Shotgun lipidomics discovered diurnal regulation of lipid metabolism linked to insulin sensitivity in non-diabetic men

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List of Abbreviations

BMI	Body mass index
CE	Cholesterol ester
CER	Ceramide
CHO	Carbohydrates
CID	Clinical investigation day
Chol	Cholesterol
DAG	Diacylglycerol
EN%	Energy percent
FA	Fatty acid
FFA	Free fatty acid
HC/HF	Isocaloric carbohydrate-rich diet until 13:30 and fat-rich diet between 16:30 and 22:00
HF/HC	Isocaloric fat-rich diet until 13:30 and carbohydrate-rich diet between 16:30 and 22:00
iAUC	incremental area under the curve
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IR	Insulin resistance
Kcal	Kilo calories
LPC (O-)	Lysophosphatidylcholine (-ether)
LPE	Lysophosphatidylethanolamines
MDA	Malondialdehyde
MS	Mass spectrometry
MSMS	Tandem mass spectrometry
MTT-HC	Carbohydrate-rich meal tolerance test
MTT-HF	Fat-rich meal tolerance test
NGT	Normal glucose tolerance

PC (O-)	Phosphatidylcholine (-ether)
PE (O-)	Phosphatidylethanolamine (-ether)
PI	Phosphatidylinositol
SFA	Saturated fatty acids
SM	Sphingomyelin
TAG	Triacylglycerol

Abstract

Context: Meal timing affects metabolic homeostasis and body weight, but how composition and timing of meals affect plasma lipidomics in humans is not well studied.

Objective: We used high throughput shotgun plasma lipidomics to investigate effects of timing of carbohydrate and fat intake on lipid metabolism and its relation to glycaemic control.

Design: 29 non-diabetic men consumed (i) a high-carb test meal (MTT-HC) at 09:00 and a high-fat meal (MTT-HF) at 15:40; or (ii) MTT-HF at 09:00 and MTT-HC at 15:40. Blood was sampled before and 180 min after completion of each MTT. Subcutaneous adipose tissue (SAT) was collected after overnight fast and both MTTs. Prior to each investigation day, participants consumed a 4-week isocaloric diet of the same composition: (1) high-carb meals until 13:30 and high-fat meals between 16:30 and 22:00 or (2) the inverse order.

Results: 12h-daily lipid patterns showed a complex regulation by both the time of day (67.8%) and meal composition (55.4%). A third of lipids showed a diurnal variation in postprandial responses to the same meal with mostly higher responses in the morning than in the afternoon. Triacylglycerols containing shorter and more saturated fatty acids were enriched in the morning. SAT transcripts involved in fatty acid synthesis and desaturation showed no diurnal variation. Diurnal changes of seven lipid classes were negatively associated with insulin sensitivity, but not with glucose and insulin response or insulin secretion.

Conclusions: This study identified postprandial plasma lipid profiles as being strongly affected by meal timing and associated with insulin sensitivity.

Précis

We studied effects of timing of carbohydrate and fat intake in a cross-over trial using plasma lipidomics. Postprandial lipid responses showed diurnal variation associated with insulin sensitivity.

Introduction

A dysfunctional lipid metabolism it is a hallmark for insulin resistance and a risk factor for many metabolic diseases. Circadian clocks - self-sustained ~24 h rhythms in behaviour, physiology and metabolism - play an important role in lipid homeostasis (1-3). In particular, 24 h rhythms in lipid metabolism are suggested to optimize energy storage and utilization (1-3). In constant conditions, a large part of the lipidome shows circadian rhythmicity in human plasma (4) and skeletal muscle (5).

Circadian misalignment and eating at the usual rest time (e.g. during shift work) lead to dysregulation of circadian rhythmicity and is associated with obesity, metabolic syndrome and dyslipidemia (2,6). A range of experimental studies confirm that timing of food intake plays an important role for metabolic homeostasis and body weight regulation (7,8) and affects the diurnal regulation of lipids. In simulated shift work, consumption of meals during the biological night induces higher postprandial triacylglycerides (TAG) levels in comparison with their consumption at daytime (9). Similarly, mice fed a high-fat diet during the inactive phase gain weight faster compared with mice fed during the active phase (10). In contrast, restricting a high fat diet to the active phase is protective against obesity and glucose intolerance (11,12) and decreases hepatic TAG levels (13).

In addition, the time at which the main meal is consumed influences the risk of obesity and the success of weight loss therapy. In 2013, a weight loss trial based on a Mediterranean diet conducted in an obese Spanish population showed that food timing was a predictive factor of weight loss success: late lunch eaters, who ate their lunch after 15.00, lost less weight on a hypocaloric diet than early eaters, who ate their lunch before 15.00 (14). A similar weight loss study with a 12-week follow-up showed that overweight and obese individuals consuming higher energy for dinner, compared to breakfast, lost less weight and had higher overall daily glucose, insulin, ghrelin, and hunger scores (15). These studies in people with overweight and obesity indicate that loading calories at the beginning of the day may be beneficial for weight management and metabolism (16). A range of studies in normal weight individuals have also suggested that late and delayed eating is associated reduced energy expenditure /

substrate oxidation and a general deterioration in metabolic function, whilst showing no clear results regarding weight gain (16). In addition, epidemiological studies propose a beneficial effect of a carbohydrate-rich diet at the beginning of the day, which was shown to be protective against the development of diabetes and metabolic syndrome (17,18). We recently showed that a diet in which fat is mainly eaten in the morning and carbohydrates mainly in the evening (compared with the reverse order) worsens glycaemic control in people with prediabetes (19) and alters substrate oxidation and adipokine secretion (20). Few studies have explored the effect of meal timing on triglyceride and cholesterol levels in plasma (21). Developing lipidomic techniques provides a powerful and comprehensive tool for the detailed assessment of lipid metabolism and its circadian regulation. In the present study, we used a high throughput shotgun lipidomic analysis (22) to investigate the effects of timing of carbohydrate and fat intake on lipid metabolism (daily patterns of plasma lipids and gene expression in subcutaneous adipose tissue) and its relation to glycaemic control (glucose and insulin response and insulin secretion and sensitivity).

Materials and Methods

Study design and sample collection

29 non-obese men without diabetes and without shift work completed a randomized controlled, cross-over trial. Women were excluded from participating due to the interplay between circadian rhythms and the menstrual cycle (23). Details of the study design, the recruitment of participants, inclusion and exclusion criteria, clinical characteristics of study subjects and dietary interventions were published recently (19,20). The study protocol and informed consent document were approved by the Medical Ethics Committee of Charité University Medicine, Berlin, Germany (EA2/074/12), and were in accordance with the Helsinki Declaration of 1975. All subjects gave written informed consent. The study was registered at clinicaltrials.gov as NCT02487576.

