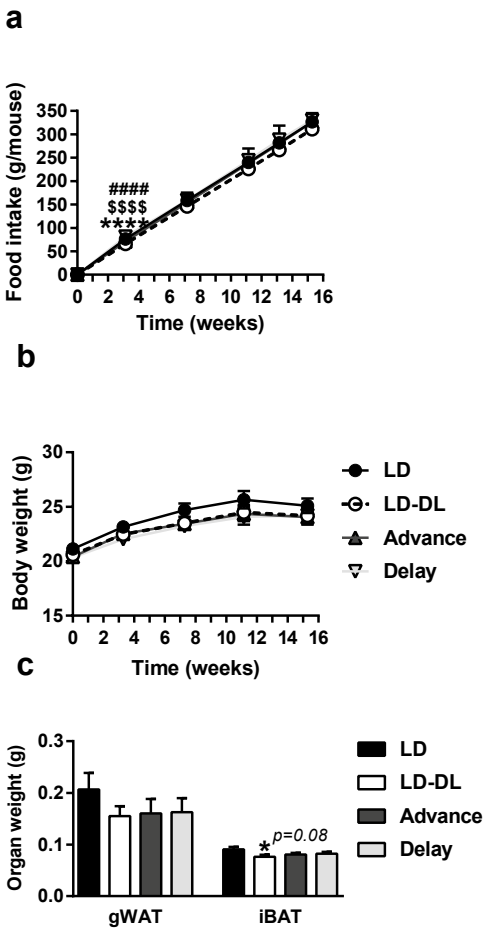
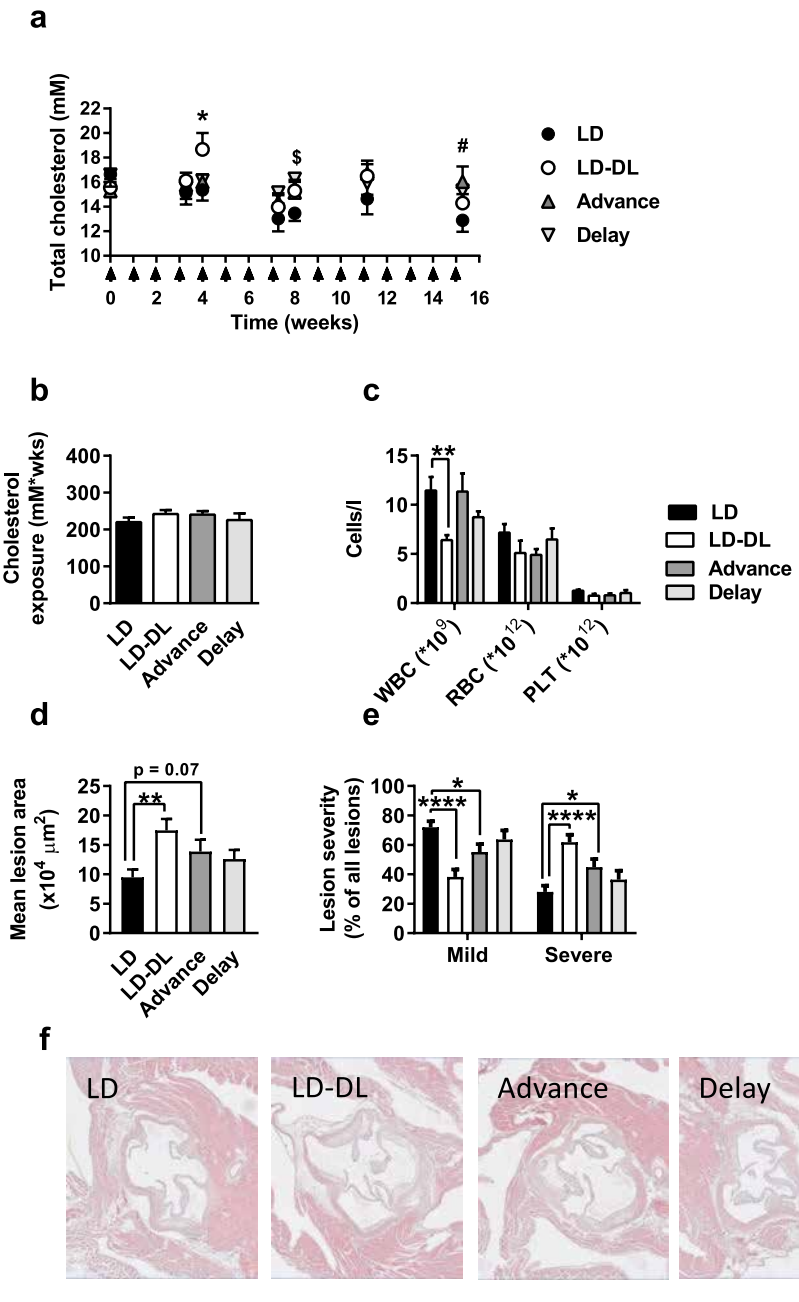


