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DISSERTATION

Human Intervention Studies on the Interplay between Peripheral Circadian Clocks and the Regulation of Metabolic Homeostasis

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ABBREVIATIONS

ANOVA	analysis of variance
ARNTL*	aryl hydrocarbon receptor nuclear translocator like
AUC	area under the curve
BMI	body mass index
CD14	CD14 molecule
CD180	CD180 molecule
CHO	carbohydrates
CLOCK	*official full name of gene**:* clock circadian regulator; *preferred full name of protein:* circadian locomotor output cycles kaput
CPT1A	carnitine palmitoyltransferase 1A
CRY	*official full name of gene:* cryptochrome circadian clock; *preferred full name of protein:* cryptochrome
DBP	D site of albumin promoter (albumin DBox) binding protein
EN%	energy percent
FASN	fatty acid synthase
FFA	free fatty acids
GIP	glucose dependent insulinotropic peptide
GLP-1	glucagon like peptide 1
HC/HF	carbohydrate-rich diet until 13.30 h and fat-rich diet between 16.30 h and 22.00 h
HFD	high fat diet
HF/HC	fat-rich diet until 13.30 h and carbohydrate-rich diet between 16.30 h and 22.00 h
HDL	high density lipoprotein
IAUC	incremental area under the curve
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
IL1B	interleukin 1 beta
LC/HFD	low carbohydrate / high fat diet
LDL	low density lipoprotein
LF/HCD	low fat / high carbohydrate diet
LPL	lipoprotein lipase
MAP1LC3	microtubule-associated protein 1A/1B-light chain 3
MTT	meal tolerance test
MTT-HC	carbohydrate-rich meal tolerance test
MTT-HF	fat-rich meal tolerance test
NAMPT	nicotinamide phosphoribosyltransferase
NFKB1	nuclear factor kappa B subunit 1
NFKBIA	NFKB inhibitor alpha
NGT	normal glucose tolerance
NR1D1	nuclear receptor subfamily 1 group D member 1

NR1D2	nuclear receptor subfamily 1 group D member 2
PBMC	peripheral blood mononuclear cells
PER	*official full name of gene:* period circadian clock; *preferred full name of protein:* period circadian protein homolog
PCG1α	*see PPARGC1A*
PPARA	peroxisome proliferator activated receptors alpha
PPARG	peroxisome proliferator activated receptors gamma
PPARGC1A	PPARG coactivator 1 alpha
PYY	peptide YY
ROR	retinoid-related orphan receptor
SAT	subcutaneous adipose tissue
SCN	suprachiasmatic nucleus
SIRT1	sirtuin 1
TEF	*official full name of gene:* TEF, PAR bZIP transcription factor; *Preferred full name of protein:* thyrotroph embryonic factor

*: Here and elsewhere: official gene names are reported according to NCBI Entrez Gene (http://www.ncbi.nlm.nih.gove/gene).

**: For genes whose proteins share the same official symbol (abbreviation) but differ in the full name, the full name for both the gene and the protein are reported (as listed in NCBI Entrez Gene), as long as both the gene and the protein are mentioned and discussed in the thesis. In the course of the thesis, the full name of either gene or protein – whatever is used first – will be reported.

1.1. ABSTRACT (German)

Die zirkadiane Uhr kontrolliert zahlreiche metabolische Stoffwechselwege. Bei Nagern beeinflussen Zeit und Komposition der Nahrungsaufnahme sie und ändern zirkadiane Genexpression und Verhalten. Bei Menschen ist dazu nicht viel bekannt. Ziel der vorliegenden Arbeit war es deshalb, den Einfluss von Änderungen der (*i*) Energieaufnahme und (*ii*) Diätkomposition auf die zirkadiane Uhr und metabolische Homöostase beim Menschen zu untersuchen. Es wurden drei Ernährungsinterventionsstudien berücksichtigt. Studie I untersuchte den Einfluss einer Gewichtsreduktion auf die Genexpression zirkadianer Gene in subkutanen Fettgewebsproben 50 übergewichtiger Probanden. Ein mittlerer Gewichtsverlust von 10,8 ± 0,4% des initialen Körpergewichtes führte zu einer Erhöhung der Genexpressionsspiegel der Gene *period circadian clock 2* (*PER2*; p<0,001) und *nuclear receptor subfamily 1 group D member 1* (*NR1D1;* p=0,031). In Studie II wurde mittels einer Rhythmusprädiktionsanalyse der Einfluss einer isokalorischen Ernährungsumstellung von einer kohlenhydratreichen, fettarmen (HC/LFD) zu einer kohlenhydratarmen, fettreichen (LC/HFD) Ernährung auf die Genexpression in Blutmonozyten und die Cortisolspiegel im Speichel bei 29 gesunden Probanden untersucht. Diese Ernährungsumstellung führte zu einer Änderung der diurnalen Oszillation von *PER1, PER2, PER3* und *TEF, PAR bZIP transcription factor* (*TEF*) mit erhöhten Expressionsspiegeln und Amplituden unter der LC/HFD Diät. Unter der LC/HFD Diät verzögerte sich auch der Zeitpunkt der minimalen Cortisolspiegel. In Studie III änderte sich der Zeitpunkt der Kohlenhydrat- bzw. Fettaufnahme im Tagesverlauf ((1) kohlenhydratreich bis 13:30 und fettreich zwischen 16:30 und 22:00 Uhr (HC/HF) *versus* (2) der inversen Reihenfolge (HF/HC)). Es wurde der Einfluss der beiden isokalorischen Ernährungsformen auf die 12h Profile von Glukose und glukoseregulierenden Hormonen bei 29 männlichen Probanden mit unterschiedlichen Stadien der Glukosetoleranz untersucht. Die HF/HC Diät erhöhte die Ganztagesspiegel an Glukose um 7,9% (p=0,026) bei Teilnehmern mit gestörter Nüchternglukose und/oder gestörter Glukosetoleranz (IFG/IGT Teilnehmer, n=11). Bei Teilnehmern mit normaler Glukosetoleranz (NGT Teilnehmer, n=18) waren die Ganztagesspiegel an *glucagon like peptide 1* (GLP-1) um 10,2% (p=0,041) erhöht. Die Verschlechterung der Glukosetoleranz am Abend war bei IFG/IGT Teilnehmern deutlich stärker ausgeprägt. Unsere Ergebnisse legen nahe, dass verminderte GLP-1 und *peptide YY* Antworten sowie erhöhte Spiegel an freien Fettsäuren dafür verantwortlich sein könnten. Vorliegende Arbeit zeigt, dass Veränderungen der Energieaufnahme und der Diätkomposition in der Lage sind, die zirkadiane Uhr beim Menschen zu beeinflussen. Darüber hinaus zeigt Studie III, dass bei IGF/IGT Teilnehmern der Zeitpunkt, zu dem Kohlenhydrate bzw. Fette aufgenommen werden, für die Glukosehomöostase entscheidend ist, jedoch nicht bei NGT Teilnehmern. Der Verzicht auf große, kohlenhydratreiche Abendessen wird daher empfohlen.

1.2. ABSTRACT (English)

The mammalian circadian clock controls numerous metabolic processes. In rodents, meal timing and composition feedback onto the circadian clock modulating circadian gene expression and behaviour. As little is known in humans, this thesis investigated the influence of changes in (*i*) energy intake and (*ii*) meal composition on circadian mechanisms and metabolic homeostasis in humans. Three dietary intervention studies were considered. In Study I, weight-loss induced changes in gene expression levels of core clock genes were determined in subcutaneous adipose tissue of 50 overweight subjects. A mean weight loss of 10.8 ± 0.4 % of the initial body weight led to an increase in gene expression of period circadian clock 2 (*PER2*; $p<0.001$) and nuclear receptor subfamily 1 group D member 1 (*NR1D1*; $p=0.031$). In Study II, three/six-time point rhythm prediction analysis was used to determine the effect of an isocaloric dietary switch from a carbohydrate-rich, low fat diet (HC/LFD) to a low carbohydrate, fat-rich diet (LC/HFD) in blood monocytes and salivary cortisol levels in 29 healthy subjects. This dietary switch induced an alteration of diurnal oscillation of *PER1*, *PER2, PER3* and TEF, PAR bZIP transcription factor (*TEF*) with increased expression levels and amplitudes on the LC/HFD diet. Nadir in 24h salivary cortisol levels was also delayed on the LC/HFD diet. In Study III, the effect of a diurnal distribution of carbohydrates and fat on 12h profiles of glucose and glucose-regulating hormones was investigated in 29 male subjects with different stages of glucose tolerance. Two isocaloric diets were used: (1) carbohydrate-rich meal until 13.30h and fat-rich meal between 16.30h and 22.00h (HC/HF) *versus* (2) the inverse sequence of meals (HF/HC). On the HF/HC diet, whole-day glucose level was increased by 7.9 % ($p=0.026$) in subjects with impaired fasting glucose and/or impaired glucose tolerance (IFG/IGT subjects, n=11); and whole-day glucagon like peptide 1 (GLP-1) was increased by 10.2 % ($p=0.041$) in subjects with a normal glucose tolerance (NGT subjects, n=18). The afternoon decline in glucose tolerance was more pronounced in IFG/IGT subjects, which was associated with stronger declines in GLP-1 and peptide YY responses and elevated postprandial free fatty acid levels. The thesis reveals changes in energy intake and meal composition modulate circadian clock and metabolic homeostasis in humans. Particularly, time of carbohydrate intake seems decisive for glycaemic control in IFG/IGT subjects but not in NGT subjects. Large, carbohydrate-rich dinners might need to be avoided by people with an impaired glucose metabolism.

2. INTRODUCTION

Numerous behavioural, physiological and metabolic processes are most active once every 24 hours. Examples range from the sleep-wake cycle over rhythms in body temperature and blood pressure to changes in hormone levels [1]. In scientific terms, these processes show circadian (Latin: *circa diem*; meaning “about a day”) rhythms [2], allowing organisms to synchronize with the environment, as they ensure the adaptation to the daily changes in light and darkness [3].

2.1. Molecular mechanism and hierarchical structure of the circadian clock

Genetically, circadian rhythms are encoded by interlocking transcriptional-translational feedback loops [4] that drive daily oscillation in gene expression [5], a process that is associated with circadian changes in mRNA processing, chromatin structure and protein turnover and activity [6].

Predominantly, two feedback loops function together to produce robust 24 h rhythms of gene expression [4]. The core feedback loop consists of four integral clock proteins: the activators circadian locomotor output cycles kaput (CLOCK) and aryl hydrocarbon receptor nuclear translocator like protein (ARNTL; also known as BMAL1) and the repressors period circadian protein homolog (PER) and cryptochrome (CRY). CLOCK and ARNTL are subunits of the heterodimeric transcription factor CLOCK/ARNTL [7], driving the transcription of *PER* (*PER1, 2, 3*) and *CRY* (*CRY1, 2*) as well as other target genes [8,9]. The resulting PER and CRY proteins accumulate, heterodimerize and inhibit the transcriptional activity of CLOCK/ARNTL [4] thereby repressing the transcription of their own genes. Ubiquitination and degradation of PER and CRY proteins allow a new cycle to start [8]. Along with the degradation rate of PER and CRY proteins, a second feedback loop ensures proper circadian timing, by regulating *ARNTL* [10] and possibly *CLOCK* transcription [11]. It consists of the activators retinoid-related orphan receptors (RORα, β, γ) and the repressors REV-ERBα and REV-ERBβ [4]; of which the latter are encoded by the nuclear receptor subfamily 1 group D member 1 (NR1D1) and member 2 (NR1D2), respectively.

In mammals, the circadian clock system is organized in a hierarchy of oscillators sharing the same molecular setup [9,12]. Via endocrine and systemic cues the master clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus [13] passes

information on to the many peripheral clocks that are found in every cell of the mammalian organism.

2.2. Metabolic processes are under circadian control

The circadian clock mechanism establishes rhythms in gene expression of clock-controlled genes [4] on transcriptional, translational and post-translational level [14], allowing local, tissue-specific control over physiology [4]. The proteins of numerous clock-controlled genes are themselves transcription factors, including D site of albumin promoter (albumin DBox) binding protein (DBP), thyrotroph embryonic factor (TEF), peroxisome proliferator-activated receptors alpha (PPARα) and peroxisome proliferator-activated receptors gamma (PPARγ) [15], suggesting that they convey timing cues to downstream targets by rhythmic transcriptional regulation [4].

In mammals, 10 % of the entire transcriptome in liver, heart, adipose and other tissue display circadian rhythmicity [16,17]. Consequently, numerous components of the glucose, protein and lipid metabolism [18], detoxification pathways and inflammatory response are under circadian control [19]. Examples range from a carbohydrate preference early in the active phase over a peak of glucose uptake in the middle to a peak of glycogen synthesis at the end of the active phase [18,20]. Intestinal lipid transport, lipogenesis, lipolysis and adipokine secretion show circadian oscillation [21].

2.3. Metabolic processes feedback onto the circadian clock

The rhythm imposed by the circadian clock is endogenous and persists in the absence of environmental information [2]. However, along with light input, metabolic and meal-induced signals have the capacity to feedback onto the circadian clock modulating circadian gene expression and behaviour [22,23].

In rodents, recent studies provide evidence that timing of food intake, primarily a high fat diet (HFD), alters the clock machinery and metabolism [6,24-29]. Feeding mice only during the light phase (i.e. sleep phase for the nocturnal animals) uncoupled the circadian clocks in peripheral tissues from the central pacemaker [25] and led, on a HDF, to increased adiposity [24]. Attenuated rhythms in food intake and altered expression of clock genes and clock-controlled genes might explain the increase in adiposity, when a high proportion of calories are consumed during the light phase [28]. A more recent study revealed that a HFD induces transcriptional reprogramming within the clock, reorganizing the relationships between the circadian metabolome and

transcriptome [6]. Interestingly, the effects of the HFD on the circadian clock are reversible [30]. Restricting the HFD to a time window of eight to twelve hours daily protected the mice against obesity, hyperinsulinemia, hepatic steatosis and inflammation [26,27]. Interestingly, a study in mice revealed that consumption of a carbohydrate-rich diet at the beginning and a HFD at the end of the active phase led to increased weight gain, adiposity and glucose intolerance [31].

In addition to a HFD, nutrient signalling by glucose [32,33], as a result by insulin [33,34], and by amino acids / polyamines [35] entrains central and peripheral circadian clocks in rodents.

2.4. Aim of this PhD Thesis

In spite of the accumulative evidence on the influence of food-induced stimuli on the circadian timing system and metabolic homeostasis in rodents, very little is known in humans. Thus, this PhD thesis aimed at unravelling the role of circadian mechanisms in the response of human metabolism to food intake. To this end, the significance of changes in (*i*) energy intake and (*ii*) meal composition for the circadian clock mechanism and metabolic homeostasis was investigated in humans.

Following objectives were addressed in three dietary intervention studies:

In study I (**Pivovarova *et al.*, 2016**) it was investigated whether a weight loss affected gene expression levels of core clock genes in human subcutaneous adipose tissue (SAT). It was further explored whether alterations in gene expression were associated with metabolic parameters.

In study II (**Pivovarova *et al.*, 2015**) the effect of a dietary switch from a carbohydrate-rich diet to a fat-rich diet on the central clock (as indexed by salivary cortisol and melatonin levels) and the peripheral clock (as assessed by clock gene expression in blood monocytes) was studied in healthy humans.

In study III (**Kessler *et al.*, 2017**) the effect of a diurnal carbohydrate and fat distribution on glycaemic control was investigated in subjects with different stages of glucose tolerance. For this, two isocaloric diets were used: (1) carbohydrate-rich meals in the morning and fat-rich meals in the evening *versus* (2) the inverse sequence of meals.

3. STUDY PARTICIPANTS AND METHODS

Study designs

Samples from three dietary intervention studies were used. Details of each study are described in corresponding *Result* section.

DiOGenes is a randomized, controlled trial including weight loss and weight maintenance phases [36,37]. The study was approved by the ethics committee of Potsdam University, Potsdam, Germany and registered at www.clinicaltrial.gov (NCT00390637).

NUGAT is a controlled trial investigating the genetic determination of metabolic responses to a shift from a high carbohydrate diet to a high fat diet [38]. The study was approved by the ethics committee of Charité, Berlin, Germany and registered at www.clinicaltrial.gov (NCT01631123).

CLOCK is a randomized controlled trial with a cross-over design investigating the effect of a diurnal carbohydrate and fat distribution on glycaemic control in men [39]. The study was approved by the ethics committee of Charité, Berlin, Germany and registered at www.clinicaltrial.gov (NCT02487576).

Samples of subcutaneous adipose tissue (SAT), blood monocytes, as well as plasma and saliva samples were collected in these studies for the gene expression analysis and hormone profiling, respectively.

