Acute hyper-energetic, high-fat feeding increases circulating FGF21, LECT2 and fetuin-A in healthy men

Scott A. Willis[1,2], Jack A. Sargeant[2,3], Thomas Yates[2,3], Toshinari Takamura[4], Hiroaki Takayama[4], Vinay Gupta[1], Emily Brittain[1], Joe Crawford[1], Siôn A. Parry[5], Alice E. Thackray[1,2], Veronica Varela-Mato[1], David J. Stensel[1,2], Rachel M. Woods[1,2], Carl J. Hulston[1,2], Guruprasad P. Aithal[6,7], James A. King[1,2]

[1]School of Sport, Exercise and Health Sciences, Loughborough University, UK

[2]NIHR Leicester Biomedical Research Centre, University Hospitals of Leicester NHS Trust and the University of Leicester, UK

[3]Diabetes Research Centre, University of Leicester, UK

[4]Department of Disease Control & Homeostasis, Kanazawa University Graduate School of Medical Sciences, Japan

[5]Radcliffe Department of Medicine, University of Oxford, UK

[6]Nottingham Digestive Diseases Centre, School of Medicine, University of Nottingham, UK

[7]NIHR Nottingham Biomedical Research Centre, Nottingham University Hospitals NHS Trust and the University of Nottingham, UK

Manuscript word count: 4542

Conflict of Interest: SAW, JAS, TY, TT, HT, VG, EB, JC, SAP, AET, VVM, DJS, RMW, CJH, GPA, and JAK, no conflicts of interest.

Funding Disclosure: The research was funded by the National Institute for Health Research (NIHR) Leicester and Nottingham Biomedical Research Centres. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health and Social Care.

Address for correspondence

Dr James King

Senior Lecturer in Exercise Physiology

School of Sport, Exercise and Health Sciences

Loughborough University

Leicestershire

United Kingdom

LE11 3TU

Phone: +44(0)1509 228457

Email: j.a.king@lboro.ac.uk

Number of figures: 3

Number of tables: 3

Supplementary data submitted: Supplemental Tables 1, 2, 3 and Supplemental Figure 1 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn.

Running title: Hyper-energetic, high-fat feeding and hepatokines

Abbreviations used: Adipo-IR, adipose tissue insulin resistance index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AMPK, AMP-activated protein kinase; FGF21, fibroblast growth factor 21; GGT, gamma-glutamyltransferase; HE-HFD, hyper-energetic, high-fat diet; HOMA-IR, homeostatic model assessment of insulin resistance; ISI, insulin sensitivity index; LECT2, leukocyte cell-derived chemotaxin 2; NEFA, non-esterified fatty acid; OGTT, oral glucose tolerance test; RMR, resting metabolic rate; SeP, selenoprotein P; TAG, triacylglycerol

Abstract

Background: Hepatokines such as fibroblast growth factor 21 (FGF21), leukocyte cell-derived chemotaxin 2 (LECT2), fetuin-A, fetuin-B and selenoprotein P (SeP) are liver-derived proteins which are modulated by chronic energy status and metabolic disease. Emerging data from rodent and cell models indicate that hepatokines may be sensitive to acute nutritional manipulation; however, data in humans are lacking.

Objective: To investigate the influence of hyper-energetic, high-fat feeding on circulating hepatokine concentrations, including the time-course of responses.

Methods: In a randomised, crossover design, 12 healthy men (mean ± SD: age, 24 ± 4 years; BMI, 24.1 ± 1.5 $kg \cdot m^{-2}$) consumed a seven-day hyper-energetic, high-fat diet (HE-HFD; +50% energy, 65% total energy as fat [32% saturated, 26% monounsaturated, 8% polyunsaturated]) and control diet (36% total energy as fat), separated by three weeks. Whole-body insulin sensitivity was assessed before and after each diet using oral glucose tolerance tests. Fasting plasma concentrations of FGF21 (primary outcome), LECT2, fetuin-A, fetuin-B, SeP, and related metabolites were measured after 1, 3 and 7 d of each diet. Hepatokine responses were analysed using two-way repeated-measures ANOVA and subsequent pairwise comparisons.

Results: Compared with control, the HE-HFD increased circulating FGF21 at 1 (105%) and 3 d (121%; $P \leq 0.040$); LECT2 at 3 (17%) and 7 d (32%; $P \leq 0.004$); and fetuin-A at 7 d (7%, $P = 0.028$). Plasma fetuin-B and SeP did not respond to the HE-HFD. Whole-body insulin sensitivity was reduced after the HE-HFD by 31% ($P = 0.021$).

Conclusions: Acute high-fat overfeeding augments circulating levels of FGF21, LECT2 and fetuin-A in healthy men. Notably, the time-course of response varies between proteins and is transient for FGF21. These findings provide further insight into the nutritional regulation of

hepatokines in humans and their interaction with metabolic homeostasis. This study was registered at clinicaltrials.gov as NCT03369145.

Key words: hepatokines, high-fat diet, overfeeding, insulin resistance, FGF21, LECT2, fetuin-A, fetuin-B, selenoprotein P

Introduction

The strong observational link that exists between hepatic steatosis and altered peripheral metabolism has stimulated interest regarding metabolic cross-talk between the liver and peripheral tissues (1,2). Analogous to ‘adipokines’ and ‘myokines’, ‘hepatokines’ have recently been identified as liver-secreted proteins (predominantly or exclusively) with the capacity to exert systemic metabolic effects in an endocrine-like manner (3,4). Prominent within this novel area is a connection identified between hepatokines, insulin sensitivity and glucose metabolism (4–8). This link has highlighted hepatokines as novel targets within the management of obesity-related chronic disease (7,9,10).

The regulation of hepatokines appears to be related to long-term energy balance, as demonstrated by associations between hepatokines, adiposity and obesity-related metabolic dysfunction (4,6,7). Pre-clinical, mechanistic studies support this notion, showing that chronic high-fat overfeeding modulates hepatocyte gene expression (4,11–13). The importance of this regulatory process for systemic metabolism has been highlighted recently; where secreted factors from steatotic hepatocytes induced pro-inflammatory signalling and insulin resistance in cultured cells (4).

In addition to chronic regulatory influences, recent pre-clinical research indicates that hepatokines may also be sensitive to acute perturbations in energy balance and nutrition. For instance, leukocyte cell-derived chemotaxin 2 (LECT2) is a novel hepatokine which promotes insulin resistance in peripheral tissues (7,14) and is suppressed by acute exercise and fasting; but increases in response to chronic overfeeding (7). In a rodent weight cycling model, LECT2 was recently shown to respond dynamically to alternating periods of hypercaloric and eucaloric feeding (15). Hepatic activation of the energy-sensing kinase AMP-activated protein kinase (AMPK) has been demonstrated to modulate the responsiveness of LECT2 to energetic and/or

nutritional status (7). Interestingly, this mechanism has also been shown to modulate other important gluco-regulatory hepatokines such as fetuin-A (16) and selenoprotein P (SeP; 17); and may link positive energy balance to peripheral metabolic dysfunction (18).