Figure 5. Weekly phase shift does not increase body weight or food intake. *APOE*3-Leiden.CETP mice fed WTD were exposed to LD, weekly 12 h reversal (LD-DL), weekly 6h advance or weekly 6h delay (n=15/group). Food intake (a) and body weight (b) were determined at regular intervals. After 14 weeks, subcutaneous gonadal white adipose tissue (gWAT) and interscapular brown adipose tissue (iBAT) were weighed (c). Data is presented as means ± SEM. * (LD-DL vs LD), $ (Delay vs LD); ANOVA, Fischer LSD post-hoc) = p<0.05, **,$$ = p<0.01. Data is presented as means ± SEM.*

Figure 6. (right page) Weekly phase shift increases atherosclerotic development independent of cholesterol levels. *APOE*3-Leiden.CETP mice fed WTD were exposed to LD, weekly 12 h reversal (LD-DL), weekly 6h advance or weekly 6h delay (n=15/group). Plasma total cholesterol (a) was determined at regular intervals. Total cholesterol exposure was calculated (b). After 11 weeks, whole blood was collected and pooled (n=3/group) for cell count using Sysmex (c). After 15 weeks, mice were sacrificed, hearts were isolated and atherosclerotic lesion size was determined at the aortic root (d) as well as lesion severity (e). Representative histological slides are shown for each group (f). Data is presented as means ± SEM. * (LD-DL vs. LD), $ (delay vs. LD), # advance vs. LD; ANOVA, Fischer LSD post-hoc) = p<0.05, ** = p<0.01. Data is presented as means ± SEM. WBC= white blood cells, RBC=red blood cells, PLT=platelets. Arrows indicate first day of light shift.*


a
Total cholesterol (mM)
Time (weeks)
LD
LD-DL
Advance
Delay
b
Cholesterol exposure (mM*wks)
c
Cells/l
WBC (*10^9)
RBC (*10^12)
PLT (*10^12)
d
Mean lesion area (x10^4 µm^2)
p = 0.07
e
Lesion severity (% of all lesions)
Mild
Severe
f
LD
LD-DL
Advance
Delay

rhythm strength associated to either TC exposure or atherosclerotic lesion size. We found a weak positive correlation between rhythm strength and TC exposure ($p=0.02$, $R^2=0.28$) and not with atherosclerotic lesion area (Fig. 3i-j). In summary, as compared to LD exposure, 14 weeks of LL exposure attenuated circadian rhythm without increasing atherosclerosis development in dyslipidemic mice, despite raised TC exposure.