Subcutaneous adipose tissue samples

Using a cutting needle, 1 g of SAT was collected at contralateral sites at the level of the umbilical cord under sterile conditions [37]. Lidocaine was used to anesthetize the skin. Samples were removed from any debris and blood, shock-frozen in liquid nitrogen and stored at -80 °C until analysis.

Isolation of blood monocytes

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood (EDTA blood) samples by density gradient centrifugation using Ficoll-Paque^{TM} PREMIUM (GE Healthcare, USA). Subsequently, monocytes were isolated from the PBMC fraction by magnetic cell sorting using anti-CD14-coated beads [38].

RNA extraction, cDNA synthesis and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from monocytes and SAT samples using the NucleoSpin RNA II Kit (Macherei-Nagel, Germany) and the RNeasy Lipid Tissue Mini Kit (Qiagen, Germany), respectively, as described [37,38]. High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Germany) was used for synthesis of single-stranded cDNA from total RNA. ABI Prism 770 sequence detection system was used to perform quantitative real-time PCR (qRT-PCR), using Power SYBR Green PCR Master Mix (Applied Biosystems, Germany) and specific primers. mRNA levels were quantified using standard curve method. Analysed genes are listed in [37,38].

Meal tolerance tests

Meal tolerance tests (MTT) were used to determine the hormonal response to an oral nutrient load [39]. Test meals whose components were weighed to the nearest of 0.1 g contained a specific nutrient composition corresponding to the study design [39]. Participants were asked to consume the test meals within 15 minutes. Before (-5) and 30, 60, 90, 120 and 180 minutes after the test meal blood samples were drawn from the forearm vein for hormone profiling. Along with each MTT a ^{13}C-acetate breath test was performed to determine the gastric emptying rate as described in detail in [39].

Measurement of hormones

Commercial enzyme-linked immunosorbent assay was used to determine circulating pre- and postprandial levels of insulin, C-peptide, glucagon, glucose dependent insulinotropic peptide (GIP), peptide YY (PYY) as described in [39] and salivary melatonin and cortisol as described in [38]. Active glucagon like peptide 1 (GLP-1) was measured by Meso Scale Discovery Assay (USA) [39].

Statistical analysis

The statistical analyses were performed with IBM SPSS statistics (IMB, USA). Detailed descriptions of all statistical tests applied are reported in [37-39]. The three/six-time point rhythm prediction method used in Study II was established by Dr O. Pivovarova (DIfE, Potsdam) and Dr K. Jürchott (Charité, Berlin) [38]. Statistical significance was defined as $p<0.05$. Data are presented as mean ± SEM

4. RESULTS

4.1. The effect of weight loss on clock gene expression in human subcutaneous adipose tissue samples

Publication 1:

Pivovarova O., Ö. Gögebakan, S. Sucher, J. Groth, V. Murahovschi, **K. Kessler**, M. Osterhoff, N. Rudovich, A. Kramer, A.F. Pfeiffer (2016). "Regulation of the clock gene expression in human adipose tissue by weight loss." Int J Obes (Lond) 40(6): 899-906.

Here we investigated the effect of weight loss on clock gene expression in human SAT and the association of these changes with markers of glucose and fat metabolism and inflammatory response. For this, gene expression analysis was performed in adipose tissue samples of 50 overweight subjects (age 40.8 ± 0.9 years, BMI 34.2 ± 0.6 kg/m^2) before and after weight loss.

Exclusion criteria were in brief a body mass index (BMI) > 45 kg/m^2 and diabetes mellitus (type 1 and 2) [36,40]. During the weight loss phase, eligible participants followed an eight-week low calorie diet consisting of 800 kcal/day plus 200 g of vegetables a day and achieved a weight loss of at least 8 % of their initial body weight. At the start and the end of the weight loss regimen, SAT samples were taken at 09.00 h following an overnight fast [37].

The publication demonstrates that the expression of clock genes in human SAT is affected by changes in body weight, indicating that energy intake modulates peripheral circadian clocks in humans. In detail, a mean weight loss of 10.8 ± 0.4 % of the initial body weight increased the expression of *PER2* ($p<0.001$) and *NR1D1* ($p=0.031$). The statistical analysis revealed a large number of significant correlations between the expression levels of circadian and metabolic genes. In particular, genes regulating the lipid metabolism including lipoprotein lipase (*LPL*), fatty acid synthase (*FASN*), carnitine palmitoyltransferase 1A (*CPT1A*) and *PPARG* as well as genes regulating the energy homeostasis including peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PPARGC1A*) and sirtuin 1 (*SIRT1*) highly correlated with the expression levels of clock genes. Genes regulating autophagy (microtubule associated protein 1 light chain 3, *MAP1LC3*) and inflammatory response including nuclear factor kappa B (NFKB) subunit 1 (NFKB1) and NFKB inhibitor alpha (*NFKBIA*) also correlated with the expression of clock genes. These results suggest a tight crosstalk between the circadian clock mechanism and metabolic as well as inflammatory pathways. The tight

interplay may allow the adipose tissue metabolism to adapt to changes in energy homeostasis.

4.2. Changes in dietary fat and carbohydrate content alter markers of the central and peripheral clocks in humans

Publication 2:

Pivovarova O.*, K. Jurchott*, N. Rudovich*, S. Hornemann, L. Ye, S. Mockel, V. Murahovschi, **K. Kessler**, A. C. Seltmann, C. Maser-Gluth, J. Mazuch, M. Kruse, A. Busjahn, A. Kramer and A. F. Pfeiffer (2015). "Changes of Dietary Fat and Carbohydrate Content Alter Central and Peripheral Clock in Humans." J Clin Endocrinol Metab 100(6): 2291-2302. *Equal contribution

To determine the effect of changes in food composition on the central (assessed by salivary cortisol and melatonin levels) and peripheral (assessed by clock gene expression in monocytes) circadian clock, 29 healthy subjects (age 37.5 ± 17.5 years, BMI 23.2 ± 2.6 kg/m^2) were studied before and after a dietary switch from a carbohydrate-rich diet to a fat-rich diet.

Subjects with a normal glucose tolerance were eligible for participation. Eligible participants first followed an isocaloric carbohydrate-rich, low fat diet (55 EN% carbohydrates (CHO), 30 EN% fat, 15 EN% protein) (HC/LFD) for six weeks, and then switched to a six-week isocaloric low carbohydrate, fat-rich diet (40 EN% CHO, 45 EN% fat, 15 EN% protein) (LC/HFD). Before as well as one and six weeks after the LC/HFD blood samples were drawn at 08.00 h, 11.15 h and 15.45 h for isolation of blood monocytes. Salivary cortisol and melatonin levels were determined every four hours over 24 h prior to each investigation day. The publication established a mathematical procedure of 24h rhythm prediction based on three/six time point data which was used to determine diurnal oscillations in gene expression and hormone levels [38].

The publication suggests that shifting from a high carbohydrate, low fat diet to a low carbohydrate, high fat diet altered the diurnal oscillations of the core clock genes *PER1*, *PER2* and *PER3* as well as *TEF*, with increased expression levels and amplitudes on the LC/HFD diet. One and six weeks on the LC/HFD diet also delayed the nadir, i.e. time of curve minimum, of the cortisol rhythm by 1 hour 30 minutes and 1 hour 17 minutes, respectively. Subsequent correlation analysis showed that core clock genes and their diet-induced changes correlated well with each other, but not with cortisol and

its changes, suggesting that the centrally driven hormone cortisol only plays a minor role for the circadian clock in peripheral blood.

Analysis of clock-controlled metabolic genes revealed diurnal oscillation of genes involved in the energy homeostasis including nicotinamide phosphoribosyltransferase (*NAMPT*), fat metabolism (*CPT1A* and *FASN*) and inflammatory response including *NFKBIA*, CD14 molecule (*CD14*), CD180 molecule (*CD180*) and interleukin 1-beta (*IL1B*). Of those, the inflammatory genes (*CD14*, *CD180*, *NFKBIA* and *IL1B*) showed an alteration in their diurnal oscillation in response to the dietary shift. Analysis showed correlations for core clock genes with inflammatory genes and genes involved in fat metabolism, primarily *CPT1A*. Core clock genes, particularly *CRY2* and *PER1* as well as *TEF* also correlated with levels of total cholesterol, low density lipoprotein (LDL) cholesterol and triglycerides.

In conclusion, this publication shows that modulating dietary carbohydrate and fat content alters both the central and peripheral circadian clocks in humans, and confirms the tight interplay between the circadian clock and metabolic and inflammatory pathways suggested by publication 1.

4.3. Effect of a diurnal carbohydrate and fat distribution on glycaemic control in subjects with different stages of glucose tolerance

Publication 3:

Kessler K., S. Hornemann, K. J. Petzke, M. Kemper, A. Kramer, A. F. H. Pfeiffer, O. Pivovarova* and N. Rudovich* (2017). “The effect of diurnal distribution of carbohydrates and fat on glycaemic control in humans: a randomized controlled trial.” Sci Rep 7:44170.

*Equal contribution

To analyse the metabolic effect of a diurnal carbohydrate and fat distribution, we compared the influence of a prolonged consumption of two diets with different diurnal distribution of carbohydrates and fat on glycaemic control in 29 men with different stages of glucose tolerance (age 45.9 ± 2.5 years, BMI 27.1 ± 0.8 kg/m^2).

Exclusion criteria were in brief a BMI > 35 kg/m^2 and diabetes mellitus (type 1 and 2). Eligible participants were randomized to two isocaloric, four-week diets: (1) carbohydrate-rich meals until 13.30 h (65 EN% CHO, 20 EN% fat, 15 EN% protein) and fat-rich meals between 16.30 h and 22.00 h (35 EN% CHO, 50 EN% fat, 15 EN%

protein) (HC/HF) *versus* (2) the inverse sequence of meals (HF/HC). Dietary intervention periods were separated by a four-week washout phase and followed by a 12 h clinical investigation day with two meal tolerance tests (MTT), a carbohydrate-rich (MTT-HC) and a fat-rich (MTT-HF), whose order was in accordance with the previous dietary intervention [39].

The publication suggests a differential response to the diurnal distribution of carbohydrates and fat in subjects with impaired fasting glucose and / or impaired glucose tolerance (IFG/IGT subjects, N=11) compared to normal glucose tolerant subjects (NGT subjects, N=18). In NGT subjects, both diets reduced glucose, C-peptide and glucagon levels as well as total, high density lipoprotein (HDL) and LDL cholesterol. Similarly, in IFG/IGT subjects, fasting glucose, C-peptide and HDL cholesterol declined. Consequently, for these parameters no statistical differences in diet effects and between NFG and IFG/IGT subjects were observed. Remarkably, in IFG/IGT subjects, the diets resulted in different regulation of fasting GLP-1 ($p=0.009$) and PYY ($p=0.034$); both GLP-1 and PYY were decreased ($p<0.05$) on the HC/HF diet but did not change on the HF/HC diet. Moreover, whole-day levels, as calculated by the integrated AUC over both meal tolerance tests, showed significant differences between IFG/IGT and NGT subjects: the HF/HC diet increased whole-day glucose levels by 7.9% ($p=0.026$) in IFG/IGT subjects, whereas in NGT subjects whole-day GLP-1 levels were increased by 10.2% ($p=0.041$) compared with HC/HF diet. On the HF/HC diet, whole-day free fatty acids (FFA) level was increased in IFG/IGT subjects compared to NGT subjects ($p=0.005$). To determine the underlying mechanisms we analysed the 12 h profiles of the studied parameters and compared the meal-induced response, assessed using incremental $AUC_{0\text{-}180}$ ($iAUC_{0\text{-}180}$), in the afternoon to that in the morning. The analysis revealed a decrease in glucose tolerance as the day progresses in both NGT and IFG/IGT subjects. Remarkably, the afternoon decline of glucose tolerance was more pronounced in IFG/IGT subjects, primarily on the HF/HC diet. Diminished postprandial GLP-1 and PYY responses and reduced suppression of postprandial FFA levels seem to explain the worsened afternoon decline of glucose tolerance in IFG/IGT subjects.

In conclusion, this publication reveals an unfavourable effect of the HF/HC diet on the glycaemic control in IFG/IGT subjects, but not in NGT subjects. In consideration of the decline of glucose tolerance as the day progresses, the publication provides evidence to recommend the avoidance of large, carbohydrate-rich meals in the evening, primarily by subjects with an impaired glucose metabolism.

5. DISCUSSION

The finding that both energy intake and food composition influence the molecular setup of peripheral circadian clocks in humans is highly interesting as it confirms observations in animals [6,28,30,41-44]. In animal models, dietary restriction and hypocaloric feeding have been shown to modulate behavioural and molecular circadian rhythms centrally and in the periphery [42-45]. Very recently, Katewa *et al.* could show that dietary restriction increases the magnitude of circadian gene expression and suggested that this way dietary restriction counterbalances the age-related loss in circadian oscillations, thereby increasing life span [42]. In humans, only limited data is available: a study by Loboda *et al.* showed that the human adipose tissue displays profound diurnal oscillation of core clock genes as well as metabolic and inflammatory genes [46]. However, in contrast to studies in rodents, the effect of fasting and sibutramine, a weight loss drug, on human adipose tissue was subtle [46]. Interestingly, the success of weight loss therapies has been linked to polymorphisms [47,48] and methylation pattern of core clock genes [49], one of which is the *PER2* gene [48]. Our Study I shows an increase in *PER2* and *NR1D1* expression after weight loss [37] proposing that weight loss induces a reprogramming of the circadian clock mechanism in adipose tissue in humans. However, the finding that indices of obesity (i.e. BMI, waist-to-hip ratio, waist circumference, body weight) did not correlate with the clock genes in the weight loss network suggests that circadian mechanisms may indirectly be regulated by weight loss. The clock gene expression levels and their weight-loss induced changes, in our Study I, tightly correlated with expression levels of genes involved in the fat metabolism, energy homeostasis, autophagy and inflammatory response. Of particular interest are correlations of core clock genes with *PPARG* as well as *SIRT1* and *PPARGC1A,* as their proteins are part of an energy sensing network controlling energy expenditure [50]. SIRT1, a NAD^+-dependent deacetylase, is a key metabolic sensor coordinating changes in energy homeostasis by targeting PGC1α and PPARγ, among others [50]; PGC1α, in turn, interacts with and co-activates PPARγ [51]. In mice, both SIRT1 and PGC1α have been identified as key regulators of the circadian clock mechanisms [52-54]. SIRT1 was shown to bind to the CLOCK/ARNTL heterodimer and PER2 in a circadian manner, and promotes the deacetylation and degradation of PER2 [52]. PGC1α might also stimulate the deacetylation of PER2 [55]. Collectively, in rodents the energy metabolism and the

circadian clock are well integrated possibly via PER2 which might also be true for humans, as the results of our study suggest.

Our Study II [38] revealed that dietary manipulation at the level of macronutrient content is a potent modulator of the human central and peripheral circadian clock. The switch from a HC/LFD to a LC/HFD diet induced a phase delay in salivary cortisol levels and altered the diurnal oscillations of *PER1*, *PER2*, *PER3* and *TEF*. These results are of great interest as (i) mounting evidence in rodents has suggested that a HFD is a potent modulator of circadian clocks [6,28,41] and as (ii) they show that also the human circadian clock appears sensitive to dietary manipulation at the level of macronutrients [23]. In rodents, a HFD leads to attenuated rhythms in food intake [28], a profound reprogramming of circadian oscillation, including of core clock mechanisms [6,23] and a shift in period length [28,56]. Centrally, a HFD caused a period lengthening, as measured in SCN or by locomotion activity [28,56], whereas the peripheral tissue, primarily the liver, showed a phase advance [6,30,41]. The impaired phase relationship between the central and peripheral clocks has been proposed to be one of the underlying mechanisms by which a HFD induces obesity [23]. The delay in salivary cortisol levels in our study might point towards similar mechanisms in humans. Interestingly, a desynchrony between the central circadian clock, as indexed by melatonin, and the peripheral circadian clock in peripheral blood has also been observed by Archer *et al.* upon mistimed sleep, which may suggest that hormones driven by the SCN (i.e. melatonin and cortisol) may only have a limited influence on the peripheral blood [57]. Studies directly manipulating melatonin and cortisol would be needed to further investigate their contribution on the human transcriptome [57].

Our Study II also revealed altered oscillations of inflammatory genes (*CD14*, *CD180*, *NFKBIA* and *IL1B)* in response to the dietary manipulation. This is of interest, as it (i) confirms the tight control of immune response and function by the circadian clock [19,46], and as it (ii) may propose a mechanism by which high fat meals lead to the low-grad inflammatory postprandial response observed in healthy subjects [58].