Fibroblast growth factor 21 (FGF21) is another hepatokine which is responsive to acute nutritional challenges, including fasting, protein restriction and chronic overfeeding (19–21). Notably, FGF21 has been shown to modulate glucose and lipid metabolism; by increasing glucose uptake into adipose tissue and enhancing hepatic fatty acid oxidation (22,23). More recently, FGF21 has been identified to play a key role in the integrated stress response to nutritional and cellular stresses (24); potentially serving as a compensatory mechanism to combat hepatic lipotoxicity and preserve metabolic homeostasis (21,25,26). Within two pilot experiments, we have recently shown that FGF21 is augmented after one, but not seven days of overfeeding (27). Additional research is needed to clarify these findings within an appropriately designed trial.

To date, nearly all evidence relating to hepatokines and short-term nutritional status has been conducted in pre-clinical models. Further research is therefore required to determine whether findings translate into humans; and to explore how acute perturbations in nutritional status influence other relevant hepatokines yet to receive attention. Therefore, using a population of healthy men, the present study examined the acute (one to seven days) influence of hyper-energetic, high-fat feeding on the circulating concentrations of five candidate hepatokines (FGF21, LECT2, fetuin-A, fetuin-B and SeP) which have been shown to modulate glucose and lipid metabolism and/or insulin sensitivity. We hypothesised that high-fat overfeeding would increase circulating levels of each hepatokine which may form part of an adaptive metabolic response to overnutrition.

Methods

Ethical approval and participant recruitment

After receiving approval from the Institutional Research Ethics Committee (R17-P144), 12 healthy males were recruited into the study following the provision of written informed consent. Participants were young (18 – 40 years), had a BMI between 18.5 and 27.9 $kg \cdot m^{-2}$, and did not smoke or possess diagnosed metabolic conditions. Participants were habitually active (no more than five structured exercise sessions per week) and reported being weight stable (< 2 kg body mass change) in the six months before the study. The study was registered as a clinical trial (NCT03369145) at clinicaltrials.gov before data collection commenced.

Participant pre-assessment

During a pre-assessment visit, participants were screened to determine study eligibility. Participants provided a medical history and completed a questionnaire determining acceptability of food items to be provided during the study. Normal fasting capillary blood glucose levels (< 5.5 $mmol \cdot L^{-1}$) were confirmed using a point-of-care bioanalyser (CardioChek®, Polymer Technology Systems Inc, Indianapolis, USA). Participants' BMI, waist circumference and blood pressure were determined using standardised procedures (28).

At the end of the visit, participants were provided with two accelerometers; an ActiGraph GTX (ActiGraph Corp, Pensacola, USA) and an ActivPAL3 ^{TM} (PAL Technologies Ltd, Glasgow, UK) which were subsequently worn for seven consecutive days to assess habitual physical activity and sedentary behaviour, respectively. A three-day weighed food record (two-week days and one weekend day) was also completed during this time to estimate baseline habitual energy and macronutrient intake. Food records were analysed for energy content and macronutrient composition as described previously (29).

Study design and procedures

The present study employed a randomised-counterbalanced crossover design, whereby each participant completed two, seven-day dietary interventions (hyper-energetic, high-fat diet [HE-HFD] and control diet) separated by a three-week washout period. Figure 1 provides a schematic illustration of the study design and procedures.

Insert figure 1

Within each dietary intervention, participants attended four laboratory visits which occurred on the morning of the first day of each diet (pre-diet), and subsequently on the morning after one, three and seven-days (post-diet) of each diet. Participants attended each visit following an overnight fast (≥ 10 h) and having abstained from caffeine, alcohol and exercise in the prior 24 h. During each visit, a fasting venous blood sample was obtained. At the pre- and post-diet visits, body mass and blood pressure were assessed; whilst resting metabolic rate (RMR) and substrate oxidation (30) were also measured using indirect calorimetry. Furthermore, whole-body insulin sensitivity was assessed using an oral glucose tolerance test (OGTT; 29). During the OGTT, venous blood samples were drawn from a cannula inserted into an antecubital vein (21 g; Venflon, Becton Dickinson, Helsingborg, Sweden) at the following time points: 0, 30, 60, 90 and 120 min. Within each dietary intervention, participants were strictly instructed not to alter their habitual physical activity levels. Habitual physical activity and sedentary behaviour were measured continuously throughout each intervention period using an accelerometer and inclinometer to assess compliance.

Dietary interventions

The HE-HFD and control diet were consumed across seven consecutive days within the study. In the HE-HFD, participants consumed 150% of their estimated daily energy requirement. Of the total energy content, approximately 65% was derived from fat (32% saturated fatty acids (SFA), 26% monounsaturated fatty acids (MUFA) and 8% polyunsaturated fatty acids (PUFA)), 21% was derived from carbohydrate and 14% was derived from protein (Table 1). An example of the two-day rotating menu provided to participants during the HE-HFD can be seen in Supplemental Table 3. Individuals' dietary energy requirements were calculated using published equations (31) and subsequently multiplied by a physical activity correction factor of 1.7 to account for moderate levels of habitual physical activity in males (32). This value was additionally multiplied by 1.1 to account for the thermic effect of feeding and then by 1.5 to identify 150% of participants' estimated daily energy requirement. Please note that this method of calculating energy requirements during our HE-HFD intervention produced a higher energy intake requirement than what would have been necessary if requirements were based on participants' food diaries (see Table 1).

Within the HE-HFD, all foods and energy-containing drinks were prepared by the research team and distributed to the participants. Participants were instructed to consume all foods provided to them and no additional energy-containing food or drinks. In the event of any leftovers, participants were told to return the food item so that the research team could account for the discrepancy. Dietary compliance was facilitated by the provision of daily menus, detailed cooking guidance and verbal confirmation. Other than one ham and cheese croissant which one participant did not eat; participants reported being fully compliant with the HE-HFD.

Within the control diet, participants were told to consume their habitual diet throughout the intervention. To assess compliance, participants completed a second three-day weighed food record (two-week days and one weekend day) during the control diet, which was subsequently

contrasted with the food record completed during the baseline (pre-intervention) assessment (Table 1).