Two 4-week isocaloric dietary interventions were applied in the cross-over trial: (1) a high-carb diet (breakfast and lunch) until 13:30 and a high-fat diet (snack and dinner) between 16:30 and 22:00 (HC/HF) versus (2) a high-fat diet until 13:30 and a high-carb diet between 16:30 and 22.00 (HF/HC), separated by a 4-week washout phase (**Figure 1A**). The high-carb diet consisted of 65 energy percent (EN%) carbohydrates (CHO), 20 EN% fat and 15 EN% protein; the high-fat diet was composed of 35 EN% CHO, 50 EN% fat and 15 EN% protein. The calories were evenly distributed between the morning (until 13:30) and evening (16:30 to 2:00) block; as a result, the daily macronutrient composition was 50 EN% CHO, 35 EN% fat (14 EN% saturated fatty acids) and 15 EN% protein in both diets.

Before and after each intervention period, participants reported to the outpatient study center at the German Institute of Human Nutrition (Potsdam, Germany) for the anthropometrical and metabolic examination. Body fat mass and fat-free mass were measured via BOD POD-Air displacement plethysmograph (CosMed, Fridolfing, Germany). Munich Chronotype Questionnaire (MCTQ) was used to determine the participants' chronotypes and sleeping habits.

After each intervention period (V2 and V4, **Figure 1A**), two meal tolerance tests (MTT) were performed, at 09:00 and 15:40, as per previous intervention (**Figure 1B**). Test meals were

either high in carbohydrates (MTT-HC), 835 kcal (64.8 EN% CHO, 14.8 EN% protein, 20.3 EN% fat, SFA 59.5% of total fat), or high in fat (MTT-HF), 849 kcal (35.3 EN% CHO, 15.1 EN% protein, 49.6 EN% fat, SFA 63.3% of total fat) (**Table S1,** (24)). Glycaemic indices of meals (GI), which was calculated as described previously (25), were similar between both test meals. Participants ingested the test meals within 15 min. For lipidomics analysis, blood samples were drawn at 8.35, 12.15, 15.35 and 18.55, i.e. before and 180 min after completion of each test meal, using EDTA monovettes (Sarstedt, Germany). For determination of glycemic measures (glucose and insulin), blood samples were taken before and 30, 60, 90, 120 and 180 minutes after completion of each meal. Subcutaneous adipose tissue (SAT) samples were collected periumbilically (in the region of the belly button) by needle aspiration at three time points during the investigation day (at 8.40, 12.20 and 19.00). With the aim to contain the inflammation biopsy procedures may cause locally, areas for biopsy were carefully selected to allow maximal distance between them: this usually meant the area left (‘9 o’clock’) of the belly button was selected for the first biopsy, the opposite area (‘3 o’clock’) for the second and the area in between (’12 o’clock’) for the third. For all biopsies, a skin area of approximately 2x2 cm was anesthetized with lidocaine. Next, a fine-needle (2.1 mm), connected to a vacuum syringe with sterile NaCl solution, was inserted in a 3 mm cut in the skin. The vacuum enabled the suction of small tissue pieces (approx. 2 g), which were immediately washed with NaCl solution and finally flash-frozen in liquid nitrogen and stored at -80°C until analysis.

Biochemical analyses of plasma samples

Routine laboratory markers were measured using standard methods (ABX Pentra 400; HORIBA, ABX SAS, France). Commercial ELISA was used for measurement of insulin (Mercodia, Sweden) in serum. Plasma levels of 19 free fatty acids (FFA) were determined by gas chromatography as described (26). Malondialdehyde (MDA) was measured as a marker of lipid peroxidation in plasma samples after derivatization with thiobarbituric acid (TBA) by reverse-phase HPLC coupled with fluorescence detection as described (27) (28).

Lipidomics analysis of plasma samples

Lipid extraction of plasma samples was performed at Lipotype GmbH using high throughput Shotgun Lipidomics (Dresden, Germany) technology as described (22). Briefly, samples were diluted 1:50 and an equivalent of 1 µl of undiluted sample was used for the extraction with methyl *tert*-butyl ether and methanol with Hamilton Robotisc STARlet liquid handling station. Shotgun mass spectrometry (MS) analysis was conducted on a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled to a TriVersa NanoMate robotic nanoflow ion source (Advion BioSciences, Ithaca, NY). Lipids were identified and quantified using the proprietary LipotypeXplorer software. Lipid intensities were normalized to lipid class–specific internal standards and data reported as molar amounts. Analytical quality was assessed by the inclusion of reference and blank samples. Data were corrected for batch effects and drift based on reference samples. Median coefficient of variation across all lipid molecules was 11%. Lipid species present in <70% of all samples were excluded.

Lipid species are annotated according to their molecular composition as *sum of the carbon atoms in the hydrocarbon moiety*: *sum of the double bonds*; *sum of hydroxyl groups*. For example, PI 34:1;0 denotes phosphatidylinositol with a total length of its fatty acids equal to 34 carbon atoms, total number of double bonds in its fatty acids equal to 1 and 0 hydroxylations.

Lipid subspecies annotation contains additional information on the exact identity of their acyl moieties and their *sn*-position (if available). For example PI 18:1;0_16:0;0 denotes phosphatidylinositol octadecenoic (18:1;0) and hexadecanoic (16:0;0) fatty acids, for which the exact position (*sn*-1 or *sn*-2) in relation to the glycerol backbone cannot be discriminated (underline "_" separating the acyl chains). On contrary, PC O-18:1;0/16:0;0 denotes an ether-phosphatidylcholine, where an alkyl chain with 18 carbon atoms and 1 double bond (O-18:1;0) is ether-bound to *sn*-1 position of the glycerol and a hexadecanoic acid (16:0;0) is connect via an ester bond to the *sn*-2 position of the glycerol (slash "/" separating the chains

signifies that the *sn*-position on the glycerol can be resolved). Lipid identifiers of the SwissLipids database (29) (http://www.swisslipids.org) are provided (30).

Gene expression analysis of adipose tissue

Total RNA was purified from SAT samples using the miRNeasy Lipid Tissue Mini Kit (Qiagen, Germany). RNA concentration was measured using an ND-1000 spectrophotometer (Nanodrop, PeqLab). Single-stranded cDNA was synthesized with miScript II RT Kit (Qiagen, Germany). QPCR was performed by ViiA 7 sequence detection system using Power SYBR Green PCR Master Mix (Applied Biosystems, USA) and specific primers. Gene expression was assessed by the standard curve method and normalized to the reference gene beta-glucuronidase (GUSB). Primer sequences are stated in repository (31).

Statistical analysis

Statistical analyses were performed with SPSS v.20 (SPSS, Chicago, IL) and R (version 3.4.2). Repeated-measures ANOVA (RM-ANOVA) was used to assess the effects of the diets, time of the day, and the diet × time interaction using the aov() in R.

To compare whether lipids show similar or different daily profiles upon both diets, a correlation approach was established. In detail, average concentrations for each lipid and diet over all subjects were calculated and correlation analysis between the diets performed. To avoid a bias caused by producing flatter curves, we established a variable (called *diff*) that is indicative of the magnitude of coherence within the cohort and calculated as follows:

$$diff = (max - min)_{HC/HF} + (max - min)_{HF/HC}$$

Diff indicates how much a curve is the sum of similar curves. If diff-level is low, individual samples can be very different. Only lipid species with high coherence within the cohort (diff>1.5) were included in the correlation analysis.