Weekly light shifts do not affect total food intake nor body weight

Previously, we showed that 5 weeks of LL exposure severely decreases rhythm strength in mice [27]. In the present experiment, we observed that after 14 weeks of LL a large subset of mice retained a strong rhythmicity, albeit with increased period length (Fig. 1). We reasoned that chronic LL exposure may have led to adaptation of mice to the light schedule. Additionally, constant light does not model human shift work, as in shift work light-dark cycles are maintained but shifted repeatedly depending on the shift rotation. This induces a repeated stress on the biological clock system, as the internal clocks need to adapt to the environmental light condition with every shift. For these reasons, we conducted a second experiment in which mice were subjected to either a weekly changing schedule of 6 h advance (advance), 6 h delay (delay) or 12 h light-dark reversal (LD-DL), compared to regular 12h light-dark (LD) cycles. Typical physical activity patterns are shown in (Fig. 4).

Similar to LL exposure, weekly shifted schedules transiently affected food intake of the mice in the first 3 weeks of the intervention. Compared to LD (76.1 ± 0.7 g/mouse), cumulative food intake measured during the first three weeks was lower in LD-DL (66.1 ± 0.7 g/mouse; $p<0.0001$) and advance (71.1 ± 0.7 g/mouse; $p=0.0001$) groups and higher in the delay group (81.1 ± 0.7 g/mouse; $p=0.0001$) (Fig. 5a). However, at subsequent time points, these differences did not persist. Also, body weight (Fig. 5b) and gonadal white adipose tissue mass did not differ between the different light schedules (Fig. 5c). Interestingly, compared to LD, iBAT weight decreased in LD-DL (0.076 ± 0.004 *vs.* 0.090 ± 0.005 g; $p=0.015$) and tended to decrease in the advance group (0.080 ± 0.003 g; $p=0.084$) (Fig. 5c).

Weekly light shifts increase atherosclerotic development independent of cholesterol levels

TC plasma levels were determined every 3-4 weeks. We did not observe consistent differences between the different groups with respect to TC levels (Fig. 6a), which was reflected by similar TC exposure between groups (Fig. 6b). After 11 weeks of light shifts, white blood count in peripheral blood was measured. In line with the decrease in white blood cells due to LL exposure, we observed also a decrease in white blood cell count due to LD-DL exposure ($6.4 \pm 1.5*10^9$ *vs.* $11.5 \pm 1.4*10^9$ cells/l; $p=0.010$). However, advance or delay did not change immune cell counts in peripheral blood and platelet count was not affected (Fig. 6c).

After 15 weeks of light schedule intervention, we determined atherosclerosis development. Compared to LD mice ($9.5 \pm 1.3*10^4$ mm^2), the delay group did not show differences in atherosclerotic plaque size in the aortic root ($12.6 \pm 1.5*10^4$ mm^2; $p=0.207$).

In contrast, 6h advance light schedule tended to increase atherosclerotic plaque size (13.9 ± 1.9*10^4 mm^2; p=0.074). Strikingly, LD-DL increased plaque size by 1.8-fold compared to LD mice (17.5 ± 1.9*10^4 mm^2; p=0.002) (Fig. 6d, f). The increased plaque size in LD-DL group and 6h advance group was accompanied by a shift of plaque severity, as LD-DL and 6h advance group displayed a decrease in mild plaques and an increase of severe plaques compared to LD (Fig. 6e).

DISCUSSION

This is the first study to demonstrate that mistimed light exposure aggravates atherosclerosis development. Specifically, we show that a weekly reversal of LD cycle, and to a lesser extent 6 h phase advance, increases atherosclerotic lesion size and lesion severity without considerable changes in plasma cholesterol levels. We therefore conclude that pro-atherogenic effects of disturbed rhythms may be due to mistimed light exposure, but are dependent on the timing of the light shift.

Constant light exposure of APOE*3-Leiden.CETP mice induced lengthening of the period of circadian physical activity rhythms in all mice, while only in a subset of mice it weakened the rhythm strength. We previously observed that 5 weeks of constant light exposure of male wild type mice induced a decrease in amplitude of the output of the central biological clock in the hypothalamus, the suprachiasmatic nuclei (SCN) [27]. Also, constant light induced an analogous decrease in rhythm strength in physical activity rhythms [17, 27]. Gender differences between these studies may have contributed to a different effect of light regime on rhythm strength. The SCN is sensitive to sex hormones, and gonadectomy experiments have demonstrated that sex hormones change circadian rhythms [28].