Biochemical analyses

Blood samples were collected into ice-cooled potassium EDTA and lithium heparin monovettes (Sarstedt, Leicester, UK) and were spun immediately in a refrigerated centrifuge (Heraeus Labofuge 400R, Thermo Fisher Scientific, Massachusetts, USA) at 4°C for 10 min (2383 x *g*). Plasma was then removed and aliquoted for storage at -80°C. Commercially available enzyme-linked immunosorbent assays were used to measure plasma concentrations of FGF21 (R & D Systems, Oxford, UK), LECT2 (BioVendor, Brno, Czech Republic) , fetuin-A (R & D Systems, Oxford, UK), fetuin-B (BioVendor, Brno, Czech Republic) and insulin (Mercodia AB, Uppsala, Sweden). Plasma concentrations of full-length SeP were measured using a sol particle homogenous immunoassay, as previously reported (33,34). The mean within-batch coefficients of variation (CV) for these assays were as follows: FGF21 8.2%, LECT2 3.7%, fetuin-A 4.5%, fetuin-B 2.8%, SeP 4.0% and insulin 6.5%. Circulating concentrations of non-esterified fatty acids (NEFA), triacylglycerol (TAG), glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyltransferase (GGT) were analysed by enzymatic, colorimetric methods using a bench-top analyser (Pentra 400, Horiba Medical, Montpellier, France; all within-batch CV $\leq$ 5.0%). Insulin resistance was assessed by the homeostatic model assessment of insulin resistance (HOMA-IR; 35), adipose tissue insulin resistance index (Adipo-IR; 36) and the Matsuda Insulin Sensitivity Index (ISI; 37), as previously described.

Sample size calculation

FGF21 was *a priori* the primary hepatokine of interest, given that our previous pilot experiments suggested that FGF21 is acutely responsive to overnutrition (27). Based on these

data, we observed a 55% increase in FGF21 after just one day of high-fat overfeeding (27). Therefore, assuming a 55% increase in FGF21 during the course of the HE-HFD compared to control, a standardised difference of 1, an intra-individual correlation of 0.5, power of 80% and significance level of 0.05, we required at least 10 people to finish the present study. Twelve participants were therefore recruited to allow for possible drop-out.

Statistical analyses

All accelerometer and inclinometer data were analysed using ActiLife (version 6.13.3, ActiGraph Corp, Pensacola, USA) and activPAL3^{TM} software (version 7.2.32, PAL Technologies Ltd, Glasgow, UK), respectively. These data are presented as absolute minutes per day for sedentary behaviour, light and moderate-vigorous physical activity (MVPA), as well as percentages of wear time (Supplemental Table 1). The primary outcome of the study was FGF21, with the other assessed hepatokines (LECT2, fetuin-A, fetuin-B and SeP) assigned as key secondary outcomes of interest. Additional secondary outcomes were changes in anthropometry, metabolic rate, plasma metabolites (glucose, insulin, NEFA, TAG, ALT, AST and GGT) and indices of insulin resistance (HOMA-IR, Adipo-IR and the Matsuda ISI). Statistical analyses were performed using commercially available software (SPSS version 24.0, SPSS Inc., Illinois, USA). Total area under the curve (AUC) values for glucose and insulin during OGTTs were calculated using the trapezoidal method. Normality of distribution for all data were assessed using the Shapiro-Wilk test. Resting metabolic rate, AST, GGT, Adipo-IR and the Matsuda ISI were not normally distributed and were subsequently log transformed prior to analysis. Normality of distribution for these data were then re-assessed and confirmed. Paired *t*-tests were used to compare pre-diet differences in study variables between the two dietary interventions. Differences in dietary intake, composition and physical activity levels at baseline and during the control diet and HE-HFD were assessed using a one-way repeated-measures analysis of variance (ANOVA) with subsequent pairwise comparisons. Two-way

repeated-measures ANOVA (within-participant factors: diet [control, HE-HFD] and time [pre-diet, 1 d, 3 d and 7 d]) was used to examine differences in circulating proteins, metabolites, HOMA-IR and Adipo-IR between the two diets across the seven-day interventions. Two-way repeated-measures ANOVA (within-participant factors: diet [control, HE-HFD] and time [pre-diet, 7 d]) were also used to analyse the variables that were only assessed during the pre-diet and 7 d time periods (glucose AUC, insulin AUC, Matsuda ISI and anthropometric variables). In the event of statistically significant diet and interaction effects, *post-hoc* analyses were performed using paired *t*-tests to locate any differences for descriptive purposes. The magnitude of statistically significant effects was determined by calculating effect sizes (ES) using Cohen's *d* (38). Statistical significance was set at $P < 0.05$; adjustment for multiple comparisons of secondary outcomes was not undertaken, therefore these findings should be viewed with caution and in relation to the overall pattern of results. Data are described as means ± SD, unless stated otherwise.

Results

Participant characteristics, dietary intake and physical activity

Participant characteristics were ascertained during the pre-assessment visit. Participants were aged 24.3 ± 4.2 years, had a BMI of 24.1 ± 1.5 $kg \cdot m^{-2}$ and a waist circumference of 79.1 ± 3.3 cm. The participants' estimated energy requirement was calculated as 13.8 ± 0.5 $MJ \cdot d^{-1}$, therefore the target energy intake for participants during the HE-HFD was 20.7 ± 0.8 $MJ \cdot d^{-1}$. Table 1 shows participants' dietary intake and composition during the baseline assessment and study interventions. No differences were apparent in participants' dietary intake at baseline versus the control diet (all $P \geq 0.49$). Conversely, as intended, energy intake was greater during the HE-HFD compared with baseline and control (both $P < 0.001$). Furthermore, both the absolute fat intake and relative fat percentage were higher during the HE-HFD compared with baseline and control (all $P < 0.001$). In contrast, the percentage of energy derived from carbohydrate and protein was reduced during the HE-HFD (all $P \leq 0.001$); however, the absolute amount of protein was elevated (both $P \leq 0.003$).

As intended, no differences were apparent in any aspect of sedentary time or physical activity when measured at baseline or during the dietary interventions (all $P \geq 0.64$; Supplemental Table 1).

Insert table 1

Anthropometry, metabolic rate and substrate oxidation

Changes in anthropometry, RMR and substrate oxidation in response to the control and HE-HFD can be seen in Table 2. No pre-diet differences were observed for any variable prior to the control diet and HE-HFD (all $P \geq 0.52$). A diet by time interaction was observed for body mass and BMI (both $P \leq 0.009$), with a tendency for body mass and BMI to be higher after the HE-HFD compared to control (ES = 0.22, $P = 0.053$ and ES = 0.19, $P = 0.057$, respectively). No effects of diet or time were found for blood pressure (systolic and diastolic), RMR and substrate oxidation (fat and carbohydrate) (all $P \geq 0.19$).