To assess postprandial responses to test meals, the ratio of postprandial to preprandial concentrations was calculated. For the analysis of a diurnal variation, postprandial responses

to the same meal (MTT-HC or MTT-HF) in the afternoon and in the morning were compared with a paired Mann–Whitney U test and RM-ANOVA as indicated.

For glucose and insulin levels, incremental AUC (iAUC) were determined by trapezoidal method after subtraction of the baseline area. Early and overall indices of insulin secretion were calculated as the ratio of iAUC for insulin to iAUC for glucose ($iAUC_{ins/glu\ 0\text{-}30}$ and $iAUC_{ins/glu\ 0\text{-}180,}$ respectively). Insulin sensitivity in MTT was determined by the Gutt index (ISI $Gutt_{0\text{-}120}$) (32). HOMA-IR was calculated according to following equation. HOMA-IR [mmol*mU*L²] = glucose [mmol/L] x insulin [mU/L] /22.5, using fasting values. To analyse associations between lipid classes and parameters of glucose metabolism, univariable linear regression models with adjustment for age and BMI were used. For this analysis, diurnal variation of postprandial response was defined as Δ = afternoon value – morning value.

P values < 0.05 were considered significant in all analyses. For the multiple testing correction, the Benjamini-Hochberg (BH) method was used. All data are presented as means ± SEMs.

Results

Study population and adherence to dietary interventions

29 non-obese men (age 45.9 ± 2.5 years, BMI 27.1 ± 0.8 kg/m^2, 18 subjects with normal glucose tolerance and 11 subjects with impaired fasting glucose/glucose tolerance) participated in the randomized controlled, cross-over trial (**Table 1**). The participants' chronotype distribution and habitual sleeping habits on free and work days are shown in **Figure S1** (33). Adherence to dietary plans was good, with similar compliances for both diets (19). There was no difference in energy intake, macronutrient composition, amount of saturated fatty acids, fiber and starch as well as GI between the two diets (19). Body weight was nearly stable with no differences between the two diets (19).

Detected lipids

Plasma lipid profiles were measured in the entire cohort (i.e. in 29 men). Lipidomics analysis of plasma samples yielded on average about 10200 pmol of lipids per µl of sample. For each diet, the highest lipid concentration was detected in the postprandial samples taken 180 min after completion of the MTT-HF (**Figure S2,** (33)).

A total of 672 lipid species belonging to 14 lipid classes (cholesterol (Chol), cholesterol esters (CE), TAG, diacylglycerols (DAG), phosphatidylcholines (PC), phosphatidylcholine ethers (PC O-), phosphatidylethanolamines (PE), phosphatidylethanolamine ethers (PE O-), phosphatidylinositols (PI), lysophosphatidylcholines (LPC), lysophosphatidylcholine ethers (LPC O-), lysophosphatidylethanolamines (LPE), sphingomyelins (SM) and ceramides (CER)) were identified and quantified. Lipid species present in <70% of all samples were excluded, leaving 233 lipid species for further analysis (30). The procedure covered 98% of the total lipid amount. We also tested different cut-offs for data inclusion (50% and 90%) and found that this did not alter our conclusions.

Additionally, plasma concentrations of 19 free fatty acids (FFA) were determined in a subcohort of 10 subjects (age 44.2 ± 4.3 years, BMI 26.0 ± 1.0 kg/m^2, all with normal glucose tolerance)

(**Figure S3A, (33)**).

Daily plasma lipid profiles in response to the diets

We first investigated how the two diets affected daily profiles of circulating lipids in the course of the investigation day. Using a correlation approach, we found that LPC and PC O- lipid classes showed similar daily profiles for both diets (**Figure 2A,B**), whereas DAG, Cer, and PE O- lipid classes demonstrated highly different daily profiles between the two diets (**Figure 2C**). In a repeated measure ANOVA analysis, 158 (67.8 %) lipid species showed a time effect, 3 (1.3 %) showed a diet effect, and 129 (55.4 %) showed a time*diet interactions (all data corrected for multiple testing), suggesting that a large part of the plasma lipidome shows a complex regulation by both the MTT composition and time of day (**Table S2, (24)**).

Comparison of fasting concentrations showed no difference between the diets for any lipid species after correction for multiple testing (data not shown). Analysis of postprandial concentrations showed higher levels for 11 out of the 14 lipid classes after the MTT-HF in comparison with MTT-HC after the morning test meal (at 12.15 hr) and higher levels for only four lipid classes (i.e. LPE, TAG, DAG and PE) after the afternoon test meal (at 18.55 hr) (**Table S3, (24)).** Similarly, postprandial levels for 14 plasma FFA were higher after the MTT-HF in comparison with the MTT-HC after the morning meal, while none showed different concentrations after the afternoon meal (**Figure S3A, (33)**).

Daily MDA profiles, which we used as a marker of lipid peroxidation, were similar for both diets and showed slightly decreased levels in the afternoon but no marked postprandial changes (**Figure S3B, (33)**).

Based on lipid classes and FFA data, we hypothesized that plasma lipids showed a pronounced diurnal variation in their response to meal intake.

Diurnal variation of plasma lipids in response to MTT-HC

To test this hypothesis, we compared postprandial responses to the same meal in the morning and in the afternoon. Postprandial responses were calculated as ratios of

postprandial to preprandial concentrations. For MTT-HC, 79 lipid species showed different postprandial responses in the morning vs. afternoon in the analysis by Mann–Whitney U test, and for 68 lipids these differences remained significant after correction for multiple testing (**Figure 3A**, **Table S4, (24)**), i.e. 29.2 % of all lipid species included in the analysis. Analysis of lipid classes revealed that postprandial LPE, PE, TAG and DAG responses were more pronounced in the morning than in the afternoon (**Figure 3B**), and TAG demonstrated maximal amplitude of diurnal changes. Removal of the outlier in DAG does not change statistical significance (with outlier: $q = 1.93 \times 10^{-05}$; without outlier: $q = 2.65 \times 10^{-05}$). Additional analysis of TAG chain length revealed that postprandial responses of TAGs containing shorter fatty acids (42-49 carbon atoms) were markedly higher in the morning than in the afternoon (**Figure 3C**). In agreement with this, postprandial levels of TAG-bound short chain FAs were increased after the morning meal but did not change after the afternoon meal (**Figure S4, (33)**). No diurnal variation was found in postprandial responses of plasma FFAs after correction for multiple testing (data not shown). Analysis of TAG saturation (i.e. number of double bonds (DB)) revealed that TAGs containing more saturated (no or low number of DBs) fatty acids showed higher postprandial responses in the morning (**Figure 3D**). Very similar results were obtained if RM-ANOVA was used for the analysis (**Figure S5, (33)**). These data indicate that the lipid metabolism differently responses to the MTT-HC in the morning and in the afternoon.