Our finding that key components of the energy metabolism (i.e. *NAMPT*) and fat metabolism (i.e. *CPT1A* and *FASN*) oscillate throughout the day highlights the close relationship between the circadian clock, the energy homeostasis and fat metabolism in humans, as has already been proposed by our Study I. CPT1α is the rate-limiting enzyme for mitochondrial fatty acid oxidation [59]. Conversely, FASN is the key enzyme

of fatty acid biosynthesis [60]. In our study, expression levels of *CPT1A* peak in the morning, whereas *FASN* levels peak at the end of the day, suggesting diurnal rhythms of fat synthesis and oxidation in humans. Remarkably, a very recent study in mice proposes that mitochondrial utilization of different nutrients displays a diurnal rhythm which is regulated by the PER proteins [61]. Oscillation of primarily CPT1A, on both gene and protein level, seems to drive the diurnal rhythm in mitochondrial utilization [61]. The results of Study II may propose similar mechanisms in humans.

The results of Study II suggest that food composition functions as a potent modulator of both the peripheral and central circadian clock in humans. Potentially, insulin [33,34] and other meal-induced hormones or humoral stimuli [32,35] are involved in the circadian entrainment. This raises the question whether food intake patterns inducing high and low insulin levels at different times of the day affect metabolic homeostasis in humans. A beneficial metabolic effect of a diurnal distribution of carbohydrates and fat has previously been indicated by a murine study [31], and is often recommended by popular dietary concepts, claiming that the avoidance of carbohydrates in the evening may help prevent weight (re)gain. Study III was designed to study the effect of a diurnal carbohydrate and fat distribution on glycaemic control in humans.

Study III [39] reveals an unfavourable effect of the HF/HC diet on glycaemic control in IFG/IGT subjects, but not in NGT subjects. On the HF/HC diet, whole-day levels of glucose were increased in IFG/IGT subjects, whereas in NGT subjects they did not differ between the diets. In contrast, NGT subjects showed, on the HF/HC diet, elevated whole-day levels of the incretin hormone GLP-1, whose beneficial effect on glycaemic control has long been acknowledged [62]. Interestingly, the findings of our Study III seems to oppose the earlier report in mice suggesting that consumption of a carbohydrate-rich diet at the end of the active day leads to improved glucose tolerance and reduced body weight [31]. Comparable studies in humans show conflicting results [63-67]. Some propose a beneficial effect of carbohydrate consumption in the evening [63,64], while others suggest that particularly the consumption of carbohydrates with a high glycaemic index in the evening is most detrimental [67]. Differences in the study design, comparison groups as well as length and setup of intervention might account for the conflicting results. Noteworthy, however, the results of our Study III suggest that large, carbohydrate-rich meals might need to be avoided, primarily by IFG/IGT subjects.

Whether the same holds true for people with Diabetes mellitus needs to be clarified by future studies.

The finding of our Study III that glucose tolerance deceases as the day progresses has been suggested by previous studies on oral glucose [68] and meal [69-72] tolerance tests in the morning *versus* evening and highlights the role of the circadian clock in the regulation of glucose metabolism [20]. Remarkably, Study III reveals that IFG/IGT subjects show a more pronounced decline of afternoon glucose tolerance (primarily on the MTT-HC), as indicated by the enhanced diurnal glucose variation. Recently, it has been shown that glycaemic variability is present to an increasingly degree from NGT over IFG/IGT subjects to people with diabetes [73,74], with more pronounced intraday glucose fluctuations in latter two groups [73]. Sonnier *et al.* showed a substantially better glycaemic control in the morning and proposed that a diminished cortisol rhythm was strongly associated with a larger decline of glycaemic control in the evening [75]. Correlation analysis in our Study III, however, cannot confirm this hypothesis. Instead Study III suggests that the decline in afternoon glucose tolerance in IFG/IGT subjects is associated with decreased responses of PYY and GLP-1 and elevated postprandial FFA levels. All three exhibit diurnal variation [70,76-78] and have been linked to diabetes and insulin resistance [79,80]. Beyond that, they are discussed as one of potentially many mechanisms contributing to the progressive decrease in glucose tolerance [70,81].

The results of Study III are of great clinical importance as they increase the existing evidence on a beneficial effect of high caloric intake in the morning over high caloric intake in the evening [82,83]. Beyond that, they propose the avoidance of large, carbohydrate-rich dinners, primarily by people with an impaired glucose metabolism.

In conclusion, the main findings of this PhD thesis are that changes of energy intake and food composition are potent modulators for the circadian clocks in humans. Both weight loss and a change in dietary fat and carbohydrate content alter gene expression levels of core clock genes and, in the latter case, the circadian oscillation of the centrally driven hormone cortisol. Moreover, the thesis revealed that even changes of a diurnal carbohydrate and fat distribution alter glycaemic control in subjects with an impaired glucose metabolism, but not in NGT subjects.

Thus, the thesis provides evidence for the need to align dietary recommendations with the circadian clock mechanisms, which has so far often been neglected.

6. REFERENCES

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AFFIDAVIT

I, Katharina Keßler certify under penalty of perjury by my own signature that I have submitted the thesis on the topic ***Human Intervention Studies on the Interplay between Peripheral Circadian Clocks and the Regulation of Metabolic Homeostasis*** I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date _____ Signature

DECLARATION OF ANY EVENTUAL PUBLICATION

Katharina Keßler had the following share in the following publications:

Publication 1:
Pivovarova O., Ö. Gögebakan, S. Sucher, J. Groth, V. Murahovschi, K. Kessler, M. Osterhoff, N. Rudovich, A. Kramer, A.F. Pfeiffer. Regulation of the clock gene expression in human adipose tissue by weight loss. Int J Obes (Lond). 2016 Jun; 40(6): 899-906.

Contribution in detail: Ms Katharina Keßler significantly contributed to the gene expression analyses and helped write the paper.

Publication 2:
Pivovarova O.*, K. Jurchott*, N. Rudovich*, S. Hornemann, L. Ye, S. Mockel, V. Murahovschi, K. Kessler, A. C. Seltmann, C. Maser-Gluth, J. Mazuch, M. Kruse, A. Busjahn, A. Kramer and A. F. Pfeiffer. Changes of Dietary Fat and Carbohydrate Content Alter Central and Peripheral Clock in Humans. J Clin Endocrinol Metab. 2015 Jun; 100(6): 2291-2302.
* O. Pivovarova, K. Jurchott and N. Rudovich contributed equally

Contribution in detail: Ms Katharina Keßler significantly contributed to the data analysis and helped write the paper.

Publication 3:
Kessler K., S. Hornemann, K.J. Petzke, M. Kemper, A. Kramer, A.F.H. Pfeiffer, O. Pivovarova* and N. Rudovich*. The effect of diurnal distribution of carbohydrates and fat on glycaemic control in humans: a randomized controlled trial. Sci Rep. 2017 Mar, 7:44170
* O. Pivovarova and N. Rudovich contributed equally

Contribution in detail: Ms Katharina Keßler contributed to the designing of the trial; she conceptualised, implemented and monitored the dietary interventions; she was responsible for supervision of study participants and helped recruit them; she ran the 12 h study days in collaboration with the study doctor and study nurses; she conducted laboratory experiments and acquired data with the help of technicians; she performed the statistical analysis with the help of Dr. O. Pivovarova; she drafted and wrote the paper in close collaboration with Dr. O. Pivovarova, Prof. A.F.H. Pfeiffer and Dr. N. Rudovich; she revised the manuscript in accordance with the reviewer´s comments; she supervised the placement students, B.Sc. and M.Sc. students who contributed to the human trial.

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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SELECTED PUBLICATIONS

Publication 1:

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

Pivovarova O., Ö. Gögebakan, S. Sucher, J. Groth, V. Murahovschi, **K. Kessler**, M. Osterhoff, N. Rudovich, A. Kramer, A.F. Pfeiffer.

Int J Obes (Lond). 2016 Jun; 40(6): 899-906.

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Publication 2:

“Changes of Dietary Fat and Carbohydrate Content Alter Central and Peripheral Clock in Humans”

Pivovarova O.*, K. Jurchott*, N. Rudovich*, S. Hornemann, L. Ye, S. Mockel, V. Murahovschi, **K. Kessler**, A. C. Seltmann, C. Maser-Gluth, J. Mazuch, M. Kruse, A. Busjahn, A. Kramer and A. F. Pfeiffer.

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* O. Pivovarova, K. Jurchott and N. Rudovich contributed equally

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Publication 3:

“The effect of diurnal distribution of carbohydrates and fat on glycaemic control in humans: a randomized controlled trial”

Kessler K., S. Hornemann, K.J. Petzke, M. Kemper, A. Kramer, A.F.H. Pfeiffer, O. Pivovarova* and N. Rudovich*.

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* O. Pivovarova and N. Rudovich contributed equally

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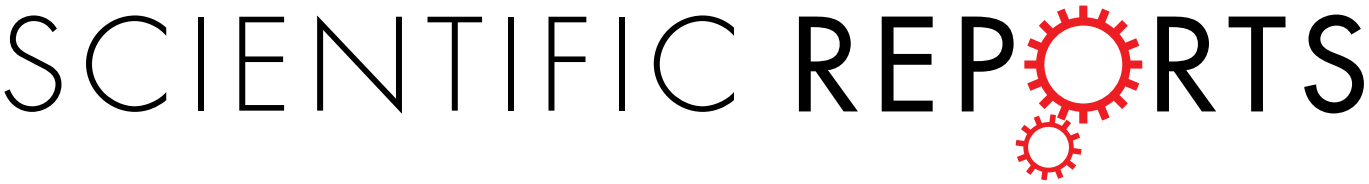

OPEN

The effect of diurnal distribution of carbohydrates and fat on glycaemic control in humans: a randomized controlled trial

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Diurnal carbohydrate and fat distribution modulates glycaemic control in rodents. In humans, the optimal timing of both macronutrients and its effects on glycaemic control after prolonged consumption are not studied in detail. In this cross-over trial, 29 non-obese men were randomized to two four-week diets: (1) carbohydrate-rich meals until 13.30 and fat-rich meals between 16.30 and 22.00 (HC/HF) *versus* (2) inverse sequence of meals (HF/HC). After each trial period two meal tolerance tests were performed, at 09.00 and 15.40, respectively, according to the previous intervention. On the HF/HC diet, whole-day glucose level was increased by 7.9% ($p = 0.026$) in subjects with impaired fasting glucose and/or impaired glucose tolerance (IFG/IGT, $n = 11$), and GLP-1 by 10.2% ($p = 0.041$) in normal glucose-tolerant subjects (NGT, $n = 18$). Diet effects on fasting GLP-1 ($p = 0.009$) and PYY ($p = 0.034$) levels were observed in IFG/IGT, but not in NGT. Afternoon decline of glucose tolerance was more pronounced in IFG/IGT and associated with a stronger decrease of postprandial GLP-1 and PYY levels, but not with changes of cortisol rhythm. In conclusion, the HF/HC diet shows an unfavourable effect on glycaemic control in IFG/IGT, but not in NGT subjects. Consequently, large, carbohydrate-rich dinners should be avoided, primarily by subjects with impaired glucose metabolism.

The control of diurnal glucose fluctuations is a crucial component of body homeostasis. Nutritional approaches are corner stones to achieving euglycaemia in diabetes and health. Today, the best dietary strategy remains unclear. The benefits of wholegrain, fruits, vegetables, nuts and legumes have been widely recognized[1]. However, recently published studies suggest that meal timing and daily eating patterns help prevent metabolic diseases[2,3], pointing towards a pivotal role of circadian rhythms in metabolism[4].

Circadian rhythms are self-sustained ~24 h rhythms in behaviour, physiology and metabolism that allow organisms the adaptation to the daily recurring day-night cycles and resultant changes in food availability[2]. Consequently, numerous processes of glucose, cholesterol and lipid metabolism, detoxification pathways and immune responses[5] display circadian oscillation.

Recent studies in rodents provide evidence that the timing of macronutrient consumption influences the circadian clock machinery and metabolism[6–10]. Under an ad libitum high fat diet, mice consume an abnormally high proportion of calories during the light phase (i.e. the sleep phase for nocturnal animals) which leads to increased adiposity and decreased glucose tolerance[8]. Remarkably, when the high fat diet was restricted to the active phase of day, mice were protected against obesity, hyperinsulinemia, hepatic steatosis and inflammation[9]. Similar results have been reported for liquid sugar and fructose intake[11,12].

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These observations support the hypothesis that consumption of fat and carbohydrates at certain time windows within the active phase might beneficially modulate metabolic homeostasis[13]. Indeed, in mice a low-fat carbohydrate-rich diet at the end of the active phase led to reduced body weight and improved glucose tolerance; conversely, mice on a high fat diet at the end of the active phase showed glucose intolerance, adiposity and features of the metabolic syndrome[14,15]. Intervention studies by Sofer *et al.* confirm the beneficial effect of carbohydrate consumption in the evening (end of the active phase)[16,17]. Eating carbohydrates mostly at dinner within a hypocaloric diet led to more pronounced weight loss, reduced hunger scores and improved metabolic status in obese subjects[16]. Similarly, carbohydrate consumption at end of the active phase resulted in the improved feeding regulation and amelioration of inflammatory parameters in mice[17]. An improvement of glycaemic control was also observed when carbohydrates were mainly eaten at dinner and protein mainly at lunch[18]. In contrast, epidemiological studies propose a beneficial effect of a carbohydrate-rich diet at the beginning of the day: an increasing carbohydrate intake at the expense of fat in the morning was shown to be protective against the development of diabetes[19] and metabolic syndrome[20].

Interestingly, a range of studies showed that identical meals result in higher postprandial elevation of plasma glucose in the evening than in the morning in healthy subjects suggesting a diurnal variation of glucose tolerance during the investigation day[21–23]. In particular, Morgan *et al.* compared the metabolic effects of varying both dietary glycaemic index (GI) and the time at which most daily energy intake was consumed[24]. Consumption of an energy-rich meal in the evening led to significantly higher glucose and insulin response compared to its consumption in the morning. Markedly, this effect was most pronounced in the evening on a high GI diet, confirming that the quality of carbohydrates at a particular time of the day acutely influences glycaemic control throughout the day[24,25].

Taken together, these studies suggest that time of day-dependent carbohydrate and fat intake alter alters metabolism both in rodents and humans. Thus the detection of the best time for carbohydrate (and fat) consumption in humans as well as the effect of a prolonged consumption of such diet for glycaemic control are of scientific and clinical interest. We therefore investigated the metabolic effect of four-week HC/HF diet (carbohydrate-rich meals in the morning and fat-rich meals in the afternoon) *versus* HF/HC diet (the inverse sequence of meals) in male subjects without diabetes. The primary objective of our study was to compare the effect of both diets on fasting and whole-day levels of glucose and glucose-regulating hormones. Our secondary objective was to analyse the effect of daily carbohydrate and fat distribution on diurnal variation in glucose tolerance. The main findings of this study are: firstly, the HF/HC diet shows an unfavourable effect on the glycaemic control in subjects with an impaired glucose tolerance, but not in subjects with a normal glucose tolerance; secondly, this effect could be explained by the stronger afternoon decline of glucose tolerance in subjects with impaired glucose metabolism.

Results

Study subjects, dietary compliance, and anthropometric parameters. In this cross-over trial, non-obese male subjects without diabetes were randomized to two isocaloric four-week diets: (1) carbohydrate-rich meals until 13.30 h and fat-rich meals between 16.30 h and 22.00 h (HC/HF) *versus* (2) the inverse sequence of meals (HF/HC) (Fig. 1). Within each diet energy intake was equally distributed between the morning and afternoon. Interventions were followed by 12 h investigation days with carbohydrate-rich (MTT-HC) and fat-rich (MTT-HF) meal tolerance tests (MTT), provided either at 09.00 or 15.40, according to the participant's previous dietary intervention (Fig. 1).

Between January 2014 and July 2015, 32 men started the trial. Three participants dropped out (Supplemental Figure 1). 29 subjects (age 45.9 ± 2.5 years, body mass index (BMI) $27.1 \pm 0.8\,kg/m^2$) completed the trial. 18 subjects were normal glucose tolerant (NGT); 11 showed an impaired fasting glucose (IFG) and/or an impaired glucose tolerance (IGT) (Table 1). Compared with NGT group, IFG/IGT subjects showed higher fasting triglyceride levels ($p = 0.044$) and a tendency towards an increased fasting glucose levels ($p = 0.064$) (Table 1). Chronotype distribution of study subjects is shown in Supplemental Fig. 2A.

Adherence to dietary plans was good, with very similar compliances for the HC/HF diet and the HF/HC diet. For the HC/HF diet, 12073.4 ± 442.3 kJ were consumed in the course of a day, consisting of 49.1 ± 0.7 energy percent (EN%) carbohydrates (CHO), 36.3 ± 0.7 EN% fat and 14.7 ± 0.2 EN% protein. For the HF/HC diet, 11826.3 ± 415.1 kJ were consumed in the course of a day, consisting of 48.7 ± 0.7 EN% CHO, 36.7 ± 0.8 EN% fat and 14.6 ± 0.2 EN% protein. There was no difference between the two diets regarding energy intake, macronutrient composition, amount of saturated fatty acids, fibre and starch as well as GI (Supplemental Table 1). A detailed fragmentation of the compliance for the morning part (06.00–13.30) and the afternoon part (16.30—22.00) is shown in Supplemental Table 1. No major problems consuming the prescribed foods were reported.