Insert table 2

Hepatokine responses to high-fat overfeeding

Pre-diet fasting plasma concentrations of FGF21, LECT2, fetuin-A, fetuin-B and SeP were similar prior to the control diet and HE-HFD (all $P \geq 0.30$). A main effect of diet was found for FGF21 and LECT2 (both $P \leq 0.024$), and a diet by time interaction was found for FGF21, LECT2 and fetuin-A (all $P \leq 0.011$; Figure 2A-C). Subsequently, fasting plasma FGF21 concentrations were higher at 1 d and 3 d within the HE-HFD compared with control (both ES ≥ 1.67, $P \leq 0.040$). Furthermore, in comparison to the control diet, fasting plasma LECT2 concentrations were elevated at 3 d and 7 d within the HE-HFD (both ES ≥ 0.69, $P \leq 0.004$; Figure 2B). Plasma fetuin-A concentrations were also higher at 7 d of the HE-HFD when compared to the control diet (ES = 0.50, $P = 0.028$; Figure 2C). Fasting plasma fetuin-B and SeP concentrations were not different between trials or across time ($P \geq 0.52$; Figure 2D/E). The raw data for the fasting plasma hepatokine responses to the two dietary interventions can be found in Supplemental Table 2.

Insert figure 2

Metabolic responses to high-fat overfeeding

Fasting plasma metabolite responses during the control diet and HE-HFD are presented in Table 3. Pre-diet concentrations of all fasting metabolites and indices of insulin resistance were similar prior to the two dietary interventions (all $P \geq 0.18$). A main effect of diet was observed for fasting plasma glucose, TAG and HOMA-IR (all $P \leq 0.049$) and a diet by time interaction was observed for fasting plasma glucose and TAG (both $P \leq 0.001$). When compared to the control diet, fasting plasma glucose concentrations were higher at 1, 3 and 7 d of the HE-HFD (all ES ≥ 0.38, $P \leq 0.033$); whilst fasting plasma TAG concentrations were reduced at 3 d and 7 d of the HE-HFD (both ES ≥ 0.85, $P \leq 0.005$). Furthermore, HOMA-IR was greater at 3 d and 7 d of the HE-HFD compared with control (both ES ≥ 0.99, $P \leq 0.028$). No differences were observed in the fasting plasma insulin, NEFA, ALT, AST, GGT and Adipo-IR responses to the two dietary interventions (all $P \geq 0.09$).

Insert table 3

The postprandial metabolic responses to the 2 h OGTTs before and after the control diet and HE-HFD are shown in Figure 3. Glucose AUC, insulin AUC and the Matsuda ISI were not different at baseline (all $P \geq 0.30$). There were no main effects of diet, or diet by time, for the glucose and insulin AUC. However, significant main effects of diet, and diet by time, were

observed for the Matsuda ISI (both $P \leq 0.036$), which was lower after the HE-HFD when compared to control (ES = 0.62, $P = 0.021$), indicating a reduction in whole-body insulin sensitivity.

Insert figure 3

Discussion

The aim of this study was to determine the sensitivity of hepatokines to short-term perturbations in energy balance induced by a controlled period of high-fat overfeeding; and to examine the time-course of responses over seven days. The primary findings identified within this study are that both circulating FGF21 and LECT2 respond dynamically (within 1-3 days) to hyper-energetic, high-fat feeding in healthy humans; whilst small elevations in fetuin-A begin to occur after seven days. Conversely, fetuin-B and SeP are unresponsive to this nutritional challenge.

Despite a constant level of increased energy and fat intake during the seven-day HE-HFD, we observed a striking increase in circulating FGF21 concentrations after one day of the HE-HFD; which was marginally increased further after three days; before declining to pre-diet levels after seven days. In agreement, we previously observed within two separate pilot experiments that circulating FGF21 levels were increased after a one-day-, but not a seven-day, high-fat overfeeding model (27); whilst other authors have also reported elevated circulating FGF21 in response to both three (39) and five days (40) of high-fat overfeeding. Our data extend these findings by reporting a novel time-course for FGF21 in which circulating concentrations are rapidly increased in response to a HE-HFD which is transient and returns to pre-diet levels after seven days. Conversely, Lundsgaard and colleagues (41) reported only a tendency (P = 0.073) for circulating FGF21 to be increased after a three-day HE-HFD, with high-carbohydrate overfeeding inducing a substantially greater response (2 vs 8-fold increase). Despite the authors overfeeding their participants to a greater degree (+75% vs +50% estimated energy requirements), the greater increase in FGF21 seen in the present study after three days of high-fat overfeeding may be related to the higher saturated fatty acid composition of the diet (32% vs 10% energy), which has been found to be more metabolically harmful than a predominantly unsaturated fat diet (42).

FGF21 modulates whole-body lipid metabolism through inhibiting adipose tissue lipolysis and enhancing hepatic fat oxidation (43–46) via the fatty-acid induced activation of peroxisome proliferator-activated receptor-α. Consequently, the augmented circulating FGF21 levels in the present study could represent a compensatory mechanism to counteract the excessive fatty acid influx during the HE-HFD (39,40). Alternatively, this response could be attributed to FGF21's role in the integrated stress response (24). FGF21 production is elevated in response to hepatic lipotoxicity and endoplasmic reticulum stress through the PERK-eIF2α-ATF4 pathway (25,26). Notably, this pathway is activated by the consumption of a high-fat diet (47,48); particularly diets high in saturated fatty acids (49). Therefore, the rapid induction of FGF21 may be an adaptive response to maintain metabolic function in a state of nutritional and energetic stress; however, the exact physiological role in humans needs to be explored further.

It is unclear why the elevation in circulating FGF21 did not persist after seven days. A speculative hypothesis could be related to the sample of healthy and 'metabolically flexible' participants being able to increase their sensitivity to FGF21, thereby normalising circulating concentrations. Recently, it was shown that adipose tissue expression of FGF21 receptors is altered after 60 hours of fasting in humans (50); therefore, this time-frame could be feasible. Further mechanistic studies are required to understand the novel time-course observed in the current study.

In response to the HE-HFD, we also observed a progressive and sustained increase in circulating LECT2 concentrations across seven days. Specifically, circulating LECT2 tended to be higher than control after one day of high-fat overfeeding but was substantially elevated after three and seven days. This finding has extended knowledge about LECT2 by demonstrating that it is responsive to short-term changes in energy balance in humans; in addition to chronic energetic status (51–54).

This novel finding is consistent with recent data obtained from rodent models (7,15). Specifically, Chikamoto et al (15) showed that LECT2 responds dynamically to alternating periods of hypercaloric and eucaloric feeding in a rodent weight cycling model. Furthermore, in C57BL/6J mice, Lan et al (7) reported an approximate doubling of circulating LECT2 in the fed vs fasted state; and in response to one week of a high-fat diet. When contrasting data obtained from rodents and humans, it is interesting to note that individual meals (augment) and single bouts of exercise (suppress) modulate circulating LECT2 in animals (7) but not in humans (28,55). This difference may reflect the relatively greater metabolic stimulus provoked by these interventions in rodents.