Diurnal variation of plasma lipids in response to MTT-HF

We further investigated if postprandial responses to MTT-HF also exhibited a diurnal variation. For MTT-HF, 100 lipid species showed different postprandial responses in the morning vs. afternoon, and for 71 lipids these differences remained significant after correction for multiple testing (**Figure 4A, Table S5, (24)**), i.e. 30.5 % of all analysed lipid species. Similar to MTT-HC, MTT-HF led to more pronounced responses of LPE, Chol, TAG, PE and DAG in the morning in relation to the afternoon, although the diurnal pattern showed a high variability across subjects (**Figure 4B**). Removal of the two outliers in DAG does not

change statistical significance (with outliers: q =0.011; without outliers: q = 0.029). Again, TAGs containing shorter fatty acids (42-49 carbon atoms) showed higher postprandial responses in the morning (**Figure 4C**). Postprandial levels of TAG-bound short chain FAs were increased both after the morning meal and after the afternoon meal, and this increase was more pronounced in the morning (**Figure S4, (33)**). Postprandial responses of plasma FFAs showed no diurnal variation (data not shown). Similar to MTT-HC, TAGs with no or one DB in fatty acids showed higher postprandial responses to MTT-HF in the morning (**Figure 4D**). Again, similar results were obtained by using RM-ANOVA (**Figure S6, (33)**). In line with the results for MTT-HC, these data indicate that the response to MTT-HF depends on the time of day.

Diurnal patterns of transcript levels of key enzymes in fatty acid metabolism

The diurnal variation in the chain length and DB number of TAG-bound fatty acids may be explained by circadian rhythms in enzymes, which are involved in fatty acid synthesis and desaturation and which were previously described in human muscle and in mice (5,34). We therefore assessed diurnal patterns of gene expression in SAT samples using quantitative PCR. Core clock genes showed a strong diurnal variation in SAT, with *ARNTL* (*BMAL1*) expression being higher and *PER1* and *NR1D1* levels being lower in the afternoon (**Figure 5A**), which is in line with our previous observations (35). Transcripts involved in fatty acid synthesis (fatty acid synthase (FASN), acetyl-CoA carboxylase (ACACA), long chain fatty acyl-CoA synthetase 1 (ACSL1)), elongation (ELOVL5) and desaturation (stearoyl-CoA desaturase (SCD), fatty acid desaturases (FADS1, FADS2)) generally showed increased levels after the MTT-HC in comparison with MTT-HF (**Figure 5B**). However, their postprandial expression did not differ depending on whether the same meal was consumed in the morning or in the afternoon. Hence, diurnal variation in the chain length and DB number of the TAG-bound fatty acids is unlikely to be explained, at least on the mRNA expression level, by circadian rhythms in these enzymes.

Associations between diurnal variation of plasma lipids and parameters of glucose metabolism

A range of studies demonstrated an association of specific plasma lipids with diabetes risk and parameters of the glucose metabolism (36-39), and insulin is a main regulators of various aspects of the lipid metabolism, including synthesis of fatty acids in the liver and triglyceride storage in adipose tissue (40). We therefore investigated if diurnal changes of postprandial lipid response are associated with diurnal variation of glucose metabolism, i.e. of glucose (iAUCglu $_{0\text{-}180}$) and insulin (iAUCins $_{0\text{-}180}$) response, of early and overall insulin secretion indices ($iAUC_{ins/glu\ 0\text{-}30}$ and $iAUC_{ins/glu\ 0\text{-}180,}$ respectively) and of Gutt's insulin sensitivity index (ISI $Gutt_{0\text{-}120}$).

For both MTT-HC and MTT-HF, postprandial glucose and insulin response were markedly higher in the afternoon, and insulin secretion and sensitivity decreased as the day progressed (**Figure 6A**). Diurnal variation of postprandial response was defined as Δ = afternoon value – morning value. We observed a negative association between the diurnal change of Gutt's insulin sensitivity index and 7 out of the 14 lipid classes (i.e. PC, PI, LPC, PC O-, SM, CE, DAG) in the context of MTT-HF (**Figure 6B**). No correlations with other parameters of glucose metabolism for MTT-HF or for MTT-HC were found.

Discussion

In this study, we used a high throughput shotgun lipidomic analysis to investigate how the intake of carbohydrate and fat at different times of the day affect plasma lipid patterns in humans. Our study provided a unique possibility to investigate dynamic changes of the lipid profile throughout the day and its response to meals of different composition.

Our study revealed four major findings. Firstly, daily patterns of circulating lipids show a complex regulation by both the meal composition and the time of day. Notably, most lipids showed higher postprandial concentrations after the MTT-HF in comparison with MTT-HC, but the differences between the meals were less pronounced in the afternoon. Our data characterised the human plasma lipidome as a highly flexible system which rapidly responses to food intake. Similarly, recently published studies showed that the postprandial plasma lipidome is influenced by the composition of the consumed meal, e.g. by the source of dietary fat (41), and other factors such as physical activity and overall fitness (42).

Secondly and this is the main finding of our study, a third of all lipid species showed a marked diurnal variation in their postprandial responses for both high-carb and high-fat meals, which to our knowledge has not been described before. Indeed, for both meal compositions, postprandial TAG, PE, LPE and DAG responses were more pronounced in the morning than in the afternoon. For MTT-HF, the meal-induced change of Chol was also larger in the morning. Circadian regulation of different categories of lipids, including fatty acids, glycerolipids, glycerophospholipids, sphingolipids and sterol lipids was previously described in constant routine experiments (by sustained wakefulness in bed and hourly equicaloric snacks) (2). Targeted lipidomic approach showed that 13 % of lipid species analysed were rhythmic in constant routine but rhythms showed marked variation across subjects (4). Circadian rhythmicity was also shown for 17 % of all lipids in the mouse liver (13) and in its intracellular organelles (nucleus and mitochondria) (43) as well as in human skeletal muscle (5). In constant routine, TAG and DAG compose the majority of the oscillating lipids which increase in humans during the night and show the highest level near the wake time (4,13). In contrast, some PCs oscillate in antiphase with highest levels in the afternoon and evening

(2,4). The lipid peroxidation marker MDA also shows diurnal variation (44) which is in line with our observations.

In contrast, our study showed, for the first time, diurnal variation in postprandial responses of plasma lipids. Multiple circadian processes could contribute to this phenomenon. Plasma lipids assessed in our study could represent products of both the dietary fat catabolism and lipid synthesis in different tissues which underwent circadian regulation. Lipid hydrolysis, absorption, secretion from enterocytes and transport from the gut, lipid biosynthesis in the intestine and in the liver, lipolysis and fatty acid oxidation are all under the circadian control. In particular, diurnal regulation of postprandial lipoprotein levels (e.g. shown for low density lipoprotein) (20) could be involved in diurnal variation in lipid postprandial responses.

On the molecular level, circadian control of lipid metabolism is performed by core clock genes which induce circadian rhythms of transcription factors and key metabolic enzymes (2,45). Remarkably, clock-driven transcription factors such as RORs, PPARs, PGC-1α, REV-ERBs and SREBP also function as lipid sensors providing a molecular link between the circadian clock and lipid metabolism (1-3). Moreover, meal intake and composition can also directly affect rhythms of clock genes (35,46) via metabolites and meal-induced hormones(47). Future studies combining lipidomic analysis and gene expression arrays are needed to understand the molecular mechanisms underlying diurnal variation of the postprandial lipidome in humans.