Despite of extensive dietary advice, body weight slightly declined after both diets (−0.7% for NGT on the HC/HF diet, non-significant on the HF/HC diet and for IFG/IGT) without difference between the diets (Tables 2 and 3).

Fasting parameters in response to the HC/HF diet *versus* the HF/HC diet. After four weeks of intervention, both the HC/HF diet and the HF/HC diet reduced fasting levels of glucose, C-peptide, glucagon and lipid parameters (total, HDL and LDL cholesterol) in NGT subjects (Table 2). Similarly, fasting levels of glucose, C-peptide, and HDL cholesterol declined in IFG/IGT subjects (Table 3). For these parameters, no significant differences between the effects of the HC/HF and the HF/HC diet and between NGT and IFG/IGT groups were observed. Interestingly, fasting glucagon like peptide 1 (GLP-1) and peptide YY (PYY) levels showed a diet effect in IFG/IGT subjects ($p = 0.009$ and $p = 0.034$, respectively), but not in NGT subjects. Indeed, in the IFG/IGT group, the HC/HF diet induced a strong reduction of GLP-1 level by 45%, whereas no significant change after the HF/HC intervention was found. Fasting PYY also declined after the HC/HF diet by 4.5% and was not altered after the HF/HC diet (Table 3). In the NGT group, post-intervention fasting glucose dependent insulinotropic peptide (GIP) levels were higher after the HC/HF diet relative to the HF/HC diet, but its diet-induced changes did not

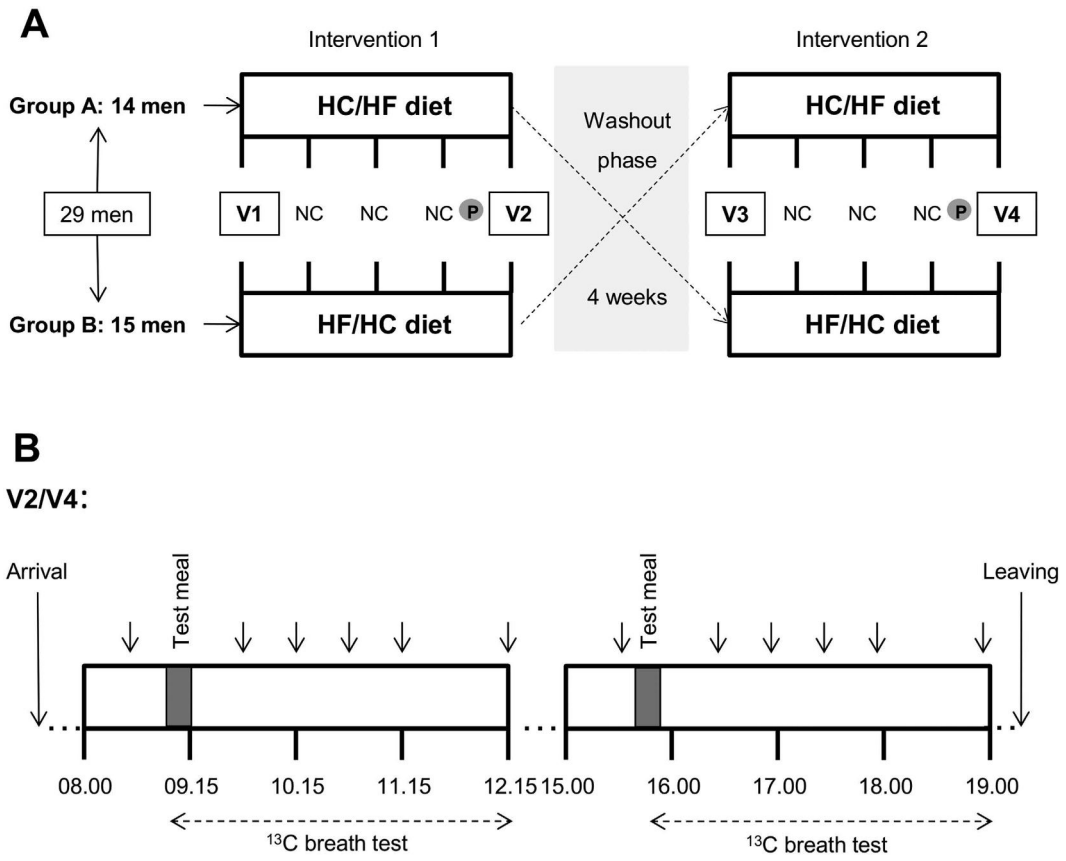

Figure 1. Study design. (**A**) In this cross-over study, two four-week dietary intervention periods were separated by a washout phase. HC/HF diet, isocaloric carbohydrate-rich meals until 13.30 and isocaloric fat-rich meals between 16.30 and 22.00; HF/HC diet, reversed order of meal sequence; V, visit; NC, nutritional counselling; P, pedometer. (**B**) Clinical investigation day. At 09.00 and 15.40 a standardized test meal – fat-rich or carbohydrate-rich – (grey bars) was provided according to participant's previous intervention. Blood samples (arrows) were drawn and a ^{13}C breath test (dotted arrows) performed.

differ between interventions (Table 2). No effect on insulin sensitivity measured by HOMA-IR was observed in either of the groups (Table 2 and 3).

Whole-day levels in response to the HC/HF diet *versus* the HF/HC diet. To determine the effect of both diets on whole-day levels, integrated areas under the curve (AUC) over both MTT (AUCday) were calculated as described in Methods. Again, different diet effects in NGT and IFG/IGT subjects were found. AUCday for glucose was increased by 7.9% on the HF/HC diet compared with the HC/HF diet in IFG/IGT subjects, but not in NGT subjects (Table 4). Conversely, a 10.2% increase in whole-day GLP-1 was observed on the HF/HC diet in NGT subjects, but not in IFG/IGT subjects. On the HF/HC diet, AUCday for free fatty acids (FFA) was significantly increased in IFG/IGT subjects compared to NGT subjects (Table 4). Statistical analysis revealed no difference in diet-induced changes between NGT and IFG/IGT groups. Whole-day levels of other studied parameters (Table 4) including hunger and satiety scores (Supplemental Figure 3) showed no differences between the diets and NGT and IFG/IGT subjects.

Diurnal variation of the metabolic response to MTT-HC. To analyse the effects of both diets on the diurnal variation of glycaemic control, we compared postprandial responses of glucose and glucose-regulating hormones to the same meal (MTT-HC or MTT-HF) in the morning and in the afternoon in the NGT and IFG/IGT group. For this, we defined the diurnal variation in a variable as Δ = afternoon value − morning value.

Analysis of the metabolic response to MTT-HC revealed an impairment of glucose tolerance in the afternoon. For glucose, postprandial peak and the incremental area under the curve ($iAUC_{0\text{-}180}$) were markedly higher in the afternoon than in the morning. Importantly, afternoon increase of $iAUC_{0\text{-}180}$ for glucose was much more pronounced in IFG/IGT subjects compared with NGT subjects (4.5-fold vs. 2.5-fold, respectively) (Fig. 2A). This suggests a stronger decrease of glucose tolerance in IFG/IGT individuals at the end of the day. Notably, after peaking, glucose levels deceased rapidly after the morning meal, whereas in the afternoon glucose levels persisted and remained significantly higher (Fig. 2A).

For insulin, the meal-induced response was rapid and fast in the morning, with peak levels at 30 minutes, while in the afternoon, insulin secretion peaked later (approximately at 2 h). Yet, the overall insulin secretion ($iAUC_{0\text{-}180}$) was increased in the afternoon only in IFG/IGT subjects (Fig. 2B). Early and overall indices of insulin

	All	NGT subjects	IGT/IFG subjects	p-value*
N (% male)	29 (100)	18 (100)	11 (100)	
Age [years]	45.90 ± 2.54	43.83 ± 3.34	49.27 ± 3.83	0.306
Anthropometric measurements				
Chronotype [MSF-Sc]	3.44 ± 0.19	3.42 ± 0.27	3.45 ± 0.18	0.924
Weight [kg]	87.04 ± 2.85	86.06 ± 3.75	88.65 ± 4.52	0.611
BMI [kg/m^2]	27.07 ± 0.75	27.13 ± 0.95	26.95 ± 1.31	0.911
Waist circumference [cm]	93.55 ± 2.09	92.06 ± 2.37	96.00 ± 3.95	0.396
Waist-to-hip ratio	0.91 ± 0.01	0.90 ± 0.01	0.94 ± 0.03	0.100
Lipid metabolism				
Total cholesterol [mmol/l]	5.24 ± 0.18	5.24 ± 0.17	5.25 ± 0.40	0.978
HDL cholesterol [mmol/l]	1.20 ± 0.04	1.24 ± 0.05	1.13 ± 0.07	0.222
LDL cholesterol [mmol/l]	3.48 ± 0.17	3.51 ± 0.17	3.42 ± 0.37	0.841
Triglycerides [mmol/l]	1.25 ± 0.14	1.08 ± 0.13	1.52 ± 0.30	**0.044**
NEFA [mmol/l]	0.49 ± 0.03	0.48 ± 0.04	0.50 ± 0.03	0.834
Glucose metabolism				
Glucose [mmol/l]	5.83 ± 0.12	5.65 ± 0.14	6.12 ± 0.21	0.064
Insulin [pmol/l]	34.27 ± 5.22	31.23 ± 6.07	39.22 ± 9.70	0.580
HOMA-IR [mmol*mU *l^{-2}]	1.55 ± 0.26	1.36 ± 0.28	1.87 ± 0.50	0.363

Table 1. Clinical characteristics of study participants. Data were collected at visit 1 (start of first intervention period). Data are shown as mean ± SEM. MSF-Sc, mid-sleep time point on free days adjusted for individual average sleep need accumulated on work days determined using MCTQ [58]; NGT, normal glucose tolerance, IFG, impaired fasting glucose, IGT, impaired glucose tolerance. *Statistical differences between group NGT and IFG/IGT.

	HC/HF diet			HF/HC diet				
	Pre	Post	Δ%	Pre	Post	Δ%	P[a]	P$_{corr}$[b]
Weight [kg]	86.0 ± 3.7	85.5 ± 3.7	−0.7*	86.1 ± 3.8	85.8 ± 3.8	−0.3	0.398	
BMI [kg/m^2]	27.1 ± 0.9	26.9 ± 0.9	−0.8**	27.2 ± 1.0	27.0 ± 1.0	−0.4	0.283	
Glucose metabolism								
Glucose [mmol/l]	5.75 ± 0.13	5.26 ± 0.08	−8.4**	5.70 ± 0.12	5.24 ± 0.09	−8.1**	0.692	0.431
Insulin [pmol/l]	32.48 ± 6.18	31.69 ± 3.45	−2.5	33.00 ± 5.51	29.44 ± 4.56	−10.2	0.584	0.560
C-peptide [μg/l]	2.22 ± 0.65	1.13 ± 0.11	−49.0	2.43 ± 0.73	1.01 ± 0.12	−58.3*	0.994	0.914
HOMA-IR [mmol· mU· l^{-2}]	1.42 ± 0.29	1.24 ± 0.14	−12.8	1.42 ± 0.25	1.15 ± 0.18	−19.4	0.576	0.567
Glucagon [pmol/l]	6.62 ± 0.81	5.66 ± 0.63	−14.4*	6.79 ± 0.84	5.75 ± 0.85	−15.4*	0.979	0.950
GIP [pg/ml]	67.28 ± 8.89	71.34 ± 8.52	6.0	62.90 ± 6.72	51.52 ± 4.72[#]	−18.1	0.368	0.383
GLP-1 [pg/ml]	1.86 ± 0.64	1.26 ± 0.40	−34.4	1.20 ± 0.28	0.75 ± 0.10	−37.5	0.701	0.632
PYY [pg/ml]	65.77 ± 8.63	56.12 ± 6.20	−14.7	59.36 ± 5.27	58.84 ± 7.09	−0.9	0.754	0.765
Lipid metabolism								
Total cholesterol [mmol/l]	5.23 ± 0.15	4.72 ± 0.17	−9.7**	5.21 ± 0.17	4.82 ± 0.21	−7.5**	0.575	0.807
HDL cholesterol [mmol/l]	1.28 ± 0.05	1.10 ± 0.04	−13.8**	1.23 ± 0.05	1.10 ± 0.04	−10.7**	0.324	0.233
LDL cholesterol [mmol/l]	3.53 ± 0.15	3.18 ± 0.16	−9.9**	3.46 ± 0.18	3.20 ± 0.21	−7.4**	0.435	0.589
Triglycerides [mmol/l]	0.95 ± 0.09	0.98 ± 0.09	−3.8	1.13 ± 0.13	1.12 ± 0.11	−0.2	0.851	0.947
NEFA [mmol/l]	0.48 ± 0.04	0.45 ± 0.03	−6.6	0.51 ± 0.04	0.42 ± 0.03	−17.4	0.115	0.139

Table 2. Fasting parameters in response to HC/HF diet and HF/HC diet in NGT subjects. *$p < 0.05$, **$p < 0.01$ for difference from baseline. [§]Pre-intervention difference between HC/HF diet and HF/HC diet, $p < 0.05$. [#]Post-intervention difference between HC/HF diet and HF/HC diet, $p < 0.05$. [a]Comparison of changes after HC/HF diet and HF/HC diet in the linear mixed model [b]Comparison of changes after HC/HF diet and HF/HC diet in the linear mixed model after correction for weight change.

secretion ($iAUC_{ins/glu\ 0\text{-}30}$ and $iAUC_{ins/glu\ 0\text{-}180}$) and Gutt index of insulin sensitivity were decreased in the afternoon both in NGT and IFG/IGT subjects without differences between groups (Table 5). C-peptide mirrored insulin levels (Fig. 2C) but showed no significant diurnal variation, neither did hepatic insulin clearance (iHIC) (Table 5).

The $iAUC_{0\text{-}180}$ for glucagon was reduced in the afternoon without differences between NGT and IFG/IGT groups (Fig. 3A). Interestingly, GLP-1 secretion showed a pronounced afternoon decline only in IFG/IGT subjects (Fig. 3C) ($p = 0.188$ for difference of Δ between NGT and IFG/IGT). Similarly, postprandial PYY secretion also showed a trend for diminished afternoon levels only in IFG/IGT subjects (Fig. 4A) ($p = 0.071$ for difference of Δ between NGT and IFG/IGT). Postprandial GIP decreased in the afternoon, but not significantly (Fig. 3B).

	HC/HF diet			HF/HC diet				
	Pre	Post	Δ%	Pre	Post	Δ%	P[a]	P_{corr}[b]
Weight [kg]	88.2 ± 4.6	87.8 ± 4.6	−0.4	88.7 ± 4.6	88.0 ± 4.5	−0.7	0.363	
BMI [kg/m^2]	26.8 ± 1.3	26.7 ± 1.3	−0.5	27.0 ± 1.3	26.8 ± 1.3	−0.7	0.394	
Glucose metabolism								
Glucose [mmol/l]	6.18 ± 0.22	5.48 ± 0.13	−11.4**	6.07 ± 0.16	5.49 ± 0.15	−9.6**	0.655	0.574
Insulin [pmol/l]	42.53 ± 11.02	33.22 ± 5.00	−21.9	45.87 ± 12.97	33.44 ± 6.79	−27.1	0.719	0.804
C-peptide [μg/l]	2.18 ± 0.44	1.25 ± 0.22	−42.6*	2.53 ± 0.65	1.25 ± 0.18	−50.6*	0.601	0. 515
HOMA-IR [mmol· mU· l^{-2}]	2.08 ± 0.56	1.38 ± 0.23	−33.8	2.15 ± 0.64	1.40 ± 0.31	−34.7	0.740	0.802
Glucagon [pmol/l]	8.53 ± 0.01	7.33 ± 1.32	−14.0	8.26 ± 0.90	7.06 ± 1.3	−14.7	0.639	0.949
GIP [pg/ml]	62.63 ± 9.24	61.76 ± 5.46	−1.4	74.47 ± 12.12	57.57 ± 8.05	−22.7	0.826	0.995
GLP-1 [pg/ml]	1.55 ± 0.35	0.85 ± 0.17	−45.0*	1.01 ± 0.23[§]	0.87 ± 0.25[#]	−13.3	**0.009**	**0.007**
PYY [pg/ml]	55.46 ± 9.33	52.45 ± 9.64	−4.5*	48.78 ± 8.65	52.46 ± 8.88	7.5	**0.034**	**0.029**
Lipid metabolism								
Total cholesterol [mmol/l]	5.19 ± 0.38	4.86 ± 0.34	−6.3	5.30 ± 0.40	4.97 ± 0.32	−6.1	0.285	0.061
HDL cholesterol [mmol/l]	1.18 ± 0.06	1.05 ± 0.05	−10.7**	1.17 ± 0.08	1.03 ± 0.05	−11.9*	0.107	**0.016**
LDL cholesterol [mmol/l]	3.34 ± 0.34	3.26 ± 0.29	−2.2	3.47 ± 0.36[§]	3.38 ± 0.27	−2.6	0.648	0.385
Triglycerides [mmol/l]	1.49 ± 0.30	1.21 ± 0.25	−18.7	1.45 ± 0.38	1.24 ± 0.24	−14.3	0.787	0.821
NEFA [mmol/l]	0.54 ± 0.05	0.46 ± 0.04	−14.6	0.45 ± 0.04	0.49 ± 0.06	8.0	0.965	0.929

Table 3. Fasting parameters in response to HC/HF diet and HF/HC diet in IGT/IFG subjects. *$p < 0.05$, **$p < 0.01$ for difference from baseline. [§]Pre-intervention difference between HC/HF diet and HF/HC diet, $p < 0.05$. [#]Post-intervention difference between HC/HF diet and HF/HC diet, $p < 0.05$. [a]Comparison of changes after HC/HF diet and HF/HC diet in the linear mixed model. [b]Comparison of changes after HC/HF diet and HF/HC diet in the linear mixed model after correction for weight change.