Pre-clinical experiments demonstrate that LECT2 directly promotes insulin resistance in skeletal muscle and adipose tissue (7,14); and our findings raise the possibility that LECT2 may contribute to changes in insulin sensitivity in response to short-term adjustments in nutrient intake and energy balance. In our study, seven days of the HE-HFD decreased whole-body insulin sensitivity by 31%, however we did not observe any correlations between LECT2 and this outcome (data not shown). An alternative suggestion is that LECT2 may respond to protect the liver from metabolic challenge (18); however, new mechanistic studies are needed to investigate these hypotheses.

The present study does not allow us to unpick the mechanisms mediating the LECT2 response to the HE-HFD; however, it is likely that hepatic AMPK activation is relevant. AMPK is an energy-sensing kinase thought to be a ‘master regulator’ of metabolic homeostasis (56); and hepatic AMPK activation has been shown to negatively regulate hepatic LECT2 expression in two separate models (7,56).

The present study identified an interaction between diet and time for circulating fetuin-A; with higher levels apparent after seven days of the HE-HFD compared with control. However, this

elevation was subtle (6.5%) and a marginal decline in fetuin-A during the control intervention contributed to this effect. This finding is consistent with our pilot data where circulating fetuin-A tended to be higher (7.3%, $P = 0.087$) than baseline after seven days of high-fat overfeeding; but was unchanged after a single day (27). Samocha-Bonet et al (57) previously demonstrated that 28 days of high-fat overfeeding increased circulating fetuin-A by approximately 16%. It therefore appears that high-fat overfeeding induces a gradual increase in circulating fetuin-A which may occur secondary to the development of hepatic steatosis (4) and gluco-lipotoxicity (58–61).

The present study investigated the influence of high-fat overfeeding on fetuin-B and SeP given their negative regulatory influences on insulin sensitivity (62) and glucose metabolism (4). In contrast to the other hepatokines measured, circulating concentrations of fetuin-B and SeP were not influenced by the HE-HFD intervention. This extends knowledge by showing that, in humans, SeP and fetuin-B are not sensitive to acute energetic challenges induced by high-fat overfeeding. Prior to this study, only one investigation had examined the short-term influence of nutritional excess on circulating levels of SeP (63); whilst no previous data was available relating to fetuin-B. Specifically, Chen et al (63) measured circulating levels of SeP before and after three days of high-fat overfeeding (+5.23 $MJ \cdot d^{-1}$, 45% fat). Consistent with the findings reported in the present study, high-fat overfeeding had no impact on circulating SeP concentrations.

Observational data show that SeP and fetuin-B are associated with various markers of adiposity and impaired metabolic health (62,64–67). It therefore appears that these hepatokines are primarily regulated by long-term changes in energy balance and metabolism. Notably, rodent studies have shown that circulating levels of SeP were elevated after several weeks of consuming a high-fat diet (10,68). The development of hepatic steatosis appears to be central to the augmentation of both SeP and fetuin-B, as recent data identified a 1.5-fold increase in

the expression of these proteins, in steatotic vs non-steatotic hepatocytes (4). Although liver fat was not measured in the present investigation, previous data suggest that the diet used in the present study is likely to have increased liver fat by approximately one-third, from a low starting point of 2-3% in healthy individuals (69). Such an increase is still below what is considered as clinically elevated liver fat; and a more prolonged and sustained period of positive energy balance may be required to elicit changes in fetuin-B and SeP.

Key strengths of this study include the crossover design and detailed scrutiny of time-course. A consideration that must be recognised was the use of estimated energy requirements as the basis for planning the HE-HFD, as underreporting of energy intake when using food diaries is extremely common (70,71). In our study, the reported control diet was approximately 2.60 $MJ \cdot d^{-1}$ less than estimated; which is in line with our previous findings (72). As the primary purpose of the present study was to assess hepatokine responses to short-term positive energy balance, we needed to ensure that a sufficient overfeed was achieved. Other considerations within this study include the lack of diet standardisation during the control period, which may have particularly influenced the fetuin-A data, as well as the potential for carryover effects in participants who undertook the HE-HFD intervention before the control intervention. Our washout period of three weeks was based on previous studies employing an identical washout (41), and retrospective analysis of the six participants who completed the HE-HFD first showed pre-diet concentrations of all plasma analytes were similar prior to the two interventions (all $P > 0.152$). The extreme nature of the HE-HFD, which was deliberately employed to perturb metabolic homeostasis, must also be recognised. Furthermore, the participant group only included healthy men and findings may therefore not generalise to women or individuals with metabolic conditions. Moreover, additional studies are needed to explore how nutritional composition (e.g. carbohydrate vs fat; saturated vs unsaturated fat) mediates hepatokine responses to overnutrition.

In conclusion, the present study demonstrates that circulating FGF21, LECT2 and fetuin-A are elevated in response to acute hyper-energetic, high-fat feeding in healthy males, whereas fetuin-B and SeP are not responsive to short-term overnutrition. Furthermore, these responses in circulating FGF21 and LECT2 occur within 1-3 days of high-fat overfeeding; however, the FGF21 response is transient. Elevated FGF21 production could be an adaptive mechanism to limit diet-induced lipotoxicity and cellular stress; whilst increased LECT2 production may contribute to the early reduction of whole-body insulin sensitivity following overnutrition. These findings broaden understanding about the regulation of hepatokines in humans and their association with metabolic homeostasis.

Acknowledgements: The authors would like to thank Miss Katharina Rüttger for her assistance during data collection and all the study participants for their commitment to the research. The authors' responsibilities were as follows—SAW, JAS, SAP, DJS, RMW, CJH, GPA, and JAK: designed the research; SAW, VG, EB, JC, and JAK: conducted the research; VVM: provided essential materials; SAW, TY, TT, and HT: analyzed data and/or performed statistical analysis; SAW, JAS, TY, SAP, AET, DJS, CJH, GPA, and JAK: wrote the paper; JAK: had primary responsibility for final content; all authors have read and approved the final manuscript.

References

1. Meex RCR, Watt MJ. Hepatokines: linking nonalcoholic fatty liver disease and insulin resistance. Nat Rev Endocrinol 2017;13:509–20.

2. Gancheva S, Jelenik T, Álvarez-Hernández E, Roden M. Interorgan Metabolic Crosstalk in Human Insulin Resistance. Physiol Rev 2018;98:1371–415.

3. Lai KKY, Kolippakkam D, Beretta L. Comprehensive and quantitative proteome profiling of the mouse liver and plasma. Hepatology 2008;47:1043–51.