Thirdly, the diurnal variation of TAG chain length and saturation, which we observed, is a novel finding. We revealed that TAGs containing shorter and more saturated fatty acids showed higher postprandial changes in the morning for both meal compositions. We hypothesized that these changes could, at least in part, be explained by circadian rhythms of enzymes involved in fatty acid synthesis, elongation and desaturation driven by the molecular clock machinery. Circadian regulation of ELOVL and ACSL transcripts were previously shown in human skeletal muscle (5). As expected, in SAT samples, we found strong diurnal variation of core clock genes. However, postprandial expression of fatty acid

metabolism genes showed no difference when the same meal was consumed in the morning or in the afternoon. Therefore, mechanisms of diurnal variation of TAG chain length and saturation in plasma require further investigation. For example, little is known about the circadian rhythms of lipid metabolism enzymes in human liver, which strongly contribute to the regulation of circulating lipids.

Fourthly, we provided novel evidence that diurnal variation of plasma lipids and glucose metabolism are associated. Similar to lipid metabolism, glucose metabolism in humans underwent circadian control (48). Our study showed that, in response to the same meal, postprandial glucose levels in the afternoon were markedly higher that in the morning (despite of the simultaneous increase of the insulin levels) suggesting a decrease in glucose tolerance as the day progresses. Similarly, indices of insulin secretion and insulin sensitivity decreased in the afternoon which is in line with previous observations (19,49,50).

Importantly, we found that diurnal variation of insulin sensitivity is associated with seven lipid classes (PC, PI, LPC, PC O-, SM, CE, DAG) in context of MTT-HF, which allows to speculate about underlying mechanisms. On one side, diurnal variation of plasma lipids might contribute to the diurnal variation of insulin sensitivity on the whole-body and cellular level (51). Ectopic accumulation of specific lipid metabolites can disrupt the normal functioning of cellular cascades and has been suggested as one hypothesis, often referred to as the lipotoxicity theory, to explain molecular mechanisms by which obesity leads to insulin resistance (IR) (52). Among these lipid metabolites are ceramides. There is good evidence linking intracellular ceramide accumulation to IR, particularly in skeletal muscle (53), and some groups have implicated plasma ceramides in the pathogenesis of IR (54,55). In addition, skeletal muscle DAG content has long been suggested to induce insulin resistance (56), but the inconclusive results from both observational and mechanistic studies have recently raised the question of how much prominence should DAGs receive in the aetiology of insulin resistance (52). Unlike ceramides, the PC class seems to be positively associated with type 2 diabetes and prediabetes (38) and, in line with our findings, might also contribute to the regulation of insulin sensitivity.

On the other side, diurnal variation of insulin action (resulting from variation of insulin secretion and insulin sensitivity) could affect various processes of lipid metabolism (lipogenesis, lipolysis, fatty acid oxidation etc.) and this way contribute to the diurnal variation of plasma lipids. Both hypotheses are presented in **Figure 6C**. The question about exact mechanisms of interaction between circadian clock, lipid metabolism and insulin sensitivity remains to be answered and requires further investigation.

Interestingly, in our study, we found no difference in fasting levels of plasma lipids between HC/HF and HF/HC diets. This finding is in agreement with our previously published data showing that the different diurnal distribution of carbohydrate and fat intake does not affect fasting triglyceride, total, HDL and LDL cholesterol as well as total FFA levels (19,20). The diets used in our study were isocaloric, and although timing of carbohydrate and fat consumption was different, the overall food composition was the same in both diets (19), which could explain the absence of dietary effects on fasting lipid levels. Nevertheless, in our study, both meal composition and meal timing strongly affected postprandial lipid level which was recently established as an important risk factor for the obesity-associated diseases such as cardiovascular disease (CVD)(57). Moreover, nonfasting lipid measurements are included in clinical guidelines in some countries for a more functional assessment of postprandial lipemia and CVD risk (58,59). This confirms the important role of meal timing in the regulation of blood lipids.

In conclusion, using a lipidomic approach, our study revealed effects of meal timing across the day on the lipid metabolism in non-diabetic humans and elucidated its association with diurnal regulation of glucose metabolism. Our results contribute to a better understanding of the interaction between diet, meal timing and the circadian clock in the regulation of lipid metabolism.

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Author contributions

KK, AK, AFHP, and OPR designed the research; KK, SH, KJP, CK, MK and DW conducted the research; KK, MJG, MD and OPR analysed data or performed statistical analysis; KK, MJG, NR, TG, KS, AK, AFHP, and OPR wrote the paper.

Data Availability

Data described in the manuscript will later be made publicly and freely available without restriction at following DOI: 10.6084/m9.figshare.9988328.

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Figure legends

Figure 1. Study design and clinical investigation day.

(A) Study design. HC/HF diet, isocaloric high-carb meals until 13.30 hr and isocaloric high-fat meals between 16.30 and 22.00 hr; HF/HC diet, reverse order of meal sequence; V, visit.

(B) Clinical investigation day. At 09.00 hr and 15.40 hr a standardized test meal – high-fat or high-carb – was provided according to participant´s previous intervention. Arrows indicate the collection of blood samples for lipidomic analysis (black) and SAT samples (grey, dashed).

Figure 2. Comparison of daily lipid profiles upon HC/HF *versus* HF/HC diet.

(A) Correlation of daily lipid profiles across lipid classes. Lipid class enrichment is shown as histogram with overlayed density plot. The distance of the peak from the dotted grey line (correlation coefficient r=0.0) indicate how similar (r>0.0) or different (r<0.0) the daily lipid profiles in each lipid class are between the two diets. Only lipid species with high coherence within the cohort (diff>1.5) were included in the analysis as described in *Methods*. **(B)** Top 10 lipid species with the highest correlation coefficients of daily profiles between HC/HF (black line) and HF/HC (grey line) diets. **(C)** Top 10 lipid species with the lowest correlation coefficients of daily profiles. Only lipid species with high coherence within the cohort (diff>1.5) were included in the analysis. (B-C) Mean z-scores with SEM are displayed.

Figure 3. Lipids with a different postprandial response in the morning *versus* afternoon for MTT-HC.

(A) Diurnal differences of lipid species. Postprandial responses of lipid species were calculated as ratios of their postprandial to preprandial concentrations. The differences between morning and afternoon values were compared with a paired Mann–Whitney U test. In the volcano plot, P-values without correction are shown on the y-axis, fold changes of means on the x-axis (morning towards the left, afternoon towards the right). Points with additional outlines show lipids significant after BH correction for multiple testing.

(B) Diurnal differences of lipid classes. Postprandial responses of lipid classes were calculated as ratios of postprandial class sums of individual species to their preprandial counterparts. The differences between morning and afternoon values were compared with a paired Mann–Whitney U test. Only differences significant after adjustment for multiple testing ($q < 0.05$) are shown. Values for individual subjects are displayed as points and connected by lines, the overall shape of the distribution is shown as a boxplot.

(C) Diurnal differences of TAG postprandial responses dependent on total TAG chain length (total number of carbon atoms in a TAG molecule). Postprandial responses of each chain length were calculated as ratios of postprandial total chain length sums of individual species to their preprandial counterparts.