	NGT			IFG/IGT		
	HC/HF diet	HF/HC diet	Δ %	HC/HF diet	HF/HC diet	Δ %
Glucose [mmol/l·6 h]	2199 ± 45	2233 ± 56	1.6	2196 ± 92	2370 ± 87	7.9*
Insulin [pmol/l·6 h]	84412 ± 9146	83926 ± 8355	−0.6	78105 ± 9176	94762 ± 20283	21.3
C-peptide [μg/l·6 h]	1696 ± 117	1740 ± 126	2.6	1748 ± 139	1870 ± 189	7.0
Glucagon [pmol/l·6 h]	2280 ± 239	2282 ± 291	0.1	2673 ± 258	2653 ± 378	−0.7
GIP [pg/ml·6 h]	190981 ± 13539	185860 ± 8762	−2.7	192525 ± 20800	207118 ± 18330	7.6
GLP-1 [pg/ml·6 h]	1113 ± 66	1227 ± 77	10.2*	1203 ± 191	1339 ± 240	11.2
PYY [pg/ml 6 h]	25838 ± 1433	26062 ± 1773	0.9	25383 ± 4627	26273 ± 4423	3.5
FFA [mmol/l·6 h]	80.3 ± 3.6	79.2 ± 3.5	−1.4	94.6 ± 8.0	100.9 ± 8.0[§]	6.7

Table 4. Whole-day levels of metabolic markers in response to HC/HF diet and HF/HC diet. For FFA, glucose and meal-induced hormone secretion (insulin, C-peptide, glucagon, GIP, GLP-1, PYY), integrated AUCs after both MTT1 and MTT2 (AUC_{day}) are shown. *$p < 0.05$ for a difference between HC/HF diet and HF/HC diet; [§]$p < 0.05$ for a difference between NGT and IGT/IFG subjects.

FFA response showed no significant diurnal variation (Fig. 4B), although afternoon $iAUC_{0\text{-}180}$ for FFA was higher in IFG/IGT subjects ($p = 0.044$) because of the reduced early postprandial suppression.

Correlation analysis of diurnal differences revealed that, in NGT subjects, diurnal variation of glucose levels (Δ $iAUC_{0\text{-}180}$) positively correlated with insulin variation ($r = 0.595$, $p = 0.009$). Remarkably, in IFG/IGT subjects, glucose excursion (Δ $iAUC_{0\text{-}180}$) did not correlate with insulin, but correlated negatively with diurnal glucagon ($r = -0.709$, $p = 0.015$), GLP-1 ($r = -0.809$, $p = 0.003$) and PYY ($r = -0.845$, $p = 0.001$) variation. Moreover, PYY decline correlated with a decrease in glucagon ($r = 0.664$, $p = 0.026$), GIP ($r = 0.618$, $p = 0.043$) and GLP-1 ($r = 0.845$, $p = 0.001$) response throughout the day.

Analysis of gastric emptying revealed no significant diurnal variation although a trend towards slower gastric emptying in the afternoon was observed (Table 5).

Diurnal variation of the metabolic response to MTT-HF. As expected, postprandial glucose, insulin, and C-peptide levels were lower and postprandial glucagon, GIP, GLP-1, PYY and FFA levels were higher after the MTT-HF compared with MTT-HC. As for MTT-HC, analysis of the metabolic response to MTT-HF revealed a decrease of glucose tolerance as the day progresses. In the afternoon, peaking of glucose levels was delayed and increased, and $iAUC_{0\text{-}180}$ strongly increased in both NGT and IFG/IGT subjects without difference between these groups (Fig. 2A). For insulin, $iAUC_{0\text{-}180}$ was significantly higher in the afternoon only in NGT subjects (Fig. 2B).

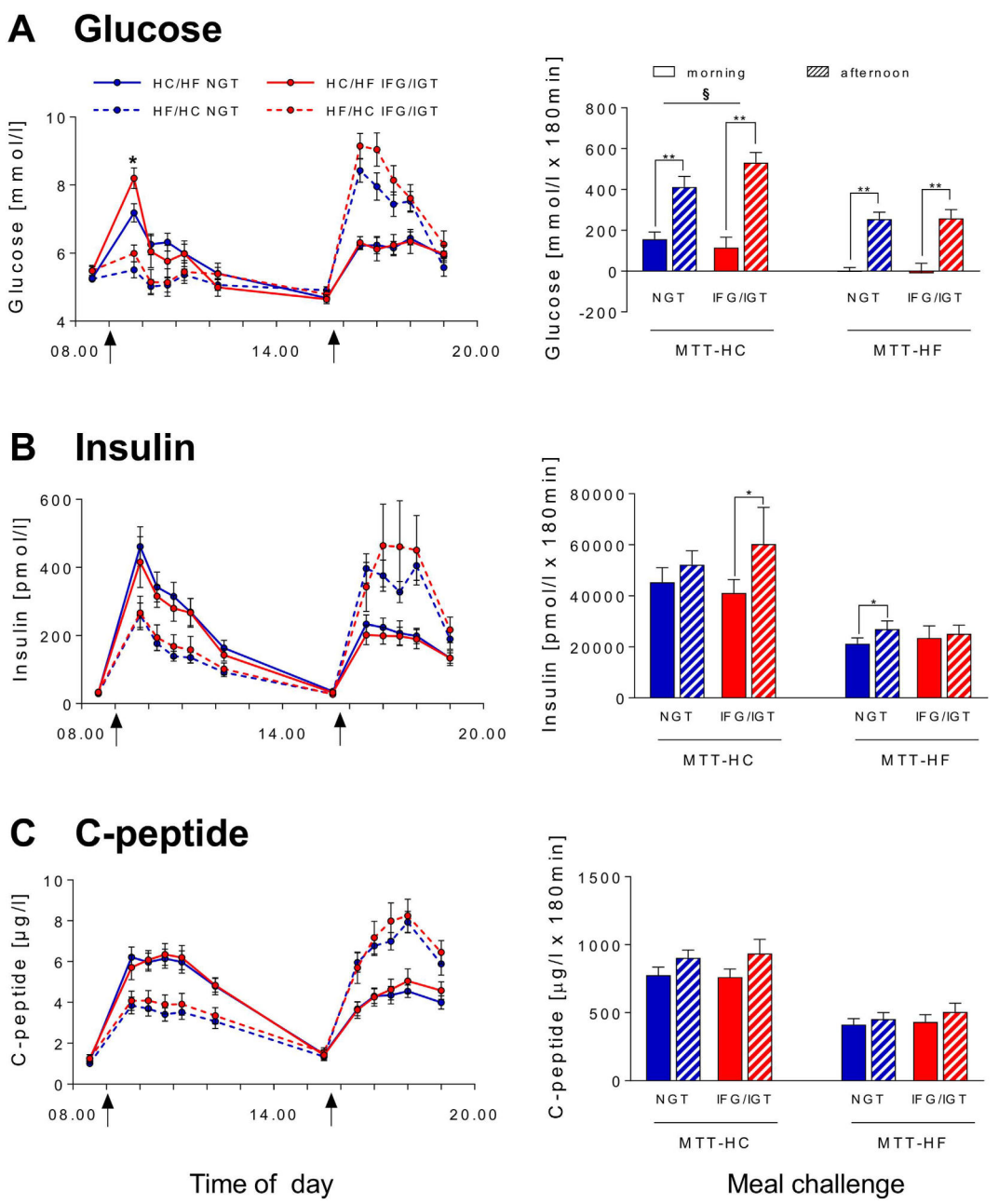

Figure 2. Effects of the HC/HF diet (solid lines) and the HF/HC diet (dotted lines) on pre- and postprandial glucose (**A**), insulin (**B**) and C-peptide (**C**) levels (left panel) and $iAUC_{0\text{-}180}$ (right panel) in NGT subjects (blue) and IFG/IGT subjects (red). Here and elsewhere: arrow - test meal; MTT-HC, carbohydrate-rich meal tolerance test; MTT-HF, fat-rich meal tolerance test. Left panel: $^{*}p < 0.05$ - NGT subjects *vs* IFG/IGT subjects for HC/HF diet; $^{\#}p < 0.05$ – NGT subjects *vs*. IFG/IGT subjects for HF/HC. Right panel: $^{*}p < 0.05$, $^{**}p < 0.01$ - afternoon *vs*. morning; $^{\S}p < 0.05$ – NGT subjects *vs*. IFG/IGT subjects for diurnal variation (afternoon value - morning value).

Both insulin and glucose rapidly decreased in the morning and remained elevated in the afternoon (Fig. 2A,B). For C-peptide, peak levels were delayed and increased, although the overall secretion was not dependent on the time of the day (Fig. 2C). In the afternoon, Gutt index showed an afternoon decline in both groups, which was significant only in NGT subjects (Table 5). Diurnal variation of insulin secretion index $iAUC_{ins/glu}$ did not reached statistical significance (Table 5). iHIC decreased in the afternoon only in NGT subjects (Table 5).

Postprandial glucagon decline in the afternoon reached statistical significance only in IFG/IGT subjects (Fig. 3A). Postprandial GIP did also not decrease significantly (Fig. 3B). Similarly to the MTT-HC, GLP-1 and PYY secretion showed pronounced afternoon decline in IFG/IGT subjects (Figs 3C and 4A) ($p = 0.188$ and $p = 0.083$, respectively, for difference of Δ between NGT and IFG/IGT). Postprandial FFA levels were higher in IFG/IGT subjects, but no significant diurnal variation was found in either of the groups (Fig. 4B).

	Meal	NGT			IFG/IGT			P-value (Δ NGT vs. Δ IFG/IGT)
		Morning	Afternoon	Δ	Morning	Afternoon	Δ	
Indices of glucose metabolism								
$iAUC_{ins/glu\ 0\text{-}30}$	HC	305.3 ± 67.6	91.0 ± 24.3	−214.3	149.1 ± 28.9[§]	73.7 ± 13.6	**−75.4****	0.122
	HF	622.9 ± 270.9	147.4 ± 23.7	−475.5	361.5 ± 115.4	114.0 ± 20.5	−247.5	1.000
$iAUC_{ins/glu\ 0\text{-}180}$	HC	384.2 ± 189.0	117.7 ± 19.4	**−266.5***	339.5 ± 142.8	114.0 ± 20.6	−225.5	0.982
	HF	184.8 ± 124.5	81.7 ± 58.2	−103.1	−116.2 ± 137.9	110.9 ± 15.2	227.1	0.191
ISI $Gutt_{0\text{-}120}$ [mg*l²/mmol*mU*min]	HC	65.5 ± 3.9	53.5 ± 3.3	**−12.0***	65.7 ± 5.5	49.8 ± 3.5	**−15.9****	0.549
	HF	88.6 ± 4.7	75.3 ± 3.2	**−13.3****	89.0 ± 7.9	78.8 ± 6.5	−10.2	0.511
iHIC [AU]	HC	6.6 ± 0.5	6.3 ± 0.4	−0.3	6.8 ± 0.6	6.3 ± 0.6	−0.5	0.600
	HF	6.7 ± 0.4	5.9 ± 0.4	**−0.8****	6.8 ± 0.5	6.8 ± 0.4	0.0	0.130
Gastric emptying								
T1/2 [min]	HC	157.6 ± 20.4	172.6 ± 17.4	−15.0	162.4 ± 18.0	197.9 ± 33.4	35.5	0.675
	HF	152.8 ± 12.8	142.9 ± 28.4	−9.9	207.7 ± 31.7	154.5 ± 29.2	−53.2	0.577
Tlag [min]	HC	108.4 ± 13.4	139.9 ± 14.9	31.5	106.5 ± 18.4	158.5 ± 25.9	52.0	0.654
	HF	110.8 ± 11.4	118.5 ± 23.2	7.7	155.0 ± 25.2	118.9 ± 24.8	−36.1	0.540
Cortisol[a] [ng/ml]	HC/HF	91.7 ± 7.8	34.6 ± 2.8	−57.1**	103.9 ± 12.6	36.9 ± 4.2	−67.0**	0.434
	HF/HC	101.0 ± 6.2	35.2 ± 2.7	−65.8**	100.1 ± 9.4	38.2 ± 5.6	−61.9**	0.716

Table 5. Indices of glucose metabolism, gastric emptying and cortisol levels in the morning *versus* afternoon in NGT and IFG/IGT subjects. T1/2 - half gastric emptying time; Tlag - time of fastest gastric emptying; iHIC – incremental hepatic insulin clearance; HC – carbohydrate-rich meal; HF – fat-rich meal. Diurnal variation in a variable is defined as Δ = afternoon value – morning value. Negative values of Δ are referred to as the afternoon decline in the given variable. *$p < 0.05$, **$p < 0.01$ for difference between morning and afternoon. [§]Difference between NGT and IFG/IGT subjects in the morning, $p < 0.05$. [a]For cortisol, values before the morning meal and after the afternoon meal are shown.

Correlation analysis of diurnal differences for MTT-HF revealed that, in NGT subjects, diurnal variation of glucose levels (Δ $iAUC_{0\text{-}180}$) correlated with response variation of insulin ($r = 0.738$, $p < 0.001$) and glucagon ($r = -0.556$, $p = 0.020$); and insulin and glucagon variations were associated with each other ($r = -0.568$, $p = 0.17$). In IFG/IGT subjects, glucose variation correlated positively with diurnal insulin pattern ($r = 0.745$, $p = 0.008$).

Diurnal variation of cortisol levels on the HC/HF diet *versus* the HF/HC diet. Because the impairment of the glycaemic control in the evening is related to the cortisol rhythm[26], we analysed diurnal variation of circulating cortisol level on both diets. As expected, cortisol levels were higher in the morning than in the afternoon (Supplemental Figure 2B). We observed no differences in diurnal cortisol variation (defined as Δ) between diets and between NGT and IFG/IGT subjects (Table 5, Supplemental Figure 2B). Moreover, no correlation of diurnal cortisol variation with afternoon increase of $iAUC_{0\text{-}180}$ for glucose and insulin and with afternoon decrease of insulin sensitivity (defined as Gutt index) was found.

Discussion

The present study is, to our knowledge, the first human trial investigating the effect of a prolonged diurnal distribution of carbohydrate and fat intake on glycaemic control. Surprisingly, we found different metabolic responses in subjects with different stages of glucose tolerance. The HF/HC diet shows an unfavourable effect on the glycaemic control in IFG/IGT subjects, but not in NGT subjects.

Indeed, in IFG/IGT subjects, whole-day glucose level was increased by 7.9% on the HF/HC diet, whereas in NGT subjects it did not differ between the diets. Notably, in NGT group, whole-day level of the incretin hormone GLP-1 was increased by 10.2% on the HF/HC diet, which, however, was not accompanied by an increase in whole-day insulin level. A differential regulation between NGT and IFG/IGT subjects is further proposed by the differences in fasting parameters between the two groups. For NGT, none of the fasting parameters showed a diet effect suggesting a compensatory mechanism in healthy subjects[27]. However, IFG/IGT subjects showed a diet effect for fasting GLP-1 and PYY levels, suggesting that people with an impaired glycaemic control are more susceptible to a diurnal carbohydrate and fat distribution. Remarkably, in neither group, we observed a different effect of the high-carbohydrate and high-fat afternoon meal on fasting triglyceride and fatty acid levels, described previously[28].

Our data on the unfavourable effect of the HF/HC diet on the glycaemic control in IFG/IGT subjects are in contrast to the results of the mouse study showing that a carbohydrate-rich diet at the end of the active phase led to improved glucose tolerance[14]. Our observation is of great clinical importance suggesting that the avoidance of carbohydrate-rich meals in the evening in people with impaired glucose tolerance should be recommended. In line with this recommendation are previous studies showing that an increasing carbohydrate intake at the expense of fat in the morning seems protective against the development of diabetes[19] and metabolic syndrome[20]. Conversely, consumption of high GI food in the evening seems most detrimental for human health[24].