4. Meex RC, Hoy AJ, Morris A, Brown RD, Lo JCY, Burke M, Goode RJA, Kingwell BA, Kraakman MJ, Febbraio MA, et al. Fetuin B Is a Secreted Hepatocyte Factor Linking Steatosis to Impaired Glucose Metabolism. Cell Metab 2015;22:1078–89.

5. Stefan N, Hennige AM, Staiger H, Machann J, Schick F, Kröber SM, Machicao F, Fritsche A, Häring H-U. Alpha2-Heremans-Schmid glycoprotein/fetuin-A is associated with insulin resistance and fat accumulation in the liver in humans. Diabetes Care 2006;29:853–7.

6. Misu H, Ishikura K, Kurita S, Takeshita Y, Ota T, Saito Y, Takahashi K, Kaneko S, Takamura T. Inverse Correlation between Serum Levels of Selenoprotein P and Adiponectin in Patients with Type 2 Diabetes. PLoS One 2012;7:e34952.

7. Lan F, Misu H, Chikamoto K, Takayama H, Kikuchi A, Mohri K, Takata N, Hayashi H, Matsuzawa-Nagata N, Takeshita Y, et al. LECT2 Functions as a Hepatokine That Links Obesity to Skeletal Muscle Insulin Resistance. Diabetes 2014;63:1649–64.

8. Markan KR, Potthoff MJ. Metabolic fibroblast growth factors (FGFs): Mediators of energy homeostasis. Semin Cell Dev Biol 2016;53:85–93.

9. Kharitonenkov A, Adams AC. Inventing new medicines: The FGF21 story. Mol Metab 2014;3:221–9.

10. Mita Y, Nakayama K, Inari S, Nishito Y, Yoshioka Y, Sakai N, Sotani K, Nagamura T, Kuzuhara Y, Inagaki K, et al. Selenoprotein P-neutralizing antibodies improve insulin secretion and glucose sensitivity in type 2 diabetes mouse models. Nat Commun 2017;8:1658.

11. Younossi ZM, Baranova A, Ziegler K, Del Giacco L, Schlauch K, Born TL, Elariny H, Gorreta F, VanMeter A, Younoszai A, et al. A genomic and proteomic study of the spectrum of nonalcoholic fatty liver disease. Hepatology 2005;42:665–74.

12. Kirpich IA, Gobejishvili LN, Homme MB, Waigel S, Cave M, Arteel G, Barve SS, McClain CJ, Deaciuc I V. Integrated hepatic transcriptome and proteome analysis of mice with high-fat diet-induced nonalcoholic fatty liver disease. J Nutr Biochem 2011;22:38–45.

13. Fu S, Fan J, Blanco J, Gimenez-Cassina A, Danial NN, Watkins SM, Hotamisligil GS. Polysome Profiling in Liver Identifies Dynamic Regulation of Endoplasmic Reticulum Translatome by Obesity and Fasting. PLoS Genet 2012;8:e1002902.

14. Jung TW, Chung YH, Kim H-C, Abd El-Aty AM, Jeong JH. LECT2 promotes inflammation and insulin resistance in adipocytes via P38 pathways. J Mol Endocrinol 2018;61:37–45.

15. Chikamoto K, Misu H, Takayama H, Kikuchi A, Ishii K, Lan F, Takata N, Tajima-Shirasaki N, Takeshita Y, Tsugane H, et al. Rapid response of the steatosis-sensing hepatokine LECT2 during diet-induced weight cycling in mice. Biochem Biophys Res Commun 2016;478:1310–6.

16. Trepanowski JF, Mey J, Varady KA. Fetuin-A: a novel link between obesity and related complications. Int J Obes 2015;39:734–41.

17. Takayama H, Misu H, Iwama H, Chikamoto K, Saito Y, Murao K, Teraguchi A, Lan F, Kikuchi A, Saito R, et al. Metformin Suppresses Expression of the Selenoprotein P Gene via an AMP-activated Kinase (AMPK)/FoxO3a Pathway in H4IIEC3 Hepatocytes. J Biol Chem 2014;289:335–45.

18. Slowik V, Apte U. Leukocyte Cell-Derived Chemotaxin-2: It's Role in Pathophysiology and Future in Clinical Medicine. Clin Transl Sci 2017;10:249–59.

19. Inagaki T, Dutchak P, Zhao G, Ding X, Gautron L, Parameswara V, Li Y, Goetz R, Mohammadi M, Esser V, et al. Endocrine Regulation of the Fasting Response by PPARα-Mediated Induction of Fibroblast Growth Factor 21. Cell Metab 2007;5:415–25.

20. Laeger T, Henagan TM, Albarado DC, Redman LM, Bray GA, Noland RC, Münzberg H, Hutson SM, Gettys TW, Schwartz MW, et al. FGF21 is an endocrine signal of protein restriction. J Clin Invest 2014;124:3913–22.

21. Markan KR, Naber MC, Ameka MK, Anderegg MD, Mangelsdorf DJ, Kliewer SA, Mohammadi M, Potthoff MJ. Circulating FGF21 Is Liver Derived and Enhances Glucose Uptake During Refeeding and Overfeeding. Diabetes 2014;63:4057–63.

22. Fisher FM, Maratos-Flier E. Understanding the Physiology of FGF21. Annu Rev Physiol 2016;78:223–41.

23. Bondurant LD, Potthoff MJ. Fibroblast Growth Factor 21: A Versatile Regulator of Metabolic Homeostasis. 2018;1–24.

24. Salminen A, Kaarniranta K, Kauppinen A. Integrated stress response stimulates FGF21 expression: Systemic enhancer of longevity. Cell Signal 2017;40:10–21.

25. Maruyama R, Shimizu M, Hashidume T, Inoue J, Itoh N, Sato R. FGF21 Alleviates Hepatic Endoplasmic Reticulum Stress under Physiological Conditions. J Nutr Sci Vitaminol 2018;64:200–8.

26. Xu X, Krumm C, So J-S, Bare CJ, Holman C, Gromada J, Cohen DE, Lee A-H. Preemptive Activation of the Integrated Stress Response Protects Mice From Diet-Induced Obesity and Insulin Resistance by Fibroblast Growth Factor 21 Induction. Hepatology 2018;68:2167–81.

27. Willis SA, King JA, Parry SA, Woods RM, Sargeant JA, Hulston CJ. The effect of acute, high-fat overfeeding on circulating hepatokine concentrations in BASES Conference 2018 – Programme and Abstracts. J Sports Sci 2018;38(sup 1):60.

28. Willis SA, Sargeant JA, Thackray AE, Yates T, Stensel DJ, Aithal GP, King JA. Effect of exercise intensity on circulating hepatokine concentrations in healthy men. Appl Physiol Nutr Metab 2019;44:1065–72.