(D) Diurnal differences of TAG postprandial responses dependent on total TAG saturation (total number of double bonds in a TAG molecule). Postprandial responses of each chain saturation were calculated as ratios of postprandial total saturation sums of individual species to their preprandial counterparts.

(C-D) Error bars show standard error of the mean (n=29). (B-D) Significances are encoded as follows: * for $q < 0.05$, ** for $q < 0.01$, *** for $q < 0.01$,**** for $q < 0.0001$.

Figure 4. Lipids with a different postprandial response in the morning *versus* afternoon for MTT-HF.

(A) Diurnal differences of lipid species. Postprandial responses of lipid species were calculated as ratios of their postprandial to preprandial concentrations. The differences between morning and afternoon values were compared with a paired Mann–Whitney U test. In the volcano plot, P-values without correction are shown on the y-axis, fold changes of means on the x-axis (morning towards the left, afternoon towards the right). Points with additional outlines show lipids significant after BH correction for multiple testing.

(B) Diurnal differences of lipid classes. Postprandial responses of lipid classes were calculated as ratios of postprandial class sums of individual species to their preprandial counterparts. The differences between morning and afternoon values were compared with a

paired Mann–Whitney U test. Only differences significant after adjustment for multiple testing ($q < 0.05$) are shown. Values for individual subjects are displayed as points and connected by lines, the overall shape of the distribution is shown as a boxplot.

(C) Diurnal differences of TAG postprandial responses dependent on total TAG chain length (total number of carbon atoms in a TAG molecule). Postprandial responses of each chain length were calculated as ratios of postprandial total chain length sums of individual species to their preprandial counterparts.

(D) Diurnal differences of TAG postprandial responses dependent on total TAG saturation (total number of double bonds in a TAG molecule). Postprandial responses of each chain saturation were calculated as ratios of postprandial total saturation sums of individual species to their preprandial counterparts.

(C-D) Error bars show standard error of the mean (n=29). (B-D) Significances are encoded as follows: * for $q < 0.05$, ** for $q < 0.01$, *** for $q < 0.01$,**** for $q < 0.0001$.

Figure 5. Gene expression levels in SAT upon HC/HF vs. HF/HC diet.

(A) Expression of core clock genes *ARNTL, PER1* and *NR1D1.*

(B) Expression of genes involved in fatty acid synthesis (*ACACA, FASN, ACSL1*), elongation (*ELOVL5*) and desaturation (*FADS1, FADS2, SCD*). Levels of the mRNA expression were measured in SAT samples in fasting state and 185 min after each test meal (HC/HF diet - black lines, HF/HC diet – grey lines, n=29). $p<0.05$, ** $p<0.01$ - HC/HF diet vs. HF/HC diet at the same time of the day (comparisons with paired Student's t test or Wilcoxon test). Data are means ± SEMs.

Figure 6. Associations between plasma lipids and parameters of glucose metabolism.

(A) Diurnal variation of postprandial responses of glucose ($iAUCglu_{0\text{-}180}$) and insulin ($iAUCins_{0\text{-}180}$), indices of early ($iAUCins/glu_{0\text{-}30}$) and overall ($iAUCins/glu_{0\text{-}180}$) insulin secretion and Gutt's index of insulin sensitivity ($ISI\ Gutt_{0\text{-}120}$) for morning (grey bars) and afternoon (black bars) meals. * $p<0.05$, ** $p<0.01$ for afternoon vs. morning meal.

(B) Associations between diurnal variations of plasma lipid classes and index of insulin sensitivity in MTT-HF.

Analysis was performed using linear regression models with adjustment for age and BMI. Only associations significant after adjustment for multiple testing ($q < 0.05$) are shown.

(C) Proposed pathways of the interaction between the circadian clock, plasma lipids and insulin sensitivity. The molecular clock machinery regulates circadian rhythms of key transcription factors and enzymes in peripheral organs involved in lipogenesis, lipolysis, fatty acid oxidation and other processes of lipid metabolism which leads to the diurnal variation of plasma lipids (DAG, CE, PC etc.) involved in the regulation of cellular insulin sensitivity (black arrows). Alternatively, diurnal variation of insulin action (insulin secretion and insulin sensitivity induced by the local internal clock) could affect various processes of the lipid metabolism (grey arrows) and this way contribute to the diurnal variation of plasma lipids.

Table 1. Clinical characteristics of study subjects

Parameters	
N (% male)	29 (100)
Age [years]	45.9 ± 2.5
BMI [kg/m²]	27.1 ± 0.8
Waist circumference [cm]	93.55 ± 2.09
Waist-to-hip ratio	0.91 ± 0.01
Total body fat [%]	25.13 ± 1.55
Fat mass (kg)	22.78 ± 1.99
Fat-free mass (kg)	74.76 ± 1.54
Total cholesterol [mmol/l]	5.24 ± 0.18
HDL cholesterol [mmol/l]	1.20 ± 0.04
LDL cholesterol [mmol/l]	3.48 ± 0.17
Triglycerides [mmol/l]	1.25 ± 0.14
FFA [mmol/l]	0.49 ± 0.03
Fasting glucose [mmol/l]	5.83 ± 0.12
Fasting insulin [pmol/l]	34.3 ± 5.2
HOMA-IR [mmol· mU· l-2][(1)]	1.55 ± 0.26
2-hour glucose in OGTT [mmol/l][(1)]	115.85 ± 21.51
NGT/IFG/IGT [n][(2)]	18/8/4

Data were collected at visit 1 (start of first intervention period). [(1)]Data were collected at screening, prior to visit 1. [(2)]11 subjects with impaired fasting glucose/glucose tolerance (discrepancy in numbers is owed to one participants showing both an impaired fasting glucose and an impaired glucose tolerance). Data are shown as mean ± SEM. FFA, free fatty acids, IFG, impaired fasting glucose, IGT, impaired glucose tolerance, NGT, normal glucose tolerance, OGTT, oral glucose tolerance test.