In our study, we did not observe an effect on body weight and adiposity. This is contrast with previous reports showing that constant light exposure increases body weight [29] and adiposity [17]. Most likely, this is due to use of different mouse models and gender. In our study, we used female APOE*3-Leiden.CETP mice on a C57Bl/6J background. It is known that female C57Bl/6J mice are less prone to diet-induced weight gain and development of insulin resistance than male C57Bl/6J mice [30] [31].

We did show that constant light exposure increased plasma TC levels, due to an increase in non-HDL-cholesterol. This effect could be explained by decreased BAT activity, since we previously observed that BAT activation increases hepatic uptake of remnants and thereby decreases TC levels in APOE*3-Leiden.CETP mice [19]. Short term LL exposure indeed decreased BAT activity [17]. In this study, we explored whether BAT was affected by long term LL but found only a lower *Ucp1* gene expression to support this hypothesis. Further studies are needed to investigate whether chronic LL exposure also decreases FA uptake by BAT. The increased plasma TC may also be a result of increased cholesterol absorption in the intestine. *Clockmt*$^{/mt}$ mice, which have a genetic disturbance of the biological clock, display increased enteric uptake of cholesterol. Consequently, these mice have

increased plasma TC levels and, when crossbred with *Ldlr-/-* mice, also display enhanced atherosclerosis development [32, 33].

Weekly reversal of light exposure aggravated atherosclerosis development without detectable changes in plasma cholesterol levels. This is striking, as previous studies in APOE*3.Leiden-CETP mice consistently showed a strong correlation between TC exposure and atherosclerosis development [34, 35]. Possibly, activation of pro-inflammatory pathways may be more important in the atherogenic effects of LD-DL exposure. We observed a decrease in total white blood cell count, which traditionally is associated with anti-atherogenic phenotype [36]. Alternatively, it may indicate more clearance of white blood cells, *e.g.* into the arterial wall. Additionally, inflammatory cells may be more polarized towards a pro-inflammatory status. In line with this hypothesis, it was previously shown that macrophages derived from *Clockmt/mt* mice have increased expression of pro-inflammatory cytokines [33]. Also, selective hematopoietic overexpression of clock gene *Rev-erbα* in *Ldlr*$^{-/-}$ mice skewed macrophages towards an M1 phenotype, thereby aggravating atherosclerotic development [37]. Lastly, phase shift increased LPS-induced mortality in mice and heightened LPS response in macrophages [38]. Together, these data strongly suggest that disturbed biological clock function induces a pro-inflammatory status, which may underlie the pro-atherogenic effect of LD-DL. The various light conditions had different effects on atherosclerosis formation. A weekly 6 h advance induced more severe lesions and tended to increase lesion size, while delay did not. This is in line with a study showing that in aged mice, advance but not delay light schedule increases mortality [21]. Adaptation to phase advance is more disturbing for the biological clock function. Moreover, 12h shifts induced more atherosclerosis than a 6 h shift. Therefore, there seems to be a correlation between the extent of disturbance to the biological clock function and atherosclerosis development.

We conclude that severe disturbance of circadian rhythm by advance light schedules and alternating LD-DL schedules accelerates atherosclerosis formation independent of plasma cholesterol levels. In human shift work, it has been shown that rotating shift work has a higher risk for CVD than fixed schedules [39]. Also, the duration of shift work has been shown to correlate to increase risk for CVD [10, 40, 41]. Our study supports a dose-dependent relationship between disturbance to the biological clock through mistimed light exposure and atherosclerosis. Together, these studies imply that employers and governments may need to rethink work schedules to minimize health risks.

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