29. Hulston CJ, Churnside AA, Venables MC. Probiotic supplementation prevents high-fat, overfeeding-induced insulin resistance in human subjects. Br J Nutr 2015;113:596–602.

30. Frayn KN. Calculation of substrate oxidation rates in vivo from gaseous exchange. J Appl Physiol 1983;55:628–34.

31. Mifflin MD, St Jeor ST, Hill LA, Scott BJ, Daugherty SA, Koh YO. A new predictive equation for resting energy expenditure in healthy individuals. Am J Clin Nutr 1990;51:241–7.

32. FAO, WHO and UNU. Human energy requirements: report of a joint FAO/ WHO/UNU Expert Consultation [Internet]. Food and Agriculture Organisation; 2005. [cited 2019 Oct 03]. Available from: http://www.fao.org/3/y5686e/y5686e00.htm.

33. Tanaka M, Saito Y, Misu H, Kato S, Kita Y, Takeshita Y, Kanamori T, Nagano T, Nakagen M, Urabe T, et al. Development of a Sol Particle Homogeneous Immunoassay for Measuring Full-Length Selenoprotein P in Human Serum. J Clin Lab Anal 2016;30:114–22.

34. Saito Y, Misu H, Takayama H, Takashima S, Usui S, Takamura M, Kaneko S, Takamura T, Noguchi N. Comparison of Human Selenoprotein P Determinants in Serum between Our Original Methods and Commercially Available Kits. Biol Pharm Bull 2018;41:828–32.

35. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28:412–9.

36. Gastaldelli A, Cusi K, Pettiti M, Hardies J, Miyazaki Y, Berria R, Buzzigoli E, Sironi AM, Cersosimo E, Ferrannini E, et al. Relationship Between Hepatic/Visceral Fat and Hepatic Insulin Resistance in Nondiabetic and Type 2 Diabetic Subjects. Gastroenterology 2007;133:496–506.

37. Matsuda M, DeFronzo RA. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. Diabetes Care 1999;22:1462–70.

38. Cohen J. Statistical Power Analysis for the Behavioral Sciences. 2nd ed. Hillsdale, NJ, USA: Lawrence Erlbaum Associates; 1988.

39. Heilbronn LK, Campbell L V., Xu A, Samocha-Bonet D. Metabolically Protective Cytokines Adiponectin and Fibroblast Growth Factor-21 Are Increased by Acute Overfeeding in Healthy Humans. PLoS One 2013;8:e78864.

40. Vienberg SG, Brøns C, Nilsson E, Astrup A, Vaag A, Andersen B. Impact of short-term high-fat feeding and insulin-stimulated FGF21 levels in subjects with low birth weight and controls. Eur J Endocrinol 2012;167:49–57.

41. Lundsgaard A-M, Fritzen AM, Sjøberg KA, Myrmel LS, Madsen L, Wojtaszewski JFP, Richter EA, Kiens B. Circulating FGF21 in humans is potently induced by short term overfeeding of carbohydrates. Mol Metab 2017;6:22–9.

42. Luukkonen PK, Sädevirta S, Zhou Y, Kayser B, Ali A, Ahonen L, Lallukka S, Pelloux V, Gaggini M, Jian C, et al. Saturated Fat Is More Metabolically Harmful for the Human Liver Than Unsaturated Fat or Simple Sugars. Diabetes Care 2018;41:1732–9.

43. Badman MK, Pissios P, Kennedy AR, Koukos G, Flier JS, Maratos-Flier E. Hepatic Fibroblast Growth Factor 21 Is Regulated by PPARα and Is a Key Mediator of Hepatic Lipid Metabolism in Ketotic States. Cell Metab 2007;5:426–37.

44. Arner P, Pettersson A, Mitchell PJ, Dunbar JD, Kharitonenkov A, Rydén M. FGF21 attenuates lipolysis in human adipocytes - A possible link to improved insulin sensitivity. FEBS Lett 2008;582:1725–30.

45. Yang C, Wang C, Ye M, Jin C, He W, Wang F, McKeehan WL, Luo Y. Control of lipid metabolism by adipocyte FGFR1-mediated adipohepatic communication during hepatic stress. Nutr Metab 2012;9:94.

46. Fisher FM, Chui PC, Nasser IA, Popov Y, Cunniff JC, Lundasen T, Kharitonenkov A, Schuppan D, Flier JS, Maratos-Flier E. Fibroblast Growth Factor 21 Limits

Lipotoxicity by Promoting Hepatic Fatty Acid Activation in Mice on Methionine and Choline-Deficient Diets. Gastroenterology 2014;147:1073–1083.e6.

47. Birkenfeld AL, Lee H-Y, Majumdar S, Jurczak MJ, Camporez JP, Jornayvaz FR, Frederick DW, Guigni B, Kahn M, Zhang D, et al. Influence of the Hepatic Eukaryotic Initiation Factor 2α (eIF2α) Endoplasmic Reticulum (ER) Stress Response Pathway on Insulin-mediated ER Stress and Hepatic and Peripheral Glucose Metabolism. J Biol Chem 2011;286:36163–70.

48. Boden G, Song W, Duan X, Cheung P, Kresge K, Barrero C, Merali S. Infusion of Glucose and Lipids at Physiological Rates Causes Acute Endoplasmic Reticulum Stress in Rat Liver. Obesity 2011;19:1366–73.

49. Leamy AK, Egnatchik RA, Shiota M, Ivanova PT, Myers DS, Brown HA, Young JD. Enhanced synthesis of saturated phospholipids is associated with ER stress and lipotoxicity in palmitate treated hepatic cells. J Lipid Res 2014;55:1478–88.

50. Nygaard EB, Ørskov C, Almdal T, Vestergaard H, Andersen B. Fasting decreases plasma FGF21 in obese subjects and the expression of FGF21 receptors in adipose tissue in both lean and obese subjects. J Endocrinol 2018;239:73–80.

51. Okumura A, Unoki-Kubota H, Matsushita Y, Shiga T, Moriyoshi Y, Yamagoe S, Kaburagi Y. Increased serum leukocyte cell-derived chemotaxin 2 (LECT2) levels in obesity and fatty liver. Biosci Trends 2013;7:276–83.

52. Yoo HJ, Hwang SY, Choi J-H, Lee HJ, Chung HS, Seo J-A, Kim SG, Kim NH, Baik SH, Choi DS, et al. Association of leukocyte cell-derived chemotaxin 2 (LECT2) with NAFLD, metabolic syndrome, and atherosclerosis. PLoS One 2017;12:e0174717.

53. Tanisawa K, Taniguchi H, Sun X, Ito T, Kawakami R, Sakamoto S, Higuchi M.

Visceral fat area is a strong predictor of leukocyte cell-derived chemotaxin 2, a potential biomarker of dyslipidemia. PLoS One 2017;12:e0173310.