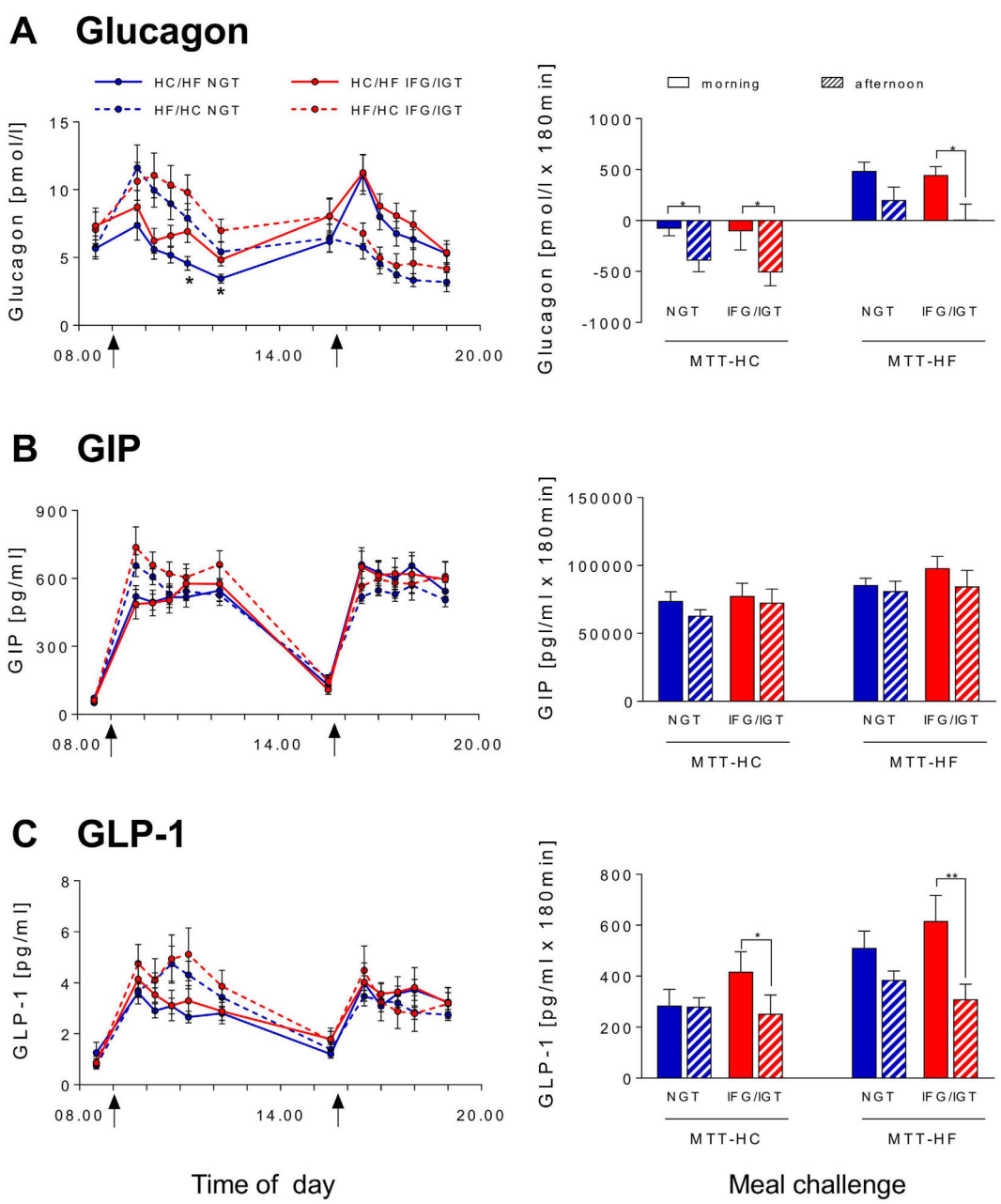

Figure 3. Effects of the HC/HF diet (solid lines) and the HF/HC diet (dotted lines) on pre- and postprandial glucagon (**A**), GIP (**B**) and GLP-1 (**C**) levels (left panel) and $iAUC_{0\text{-}180}$ (right panel) in NGT subjects (blue) and IFG/IGT subjects (red).

To understand the mechanisms of the unfavourable effect of the HF/HC diet, we compared the effects of both diets on diurnal variation of glycaemic control. In our study, postprandial glucose responses were largely increased and delayed in the afternoon independent of meal composition, confirming the decline in glucose tolerance in the evening, as demonstrated in healthy humans by a range of previous studies[21–23,29]. Importantly, the evening decline of glucose tolerance was more pronounced in IFG/IGT subjects. We observed this phenomenon only for the high-carbohydrate meal, which could be explained by the larger postprandial glucose excursion that allowed detecting diurnal differences between morning and afternoon meal. Similar results were shown in an earlier study examining oral glucose tolerance tests in large cohorts in the morning (0930 h) and early afternoon (1300–1400 h)[30]. In this study, subjects with normal fasting glucose showed a small diurnal variation in their glucose tolerance, with less homeostatic control in their afternoon test, while subjects with mildly elevated fasting glucose showed an enhanced diurnal variation[30]. Recent studies confirm the progressive increase in 24 h glycaemic variability from NGT to IFG/IGT subjects[31,32]. Sonnier *et al.* showed that insulin resistant subjects suffered from a loss of rhythm in insulin sensitivity, which was partially compensated by an enhancement of the rhythm in insulin levels[26]. Two other studies found increased 24 h profiles of glucose and C-peptide in IFG subjects[33] and first-degree relatives of type 2 diabetic patients[34], with conflicting results regarding 24 h insulin levels.

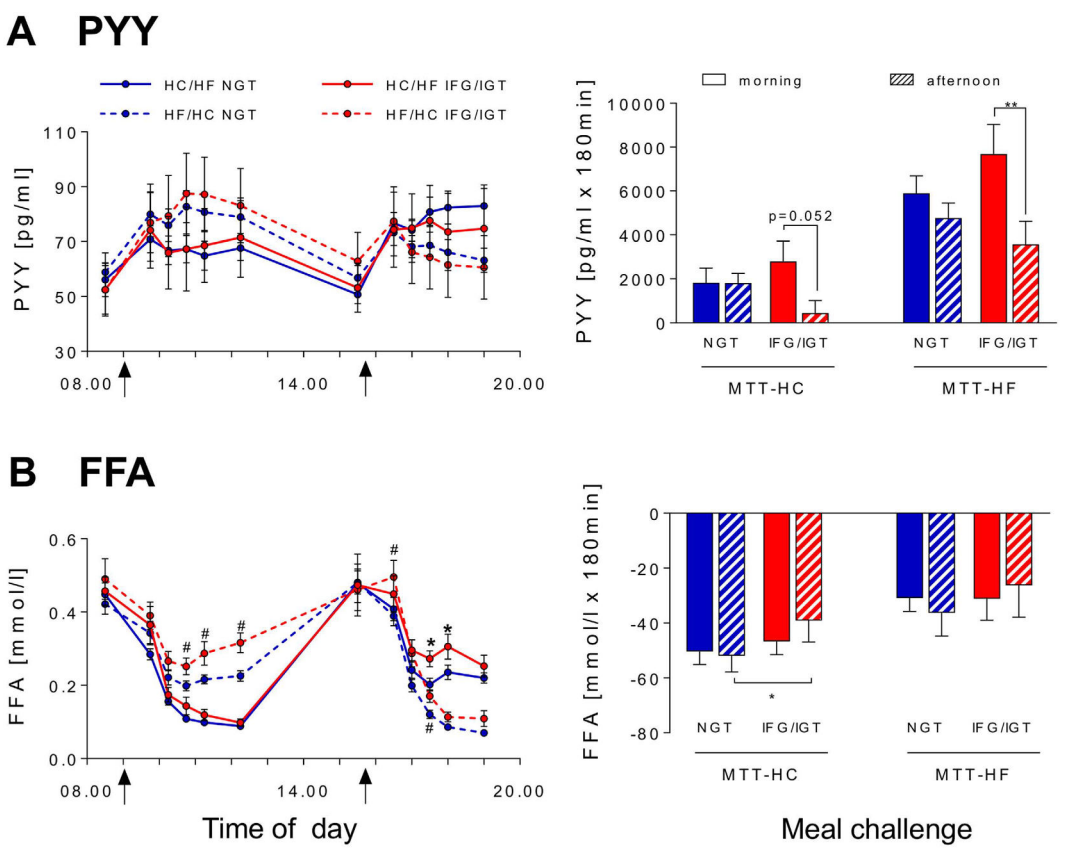

Figure 4. Effects of the HC/HF diet (solid lines) and the HF/HC diet (dotted lines) on pre- and postprandial PYY (**A**) and FFA (**B**) levels (left panel) and $iAUC_{0\text{-}180}$ (right panel) in NGT subjects (blue) and IFG/IGT subjects (red).

The higher postprandial glucose response to the high-carbohydrate meal in combination with the worsened glucose tolerance in IFG/IGT subjects in the afternoon might explain the increase in whole-day glucose level, observed on the HF/HC diet in IFG/IGT subjects. These results are of great practical importance as they extend the current evidence on the favourable effect of high caloric intake in the morning over high caloric intake in the evening[35,36]. Eating the main meal early in the day may therefore be a beneficial strategy to counteract the afternoon/evening impairment of glycaemic control.

Multiple mechanisms contribute to the reduced glucose tolerance in the evening[13,37] including decreased insulin sensitivity, elevated hepatic glucose production, and decreased β cell function. In healthy humans, both insulin sensitivity and β cell responsivity to glucose are reduced in the evening[38] whereas the data on the diurnal hepatic glucose production are controversial[22,39,40]. Consistent with previous observations[22,41], we found an increase in insulin resistance and a decrease in early insulin secretion in the afternoon.

Experiments with tissue-specific disruption of circadian rhythms in rodents have identified major roles of peripheral clocks in glucose homeostasis[42,43]. Protocols precisely controlling behavioural rhythms showed that the endogenous circadian time is a powerful determinant of glycaemic control in humans[23]. Multiple other factors modulate the rhythm of glucose tolerance in humans including sleep/wake cycle and daily variation of cortisol[44], adrenocorticotropic hormone[45], glucagon[22] and incretin secretion[21], gastric emptying, as well as fatty acid metabolism[29].

In our study, the worsened glucose tolerance in IFG/IGT subjects in the afternoon was associated with a stronger decrease of postprandial GLP-1 and PYY secretion. Interestingly, for the high-carbohydrate meal, diurnal glucose variation did not correlate with insulin secretion changes, but correlated with declines in glucagon, GLP-1 and PYY secretion in the afternoon. Further, postprandial insulin levels were strongly increased in the afternoon in IFG/IGT subjects, and the diurnal insulin variation was not associated with pattern of glucagon, GLP-1 and PYY secretion. Notably, in NGT subjects this effect was not found, and glucose pattern correlated well with diurnal changes of insulin level. This suggests that in subjects with impaired glucose metabolism the decreased incretin response in the afternoon reinforced insufficient insulin secretion with consequences of higher postprandial glycaemic levels.

GLP-1 has previously been identified as a potent modulator of the diurnal variation of glycaemic control: Lindgren *et al.* show that early postprandial secretion of both incretin hormones, GIP and GLP-1, is increased in the morning compared to the evening, suggesting that the diurnal rhythm in GIP and GLP-1 levels might be one of potentially many mechanisms accounting for the increased insulin sensitivity in the morning[21]. The mechanism of the higher incretin responses in the morning is not known, and might be associated with diurnal

variation of gastric emptying rate[46] or vagal tone. In our study, we did not find a diurnal variation of gastric emptying rate although a trend towards slower gastric emptying in the afternoon was observed for the MTT-HC. Notably, we observed a marked afternoon decline of GLP-1 secretion (more pronounced in IFG/IGT subjects), but only moderate diurnal variation of GIP secretion. There has been a long-standing debate whether reduced GLP-1 levels are a "universal characteristic" of IFG/IGT and diabetic subjects[47]. Our data indicate that diurnal variation of GLP-1 levels might be modulated by the stages of glucose tolerance.

As GLP-1, PYY displays diurnal variation peaking at 1500 h and subsequently decreasing until the early morning[48]. Recent studies suggest that the stage of glucose tolerance affects PYY secretion with diminished postprandial secretion in diabetic subjects[49]. As for GLP-1, our data propose that particularly the diurnal variation of PYY levels might be affected by the stage of glucose tolerance. Interestingly, a study in primates also suggests a diurnal variation of PYY effects: infusion of PYY reduced the initial rate of eating only during the morning meal, but not during the evening meal[50]. This phenomenon might explain why we did not detect a difference in hunger and satiety scores between NGT and IFG/IGT subjects in our study.

Further, our study revealed increased whole-day FFA levels on the HF/HC diet in IFG/IGT subjects compared to the NGT subjects. Moreover, in the afternoon, $iAUC_{0\text{-}180}$ was higher in IFG/IGT subjects after the high-carbohydrate meal than in NGT subjects. Elevated FFA levels are important players in the development of insulin resistance[51] and diurnal variation of FFA response have been shown to contribute to the decline in glucose tolerance as the day progresses[29]. However, some studies demonstrated no difference in daytime variations between NGT subjects and individuals with IGT[33,34] and therefore this question requires further research.

The next possible mechanism which might contribute in the difference of diet effect in NGT and IFG/IGT subjects is the alteration of cortisol rhythm. As a recent study found that weak cortisol rhythms are associated with greater evening declines in glucose tolerance in prediabetic subjects[26], we further investigated the possible contribution of this mechanism to the difference in diet effect in NGT and IFG/IGT subjects. Hydrocortisone infused at 1 pm (elevated at abnormal time) versus 5 am (elevated at normal time) results in increased plasma glucose and insulin levels[52]. However, our study observed no differences in diurnal cortisol variation between diets and between NGT and IFG/IGT subjects. Moreover, no correlation of diurnal cortisol variation with evening increase of glucose and insulin levels and decrease of insulin sensitivity was found. Thus, our study could not confirm that the cortisol rhythms contribute to the differences in the diurnal variation in glucose tolerance between NGT and IFG/IGT subjects as described previously[26].

Finally, strengths and limitations of the current study should be mentioned. The current study has the strength of being a randomized controlled trial with well-defined participants, which is only possible in small-scale studies. Secondly, the duration of our dietary interventions (i.e. four weeks) should be highlighted, as previous reports on a diurnal carbohydrate and/or fat distribution focused on the effect of short-term interventions. Finally, in the current study the macronutrient composition was altered without changing energy intake, by distributing the calories equally between the eating occasions (morning *versus* afternoon part). However, some limitations need to be addressed. Firstly, in spite of extensive nutritional counselling and thoughtful designing of dietary plans, both diets led to a minor weight loss. Possibly, the weight reduction was a reflection of the fact that the participants did not report their true intake in the weighed food records, which is a common problem in human studies[53]. However, the cross-over design of the study should minimize any potential effect of the weight reduction. Secondly, although the overall composition of both diets were very similar and did not show differences in macronutrient composition, fibre and starch content as well as GI, there were minor differences between the fat-rich and carbohydrate-rich diet in the morning versus evening, and we cannot completely exclude that this may influence the results of our study. Thirdly, we cannot completely exclude a contribution of the second-meal phenomenon to the regulation of meal-induced insulin and incretin response in the afternoon[54]. However, the time between MTT1 and MTT2 was long enough (~7 h) to minimize this effect. Finally, our study was conducted in overweight healthy individuals and we cannot exclude that our diets would induce other effects on the glycaemic control in people with obesity and type 2 diabetes. In obese people, it was reported that diurnal variation in glycaemic control was absent[55]. In diabetic patients, the 24 h profile of plasma glucose is impaired and characterized by hyperglycemia in the morning (dawn phenomenon) which is particularly caused by an elevated hepatic glucose production and corresponding increased early morning insulin requirements[56]. In other studies, a loss of diurnal variation after oral glucose administration was described[30] as well as a time of day-dependence with the highest tolerance at lunch time[57]. Thus, in diabetic patients, results are controversial[30,57] and require further research.

In conclusion, the present study reveals an unfavourable effect of the HF/HC diet on glycaemic control in IFG/IGT subjects, but not in NGT subjects. Consequently, considering the impairment of glucose tolerance as the day progresses, large, carbohydrate-rich dinners may potentially need to be avoided, primarily by individuals with an impaired glycaemic control.

Methods

Ethics statement. 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 in May 2015 at clinicaltrial.gov as NCT02487576.

Study participants. Study participants (18–68 years old) were recruited from Berlin-Brandenburg, Germany. The screening examination of participants included anthropometric measurements, blood sampling, an oral glucose tolerance test (OGTT), indirect calorimetry (CareFusion; Yorba Linda, USA), and interviews on lifestyle and medical history. Men with a BMI between 22 and 34.9 kg/m^2, fasting venous glucose levels <126 mg/dL and 2 h glucose levels <200 mg/dL in the 75-g OGTT were eligible for participation. Exclusion criteria were

weight changes >2 kg within past 2 months, current shift work or history of shift work and diseases or conditions that might influence the outcome of the study.