Figure 1

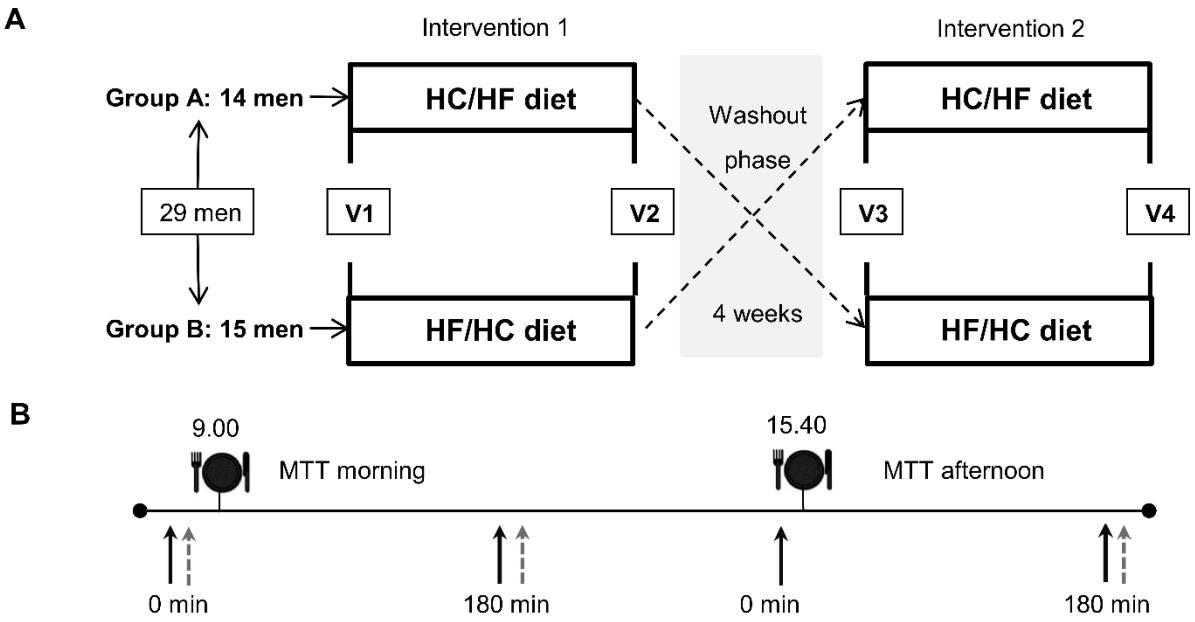

Figure 2

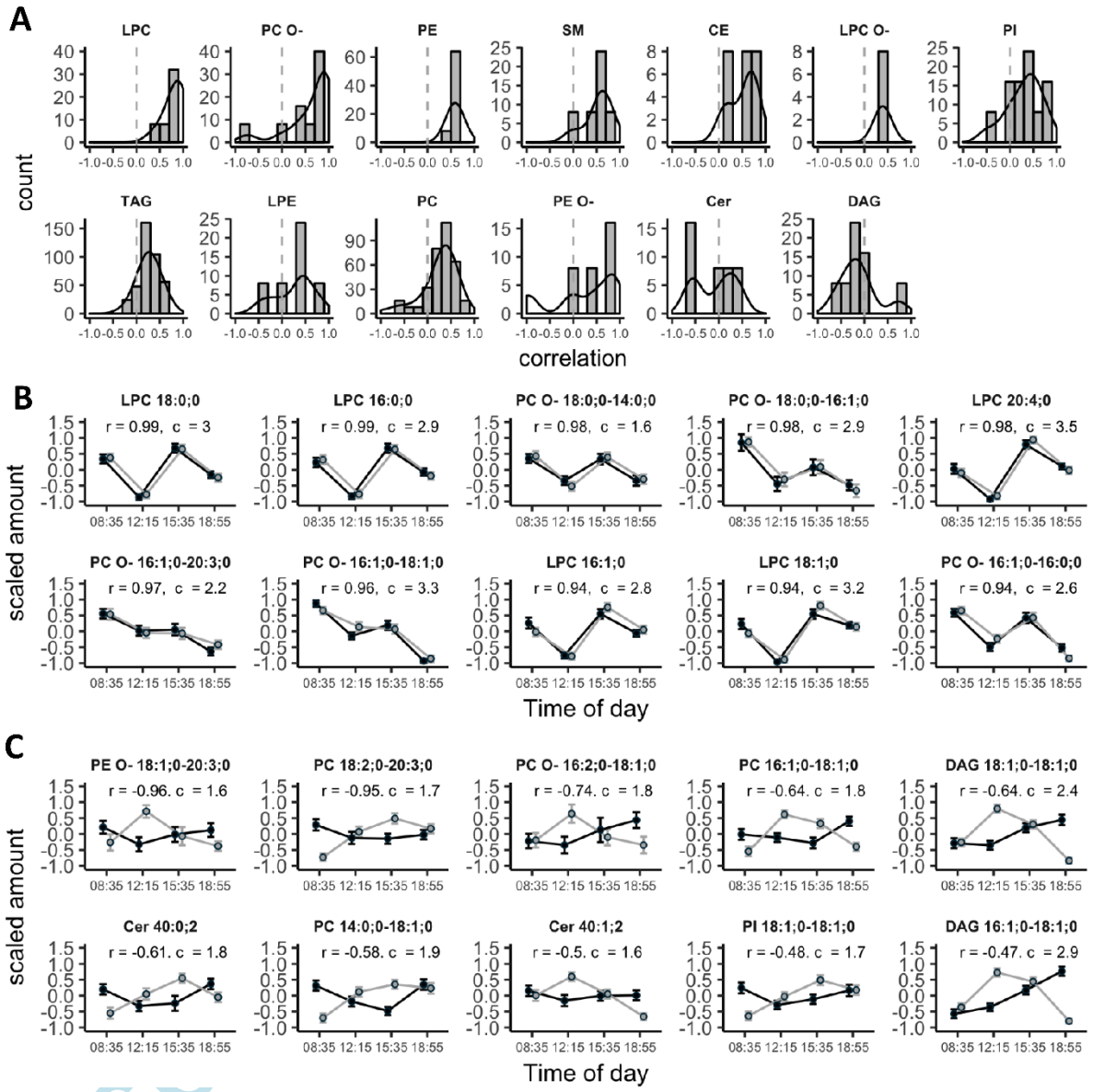

Figure 3