Study design. The cross-over study included two four-week dietary intervention periods separated by a washout phase of 31 ± 2 days (Fig. 1). Details on the randomization are stated in Supplemental Methods. The HC/HF diet consisted of isocaloric carbohydrate-rich breakfast and lunch (65 EN% CHO, 20 EN% fat, 15 EN% protein) and fat-rich snack and dinner (35 EN% CHO, 50 EN% fat, 15 EN% protein). The HF/HC diet consisted of the reversed order of meal composition.

Dietary interventions. For each participant, weighed food records for five consecutive days were analysed with PRODI 6.1 expert software (Nutriscience, Stuttgart, Germany) to determine food preferences and mean caloric intake. To ensure a good compliance, individual dietary plans were formulated for each participant meeting the macronutrient composition of both diets and considering the individual food preferences (Supplemental Tables 2 and 3). As well as possible, dietary plans were controlled for the amount of saturated fatty acids, starch and fibre as well as GI. Dietary plans were isocaloric with the product of the individual resting metabolic rate and the physical activity level and adjusted to the mean daily intake of the food record. Calories of dietary plans were evenly distributed between morning (breakfast + lunch) and afternoon (snack + dinner) leading to a daily macronutrient composition of 50 EN% CHO, 35 EN% fat (14 EN% SFA) and 15 EN% protein.

Participants were instructed to eat breakfast and lunch by 13.30 and snack and dinner 16.30–22.00. Cut-off points were chosen according to usual eating times in Germany to allow a best possible compliance. During the run-in, intervention, and washout periods, participants were asked to avoid alcohol, maintain their normal coffee consumption and follow their regular routine of wakefulness and sleep and physical activity levels. Munich Chronotype Questionnaire (MCTQ)[58] was used to determine participants' chronotypes, by determination of the mid-sleep time point on free days adjusted for individual average sleep need accumulated on work days (MSF-Sc). Activity levels were assessed at the end of each intervention using a pedometer (AS 50; Beurer Inc, Ulm, Germany)

Patient examinations. At all visits, anthropometrical measurements were performed after an overnight fast. Fasted blood samples were drawn from the forearm vein, centrifuged at 1800 g for 10 minutes 4 °C and stored at −80 °C until analysis.

At V2 and V4, two MTTs were performed in the course of the day, i.e. at 09.00 and at 15.40 (Fig. 1B, Supplemental Table 4). MTT-HC was rich in carbohydrates (64.8 EN% CHO, 20.3 EN% fat, 14.8 EN% protein) and contained 835 kcal; MTT-HF was rich in fat (35.3 EN% CHO, 49.6 EN% fat, 15.1 EN% protein) and contained 849 kcal. The chronological order of the MTTs depended on the participant's previous dietary intervention. Participants ingested the meals within 15 minutes. Blood samples were taken before (−5 minutes) and 30, 60, 90, 120 and 180 minutes after completion of each meal. A ^{13}C-acetate breath test was performed along with each MTT to determine gastric emptying rate (Supplemental Methods). Satiety and hunger scores were assessed before (−5 minutes), immediately after (0 minutes) and 180 minutes after each meal as described before[59]. Cortisol levels were determined before and 180 minutes after each meal.

Sample analyses. Routine laboratory markers were measured using standard methods (ABX Pentra 400; HORIBA, ABX SAS, France). Commercial ELISA were used for measurement of insulin, C-peptide, glucagon (Mercodia, Sweden), GIP, total PYY (Merck Chemicals GmbH, Germany) and cortisol (IBL Internation, Germany) in serum; active GLP-1 was measured by Meso Scale Discovery assay (USA). For measurement of glucagon, GIP and GLP-1, EDTA plasma with 100 μM DPPIV inhibitor and 500 KIU/ml aprotinin were used.

Sample size and power calculation. Power calculation was completed using the nQuery Advisor 6.0. For the paired parametric design and the sample size of 28 subjects, the current study provided 80% power to detect 5% difference between groups, if the effect size was 0.55. To allow discontinuation, 32 participants started the trial.

Calculations and statistical analyses. Dietary GI was calculated as described previously[60]. GI values of foods consumed were obtained from the DioGenes database[60].

Statistical analyses were performed with SPSS v.20 (SPSS, Chicago, IL). To estimate the effects of dietary treatments on anthropometrical and fasting metabolic parameters, changes from baseline (week 4 – week 0, in percent to baseline) were calculated. They were used as dependent variables in a linear mixed-effects model with treatment (HC/HF or HF/HC diets), period (first or second) and residual effect of the first experimental period over the second period as fixed factors, and subjects included as a random factor[61]. In an additional analysis, weight change from baseline was included in the linear mixed model as a covariate. Sampling distribution was analyzed using Shapiro-Wilk test. Not normally distributed data were log-transformed before analysis. The same model was used for parameter levels at the start (week 0) and at the end (week 4) of intervention. Because no effect of the period and no residual effect were observed for any measured variable, for following analyses data were pooled in HC/HF and HF/HC groups.

For FFA, glucose and meal-induced hormone secretion, areas under the curve (AUC) and incremental AUC (after subtraction of the baseline area, iAUC) were determined by trapezoidal method. Integrated AUC of both meal tolerance tests (AUCday) were calculated for analysis of whole-day levels. To compare MTTs in the morning and in the afternoon, iAUC were calculated for the total (0–180 min) responses. For fasting whole-day levels, differences between NGT and IFG/IGT subjects were calculated as the delta percentage (Δ% = (new value − basal

value)/basal value * 100). For the comparison between morning and afternoon meal, diurnal variation in a variable was calculated as Δ = afternoon value – morning value.

Insulin secretion was assessed as the ratio of iAUC for insulin to iAUC for glucose ($iAUC_{ins/glu}$). Insulin sensitivity in MTT was determined by the Gutt index (ISI $Gutt_{0\text{-}120}$)[62]. Hepatic insulin clearance (HIC) was calculated as ratio of $iAUC_{0\text{-}180}$ for C-peptide to $iAUC_{0\text{-}180}$ for insulin[63]. HOMA-IR was calculated according to following equation. HOMA-IR [mmol * mU * L^2] = glucose [mmol/L] × insulin [mU/L]/22.5, using fasting values.

For markers of satiety and hunger scores, daily levels were calculated as the average level from six time points of data collection.

Time-of-day and diet effects were estimated using two-way repeated measures ANOVA. Comparisons between two groups at each time point were performed using paired Student's t-test or Wilcoxon test, and correlations were calculated using Pearson or Spearman tests, depending on sample distribution. P values < 0.05 were considered significant in all analyses. All data are presented as means ± SEMs.

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Author Contributions

K.K., A.K., A.F.H.P., N.R. and O.P. designed the research; K.K., S.H., K.J.P. and M.K. conducted the research; K.K., N.R. and O.P. analysed data and performed the statistical analysis; K.K., N.R., A.F.H.P., and O.P. were responsible for interpretation of the data and drafting of the manuscript; K.K., S.H., K.J.P., M.K., A.K., A.F.H.P., N.R. and O.P. critically revised the manuscript for important intellectual content; O.P. is the guarantor of this work and, as such, had full access to all data in the study and takes responsibility for the integrity of the data and accuracy of the data analysis.

Additional Information

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The effect of diurnal distribution of carbohydrates and fat on glycaemic control in humans: a randomized controlled trial

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Table of Content

Randomization and blinding

Randomization of study participants was done by a statistician who had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Statistician was blinded regarding the allocation groups. Participants were matched regarding age, BMI, fasting and 2-h glucose levels in the OGTT and subsequently randomized. For randomization a random number generator was used to generate a number between 0 and 1. The obtained number was rounded leading to 0 or 1, respectively, each presenting one of the allocation groups. Allocation was disclosed to both the participant and a nutritionist as soon as available, allowing time for preparation of dietary protocols.

^{13}C-acetate breath test

A ^{13}C-acetate breath test was performed along with each meal tolerance test to determine gastric emptying rate[1]. 60 mg sodium acetate-1-13C (99 atom % 13C, Wagner Analysen Technik GmbH, Bremen, Germany) was swallowed with a sip of the provided water at the end of each meal. Breath samples were taken in duplicates at baseline and every 15 min after ^{13}C-acetate administration into 10 mL tubes (Exetainer, Labco, High Wycombe, UK) for 4-h (morning meal tolerance test) and 3-h (afternoon tolerance test), respectively, for analysis of $^{13}CO_2$ enrichments. Breath [$^{13}CO_2$] enrichments were analyzed by isotope-ratio mass spectrometry (BreathMAT, Thermo Scientific Corp., Bremen, Germany). Isotope composition of carbon was expressed in the conventional delta per mill notation [2]. Evaluation of the breath tests were performed as described[3,4]. Percentage of ^{13}C-recovery from [1-13C]-acetate in $^{13}CO_2$ was calculated according to Schoeller [5] based on delta per mill values over baseline and computed endogenous CO_2 production rate using the body surface area according to Haycock [6]. The time plot of pulmonary [$^{13}CO_2$]-excretion (% dose/h) data were used for mathematical curve fitting using nonlinear regression analysis provided by the Microsoft® Excels Solver procedure and following formula: $y = atb \cdot e\text{-}ct$, where (atb) describes the increase in [$^{13}CO_2$] recovery in breath, (e-ct) describes the washout of the [$^{13}CO_2$] from the breath, t is time in hours and a, b, and c are regression-estimated constants.

The following breath test parameters of gastric emptying were computed: half emptying time (T1/2) and time of fastest emptying (Tlag)[4,7,8].

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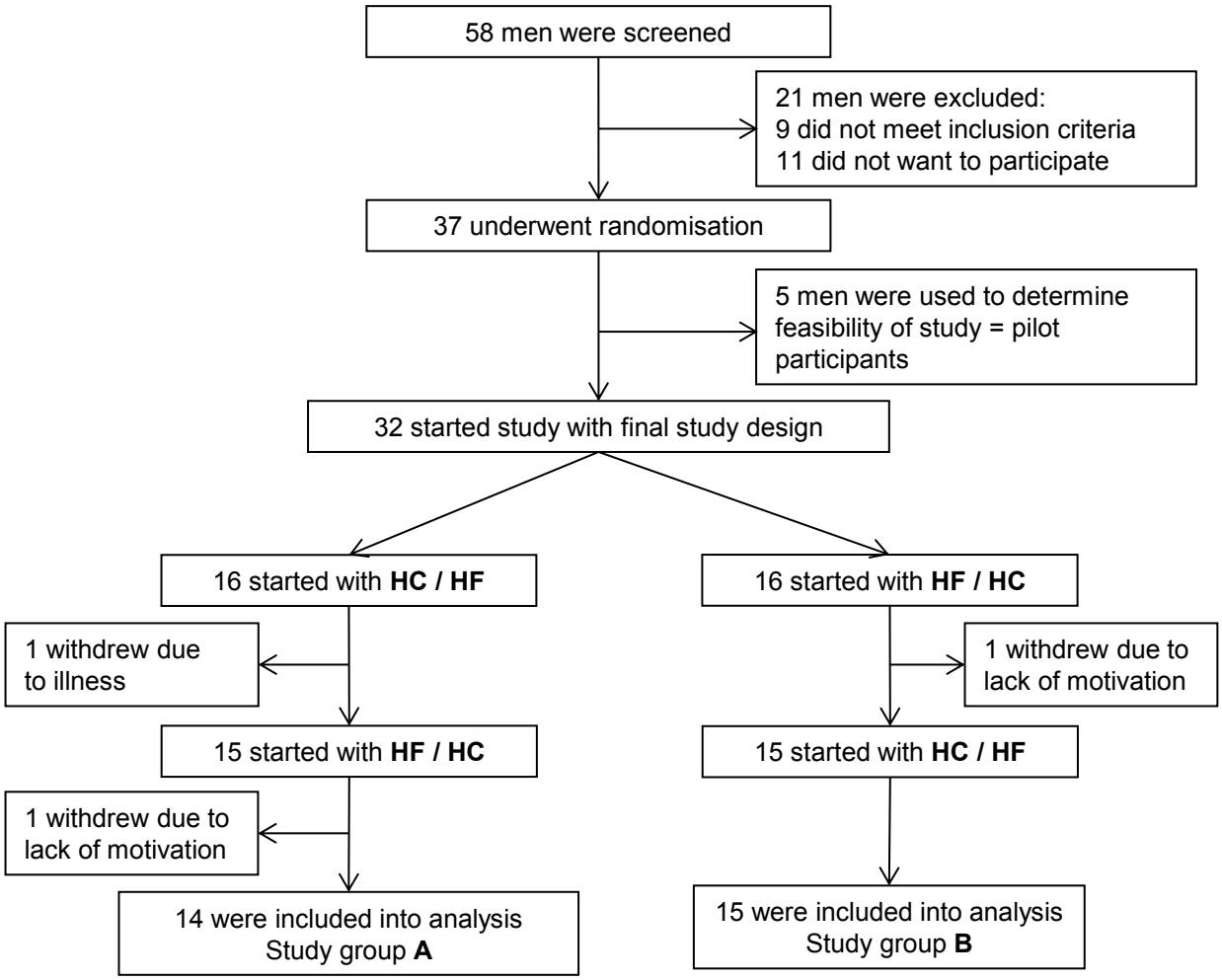

Figure S1. Flow of participants. HC/HF, isocaloric carbohydrate-rich diet in the morning and fat-rich diet in the evening; HF/HC, isocaloric fat-rich diet in the morning and carbohydrate-rich diet in the evening.

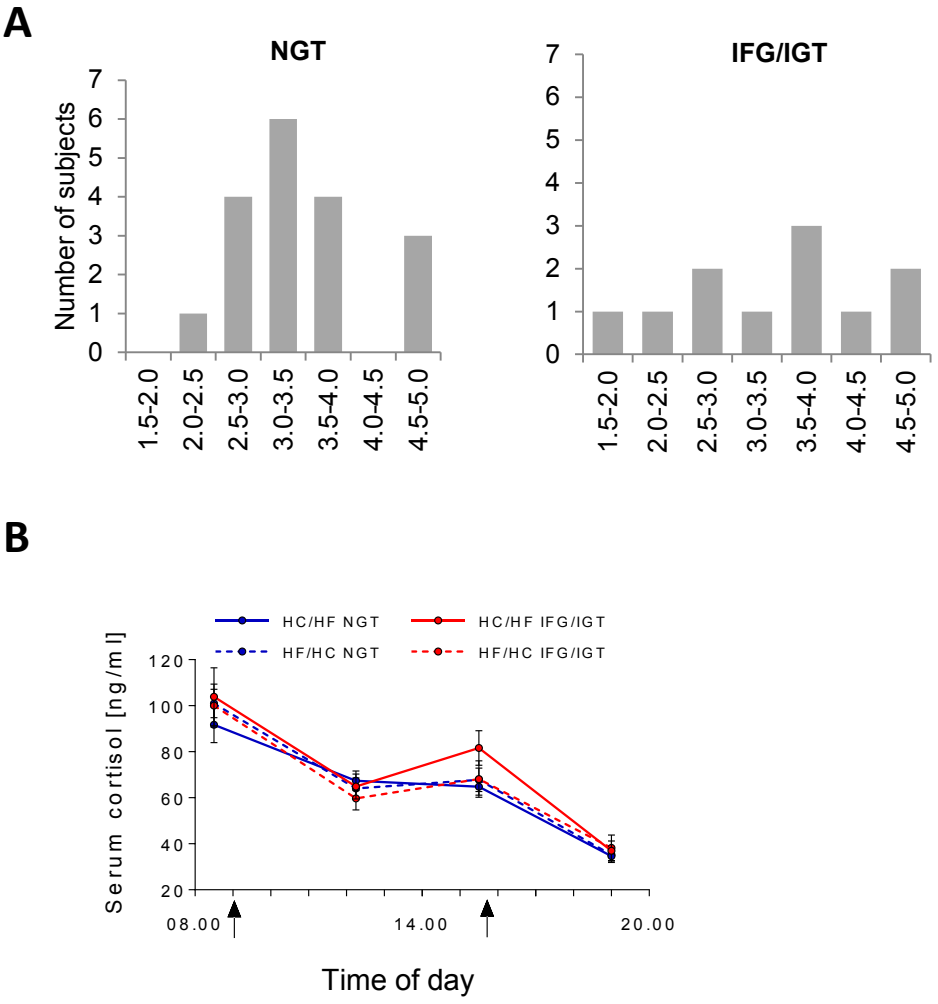

Figure S2. Circadian parameters of study participants.

(A) Chronotypes of NGT (n=18) and IFG/IGT (n=11) subjects determined using the MCTQ. Mid-sleep time-point on free days adjusted for individual average sleep need accumulated on work days (MSF-Sc) of all subjects is depicted. (B) Plasma cortisol levels measured at four time points (08.35 h, 12.15 h, 15.35 h and 19.00 h) during the investigation day.

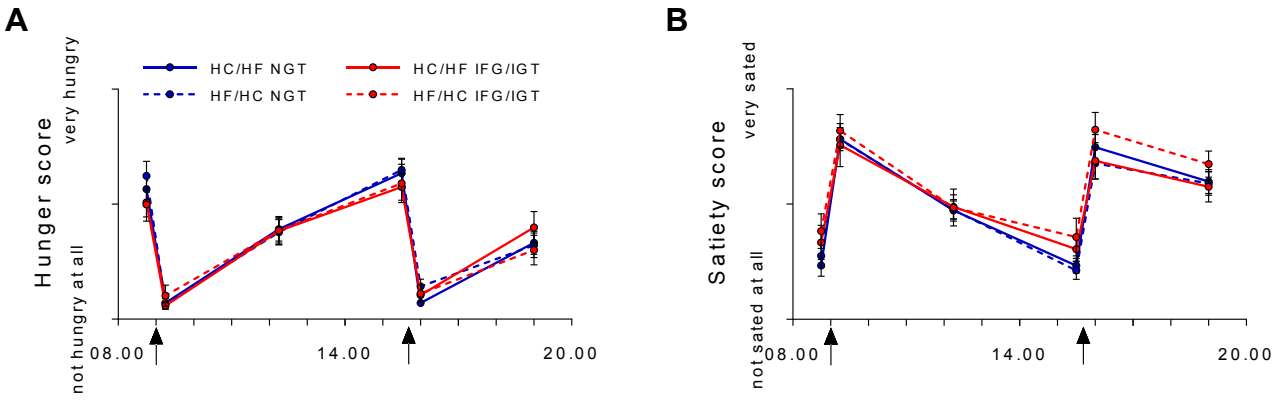

Figure S3. Hunger and satiety scores in response to the HC/HF diet and the HF/HC diet. Effects of the HC/HF diet (solid lines) and the HF/HC diet (dotted lines) on pre- and postprandial hunger (A) and satiety (B) scores in NGT subjects (blue) and IFG/IGT subjects (red). Arrow - test meal.

Table S1. Compliance during both dietary interventions

	HC/HF diet			HF/HC diet		
	06.00 – 22.00	06.00 – 13.30	16.30 – 22.00	06.00 – 22.00	06.00 – 13.30	16.30 – 22.00
Energy [KJ]	12073.4 ± 442.3	6197.1 ± 261.3[§]	5876.0 ± 212.0	11826.3 ± 415.1	6215.0 ± 252.9	5712.7 ± 207.5
CHO [EN %]	49.1 ± 0.7	64.8 ± 0.7	32.7 ± 1.2	48.7 ± 0.7	33.5 ± 0.9	65.0 ± 1.2
Fat [EN %]	36.3 ± 0.7	20.7 ± 0.5	52.4 ± 1.2	36.7 ± 0.8	51.4 ± 1.0	20.6 ± 1.0
Protein [EN %]	14.7 ± 0.2	14.4 ± 0.3	14.9 ± 0.3	14.6 ± 0.2	15.1 ± 0.3	14.4 ± 0.3
SFA [% from total fat]	42.8 ± 1.2	45.4 ± 1.1	41.8 ± 1.4	44.5 ± 1.0	43.9 ± 1.1	44.2 ± 1.4
Dietary GI	58.3 ± 0.5	59.4 ± 0.6	56.0 ± 0.9[#]	58.7 ± 0.5	59.9 ± 0.7	58.3 ± 0.5
Fibre [g]	35.5 ± 1.9	22.5 ± 1.6[§]	13.0 ± 0.7	32.9 ± 1.2	14.3 ± 0.6	18.6 ± 0.9
Starch [g]	174.3 ± 10.1	116.7 ± 8.5[§]	57.4 ± 3.3[#]	177.1 ± 6.5	79.7 ± 3.3	97.5 ± 4.8

Data are shown as mean ± SEM. [§], $p < 0.05$, carbohydrate-rich diet in the morning (06.00 – 13.30) *versus* evening (16.30 – 22.00). [#],$p < 0.05$, fat-rich diet in the morning *versus* evening). HC/HF, isocaloric carbohydrate-rich diet in the morning and fat-rich diet in the evening; HF/HC, isocaloric fat-rich diet in the morning and carbohydrate-rich diet in the evening; KJ, kilo joule; CHO, carbohydrates; EN %, energy percent; SFA, saturated fatty acids; GI, glycaemic index; GL, glycaemic laod.

Table S2. Example of a dietary plan for the HC/HF diet.

Food to be eaten		**Alternative food**
Morning block (breakfast + lunch) – to be eaten until 01.30 pm		
250 g	Orange Juice	107 g banana / 150 g apple / 174 g pear / 213 g mandarines / 141 g grapes / 266 g oranges / 174 g pineapple / 391 g strawberries / 260 g water melon / 112 g commercially available apple puree (tin)
50 g	Müsli	*Swiss style müsli*
150 g	Yoghurt (3.5% FDM)	159 g milk (3.5% FDM)
100 g	Low-fat curd	377 g semi-skimmed milk (1.5% fat) / 356 g yoghurt (1.5% fat) / 358 g buttermilk / 56 g ham / 55 g turkey breast
150 g	Apple	107 g banana / 174 g pear / 213 g mandarines / 141 g grapes / 266 g oranges / 174 g pineapple / 391 g strawberries / 260 g water melon / 112 g commercially available apple puree (tin) / 250 g fruit juice *(any kind)*
30 g	Sultanas	43 g dried apricots / 43 g dried plums / 33 g dried mango / 35 dried figs
120 g	Spaghetti (unboiled)	*equals to 300 g boiled spaghetti.* 109 g unboiled rice / 126 unboiled couscous / 126 g unboiled bulgur / 186 g bread / 104 g unboiled Chinese noodles
200 g	*Bertolli*® tomato sauce with basil	*any tomato sauce with similar composition*
10 g	Parmesan	
30 g	Milk chocolate	30 *Kitkat*® chocolate bar / 29 g *Sondey*® double choc cookies / 30 g *Snickers*® / 29 g *Bounty*® / 27 g *Twix*®
Energy [kcal]		**1400.8**
CHO [EN %]		**65.1**
Fat [EN %]		**19.9**
Protein [EN %]		**15.0**
Evening block (snack + dinner) – to be eaten between 04.30 and 10.00 pm		
200 g	Landliebe® Vanilla cream pudding	*Any kind of cream pudding with a similar composition*
40 g	Roasted peanuts	30 g walnuts / 32 g hazelnuts / 44 g roasted cashewnuts
135 g	Wheat roll	*Any kind of bread*
25 g	Butter	
45 g	Salami	32 g tea sauge spread / 47 g black pudding / 46 g calves liverwurst/ 54 g semi-hard cheese (45 % FDM) / 70 g Mozzarella / 46 g cream cheese (60 % FDM) / 49 g hummus
40 g	Ham	43 g corned beef / 40 g turkey breast / 42 g smoked pork chop / 72 g cottage cheese (20% FDM) / 30 g harz cheese / 72 g low fat curd
Energy [kcal]		**1402.3**
CHO [EN %]		**35.0**
Fat [EN %]		**50.4**
Protein [EN %]		**14.6**

Example of an individual dietary plan for the HC/HF diet adjusted for participant´s energy expenditure and food preferences. If possible 3-7 alternative food items were provided containing the same amount of the major macronutrient as the plan´s original food item. HF/HC, isocaloric fat-rich diet in the morning and carbohydrate-rich diet in the evening; Kcal, kilo calories; CHO, carbohydrates; EN %, energy percent; FDM, fat in dry matter.

Table S3: Example of a dietary plan for the HF/HC diet.

Morning block (breakfast + lunch) – to be eaten until 01.30 pm		
70 g	Wheat bread roll	*Any kind of bread*
10 g	Butter	
25 g	Nutella®	*Any kind of chocolate spread with similar composition*
90 g	Wheat bread roll	*Any kind of bread*
350 g	Tomatoes	455 g cucumber / 147 g peppers / 140 g carottes / 140 g beet root / 248 g kohlrabi / 464 g courgette / 413 g cellery / 420 g radish
120 g	Mozzarella	115 goat´s cheese / 105 g feta cheese / 82 g Gouda cheese
15 g	Olive oil	*Any kind of oil*
10 g	Vinegar	*Any kind of vinegar*
30 g	Roasted peanuts	22 g walnuts / 25 g hazelnuts / 33 g roasted cashewnuts / 126 g avocado / 113 g olives
Energy [kcal]		**1406.1**
CHO [EN %]		**34.9**
Fat [EN %]		**50.3**
Protein [EN %]		**14.8**
Evening block (snack + dinner) – to be eaten between 04.30 and 10.00 pm		
150 g	Apple	107 g banana / 174 g pear / 213 g mandarines / 141 g grapes/ 266 g oranges / 174 g pineapple / 391 g strawberries / 260 g water melon / 112 g commercially available apple puree (tin) / 250 g fruit juice *(any kind)*
107 g	Banana	150 g apple / 174 g pear / 213 g mandarines / 141 g grapes / 266 g oranges / 174 g pineapple / 391 g strawberries / 260 g water melon / 112 g commercially available apple puree (tin) / 250 g fruit juice *(any kind)*
54 g	Wine gums	59 g soft cake / 57 g lady finger / 56 g pretzel sticks / 390 g coke / 64 g marshmallow treat
130 g	Spaghetti (unboiled)	*Equals to 375 g boiled spaghetti.* 118 g unboiled rice / / 136 unboiled couscous / 136 g unboiled bulgur / 200 g bread / 112 g unboiled Chinese noodles
13 g	Olive oil	*Any kind of oil*
200 g	*Bertolli*® tomato sauce with basil	*Any tomato sauce with similar composition*
75 g	Ham	78 g corned beef / 73 g turkey breast / 77 g smoked pork chop / 74 g tuna / 134 g cottage cheese (20% FDM) / 134 g low fat curd / 91 g prawns / 506 g semi-skimmed milk / 482 g buttermilk
20 g	Parmesan	
300 g	Orange Juice (100%)	128 g banana / 180 g apple / 208 g pear / 256 g mandarines / 169 g grapes / 313 g oranges / 209 g pineapple / 469 g strawberries / 209 g water melon / 134 g commercially available apple puree (tin)
Energy [kcal]		**1400.8**
CHO [EN %]		**65.1**
Fat [EN %]		**19.9**
Protein [EN %]		**15.0**

Example of an individual dietary plan for the HF/HC diet adjusted for participant´s energy expenditure and food preferences. If possible 3-7 alternative food items were provided containing the same amount of the major macronutrient as the plan´s original food item. HF/HC, isocaloric fat-rich diet in the morning and carbohydrate-rich diet in the evening; Kcal, kilo calories; CHO, carbohydrates; EN %, energy percent; FDM, fat in dry matter.

Table S4. Composition of provided test meals.

	MTT-HC			MTT-HF		
	120 g	Wheat bread roll		119 g	Wheat bread roll	
	50 g	Wheat toast		17 g	Butter	
	12.5 g	Butter		19 g	Cheese (45 % FDM)	
	65 g	Strawberry jam		50.1 g	Philadelphia ®	
	16.7 g	Philadelphia®		150 g	Full-fat curd	
	110 g	Low-fat curd		250 g	Water	
	250 g	Water				
Energy [kcal]			**835.2**			**849.0**
CHO [EN %]			**64.8**			**35.3**
Fat [EN %]			**20.3**			**49.6**
Protein [EN %]			**14.8**			**15.1**
SFA [% from total fat]			**59.5**			**63.3**

MTT, meal tolerance test; HC, carbohydrate-rich; HF, fat-rich; FMD, fat in dry matter; CHO, carbohydrates; SFA, saturated fatty acids; kcal: kilo calories; EN %: energy percent

CURRICULUM VITAE

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

COMPLETE LIST OF PUBLICATIONS

Publications

Kessler K, Hornemann S, Petzke KJ, Kemper M, Kramer A, Pfeiffer AF, Pivovarova O*, Rudovich N* (2017). "The effect of diurnal distribution of carbohydrates and fat on glycaemic control in humans: a randomized controlled trial." Sci Rep 7:44170

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Jugdaohsingh R, **Kessler K**, Messner B, Stoiber M, Pedro LD, Schima H, Laufer G, Powel JJ, Bernhard D (2015). "Dietary Silicon Deficiency Does Not Exacerbate Diet-Induced Fatty Lesions in Female ApoE Knockout Mice." J Nutr 145(7):1498-506.

Pivovarova O*, Jurchott K*, Rudovich N*, Hornemann S, Ye L, Mockel S, Murahovschi V, **Kessler K**, Seltmann AC, Maser-Gluth C, Mazuch J, Kruse M, Busjahn A, Kramer A, Pfeiffer AF (2015). "Changes of Dietary Fat and Carbohydrate Content Alter Central and Peripheral Clock in Humans." J Clin Endocrinol Metab 100(6): 2291-2302

Kessler K, Pivovarova O, Pfeiffer AF (2014). "[Circadian clocks and energy metabolism: implications for health]." Dtsch Med Wochenschr 139(14): 684-686.

* Equal contribution

Conference contributions and presentations

"Effects of a temporal segmentation of meal composition on lipid and glucose metabolism in men"
Kessler K, Hornemann S, Petzke KJ, Kemper M, Rudovich N, Kramer A, Pfeiffer AF and Pivovarova O. Poster presentation; 51. Deutscher Diabetes Kongress 2016; Deutsche Diabetes Gesellschaft (DDG), Berlin, Germany

"Kohlenhydrate morgens, Kohlenhydrate abends – spielt dies eine Rolle?"
Kessler K. Oral presentation; Diabetes Kongress 2016, Münster, Germany

„Metabolic effects of a temporal segmentation of meal composition on substrate oxidation and glucose metabolism in men"
Kessler K, Hornemann S, Petzke KJ, Kemper M, Rudovich N, Kramer A, Pfeiffer AF and Pivovarova O. Poster presentation; 1st International CBBM Symposium 2016, Lübeck, Germany

"Day-time dependent effects of fat-rich and carbohydrate-rich meals on metabolic parameters in healthy humans"
Pivovarova O, **Kessler K**, Hornemann S, Kramer A and Pfeiffer AF. Poster presentation; 12th FENS European Nutrition Conference Berlin 2015, Berlin, Germany

"Day-time dependent effects of fat-rich and carbohydrate-rich meals on metabolic parameters in healthy humans"
Kessler K, Pivovarova O, Hornemann S, Rudovich N, Kramer A and Pfeiffer AF. Poster presentation; 31. Jahrestagung der Deutschen Adipositas Gesellschaft 2015, Berlin, Germany

"Effect of different diurnal patterns of meal composition on metabolic parameters in healthy men"
Kessler K, Hornemann S, Sticht C, Gretz N, Kramer A, Pivovarova O and Pfeiffer AFH. Poster presentation; 51st EASD Annual Meeting 2015, Stockholm, Sweden

"Clock genes are implicated in the regulation of energy homeostasis in humans"
Pivovarova O, Gögebakan Ö, Sucher S, Groth J, Murahovschi V, **Kessler K**, Osterhoff M, Rudovich N, Kramer A, Pfeiffer AFH. Poster presentation; 51st EASD Annual Meeting 2015, Stockholm, Sweden

"Day-time dependent effects of fat-rich and carbohydrate-rich meals on metabolic parameters in healthy humans"
Kessler K, Pivovarova O, Hornemann S, Kramer A and Pfeiffer AF. Poster presentation; 3rd DZD Diabetes Research School 2015, Stockholm, Sweden

"Effect of different diurnal patterns of meal composition on lipid metabolism and satiety-hunger-scores in healthy men"
Kessler K , Hornemann S, Kramer A, Pivovarova O, Pfeiffer AFH. Poster Presentation; 50. Deutscher Diabetes Kongress 2015; Deutsche Diabetes Gesellschaft (DDG), Berlin, Germany

"The CLOCK Study – a human intervention study on circadian clocks and energy metabolism"
Kessler K , Hornemann S, Kramer A, Pivovarova O, Pfeiffer AFH. Round Table Presentation; Timelines in Biology 2014; Weizmann Institute of Science, Revohot, Israel

"CLOCK Study – a human intervention study on peripheral circadian clocks and energy metabolism"
Kessler K , Hornemann S, Kramer A, Pivovarova O, Pfeiffer AFH. Poster Presentation; ESE Bregenz Summer School on Endocrinology 2014, Bregenz, Austria

"A human intervention study on the interplay between peripheral circadian clocks and energy metabolism"
Kessler K , Hornemann S, Kramer A, Pivovarova O, Pfeiffer AFH. Poster Presentation; ESE Bregenz Summer School on Endocrinology 2013, Bregenz, Austria

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