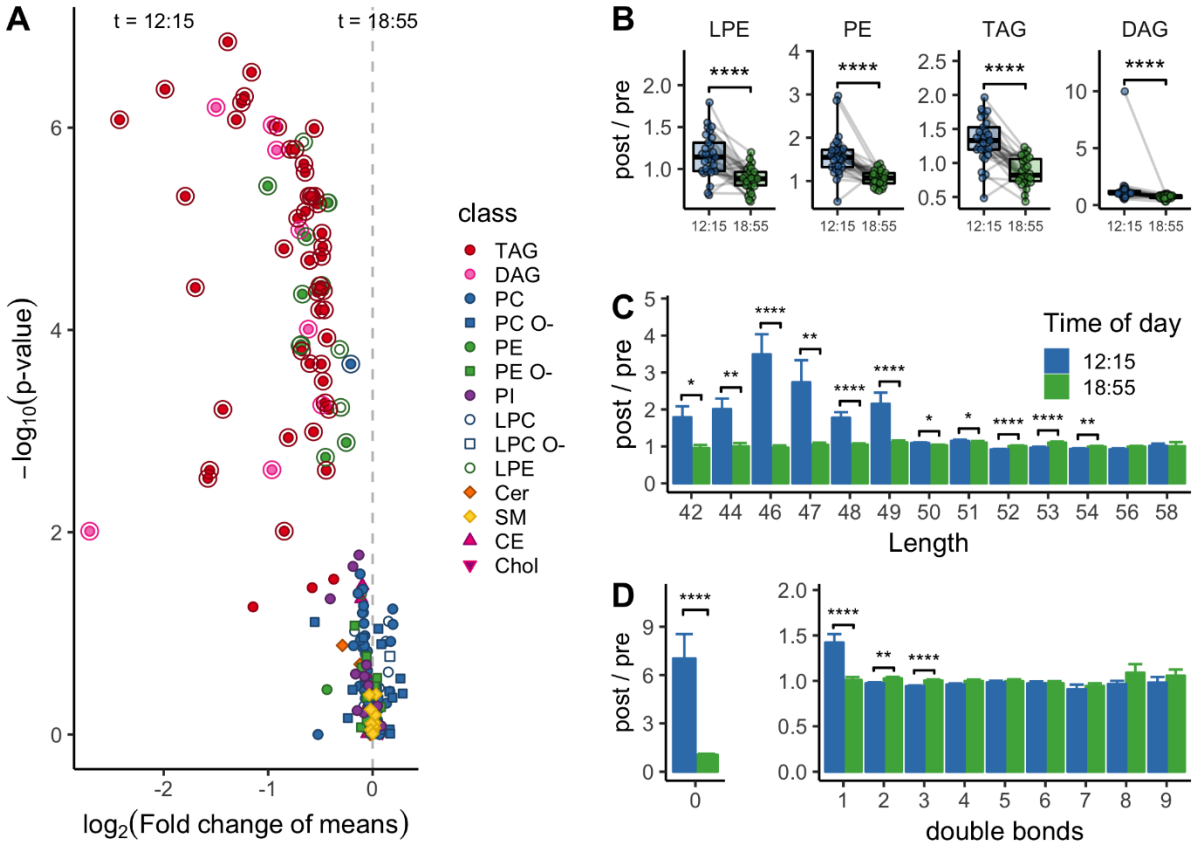

Figure 4

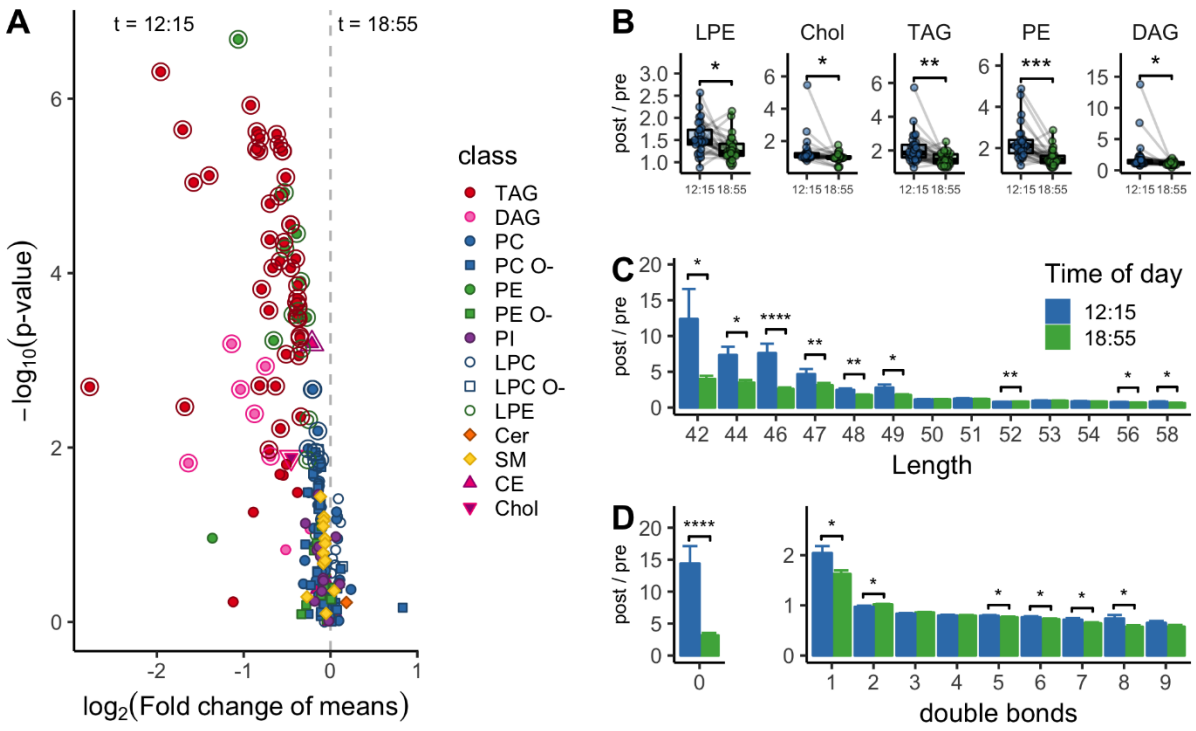

Figure 5

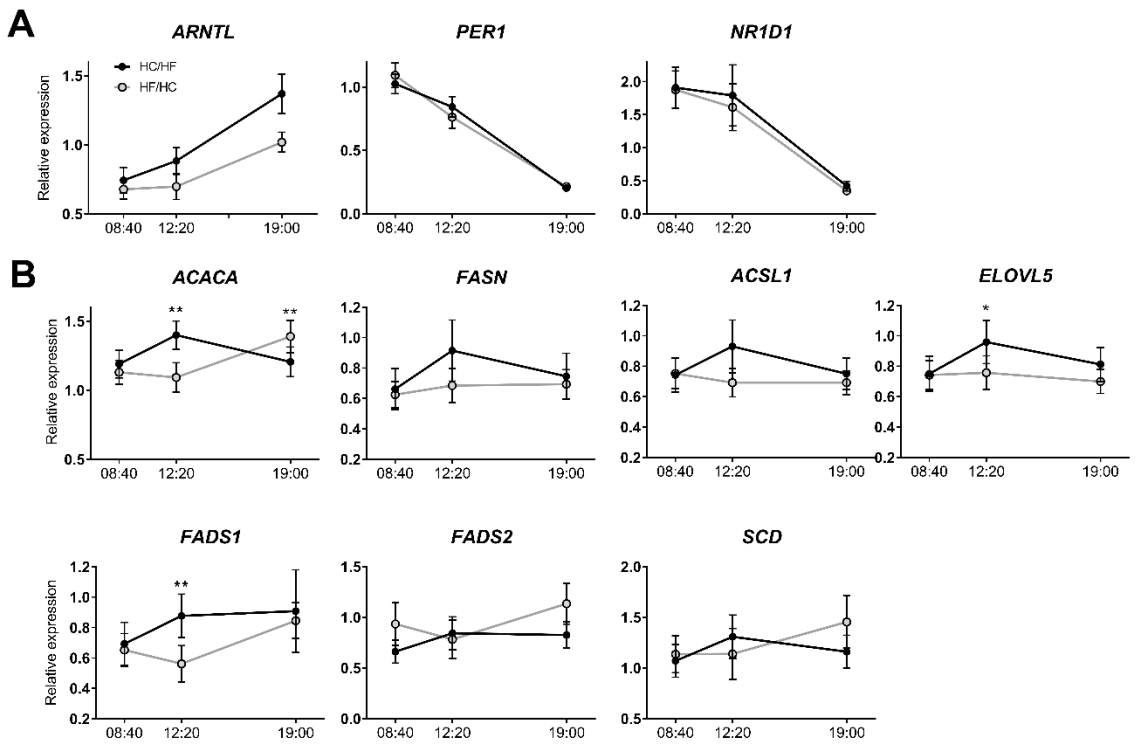

Figure 6