54. Zhang Z, Zeng H, Lin J, Hu Y, Yang R, Sun J, Chen R, Chen H. Circulating LECT2 levels in newly diagnosed type 2 diabetes mellitus and their association with metabolic parameters. Medicine 2018;97:e0354.

55. Sargeant JA, Aithal GP, Takamura T, Misu H, Takayama H, Douglas JA, Turner MC, Stensel DJ, Nimmo MA, Webb DR, et al. The influence of adiposity and acute exercise on circulating hepatokines in normal-weight and overweight/obese men. Appl Physiol Nutr Metab 2018;43:482–90.

56. Garcia D, Hellberg K, Chaix A, Wallace M, Herzig S, Badur MG, Lin T, Shokhirev MN, Pinto AFM, Ross DS, et al. Genetic Liver-Specific AMPK Activation Protects against Diet-Induced Obesity and NAFLD. Cell Rep 2019;26:192–208.e6.

57. Samocha-Bonet D, Tam CS, Campbell L V., Heilbronn LK. Raised Circulating Fetuin-A After 28-Day Overfeeding in Healthy Humans. Diabetes Care 2014;37:e15–6.

58. Lin X, Braymer HD, Bray GA, York DA. Differential expression of insulin receptor tyrosine kinase inhibitor (fetuin) gene in a model of diet-induced obesity. Life Sci 1998;63:145–53.

59. Takata H, Ikeda Y, Suehiro T, Ishibashi A, Inoue M, Kumon Y, Terada Y. High glucose induces transactivation of the alpha2-HS glycoprotein gene through the ERK1/2 signaling pathway. J Atheroscler Thromb 2009;16:448–56.

60. Dasgupta S, Bhattacharya S, Biswas A, Majumdar SS, Mukhopadhyay S, Ray S, Bhattacharya S. NF-κB mediates lipid-induced fetuin-A expression in hepatocytes that impairs adipocyte function effecting insulin resistance. Biochem J 2010;429:451–62.

61. Jung TW, Youn B-S, Choi HY, Lee SY, Hong HC, Yang SJ, Yoo HJ, Kim B-H, Baik SH, Choi KM. Salsalate and adiponectin ameliorate hepatic steatosis by inhibition of the hepatokine fetuin-A. Biochem Pharmacol 2013;86:960–9.

62. Misu H, Takamura T, Takayama H, Hayashi H, Matsuzawa-Nagata N, Kurita S, Ishikura K, Ando H, Takeshita Y, Ota T, et al. A Liver-Derived Secretory Protein, Selenoprotein P, Causes Insulin Resistance. Cell Metab 2010;12:483–95.

63. Chen M, Liu B, Wilkinson D, Hutchison AT, Thompson CH, Wittert GA, Heilbronn LK. Selenoprotein P is elevated in individuals with obesity, but is not independently associated with insulin resistance. Obes Res Clin Pract 2017;11:227–32.

64. Yang SJ, Hwang SY, Choi HY, Yoo HJ, Seo JA, Kim SG, Kim NH, Baik SH, Choi DS, Choi KM. Serum Selenoprotein P Levels in Patients with Type 2 Diabetes and Prediabetes: Implications for Insulin Resistance, Inflammation, and Atherosclerosis. J Clin Endocrinol Metab. 2011;96:E1325–9.

65. Choi HY, Hwang SY, Lee CH, Hong HC, Yang SJ, Yoo HJ, Seo JA, Kim SG, Kim NH, Baik SH, et al. Increased Selenoprotein P Levels in Subjects with Visceral Obesity and Nonalcoholic Fatty Liver Disease. Diabetes Metab J 2013;37:63.

66. Zhu J, Wan X, Wang Y, Zhu K, Li C, Yu C, Li Y. Serum fetuin B level increased in subjects of nonalcoholic fatty liver disease: a case-control study. Endocrine 2017;56:208–11.

67. Peter A, Kovarova M, Staiger H, Machann J, Schick F, Königsrainer A, Königsrainer I, Schleicher E, Fritsche A, Häring H-U, et al. The hepatokines fetuin-A and fetuin-B are upregulated in the state of hepatic steatosis and may differently impact on glucose homeostasis in humans. Am J Physiol Metab 2018;314:E266–73.

68. Onishi S, Kitazawa H, Meguro S, Tokimitsu I. Green tea extracts reduce leukocyte cell–Derived chemotaxin 2 and selenoprotein P levels in the livers of C57BL/6J mice fed a high-fat diet. Biosci Biotechnol Biochem 2018;82:1568–75.

69. Wulan SN, Schrauwen-Hinderling VB, Westerterp KR, Plasqui G. Liver fat accumulation in response to overfeeding with a high-fat diet: a comparison between South Asian and Caucasian men. Nutr Metab 2015;12:18.

70. Macdiarmid J, Blundell J. Assessing dietary intake: Who, what and why of under-reporting. Nutr Res Rev 1998;11:231.

71. Salle A, Ryan M, Ritz P. Underreporting of Food Intake in Obese Diabetic and Nondiabetic Patients. Diabetes Care 2006;29:2726–7.

72. Parry SA, Smith JR, Corbett TRB, Woods RM, Hulston CJ. Short-term, high-fat overfeeding impairs glycaemic control but does not alter gut hormone responses to a mixed meal tolerance test in healthy, normal-weight individuals. Br J Nutr 2017;117:48–55.

.

Table 1. Dietary intake and composition at baseline and during the seven-day control and hyper-energetic, high-fat diets.

	Baseline (Pre-intervention)	**Control diet**	**HE-HFD**	***P*-value** (Diet effect)
Energy (MJ·d^{-1})	11.5 ± 1.4	10.9 ± 2.0	20.9 ± 0.8^{ab}	**<0.001**
Fat				
Grams per day	111 ± 25	102 ± 27	356 ± 15^{ab}	**<0.001**
% energy	36.9 ± 8.0	35.7 ± 6.7	65.0 ± 0.6^{ab}	**<0.001**
SFA (% energy)	13.7 ± 2.9	13.2 ± 2.6	31.5 ± 0.5^{ab}	**<0.001**
MUFA (% energy)	15.9 ± 5.2	15.7 ± 4.4	25.7 ± 0.5^{ab}	**<0.001**
PUFA (% energy)	7.3 ± 2.0	6.9 ± 2.0	7.8 ± 0.5	0.44
Carbohydrate				
Grams per day	301 ± 75	288 ± 70	256 ± 10	0.07
% energy	44.1 ± 9.3	45.3 ± 8.1	20.7 ± 0.4^{ab}	**<0.001**
Protein				
Grams per day	126 ± 29	117 ± 44	176 ± 5^{ab}	**<0.001**
% energy	19.0 ± 2.7	18.9 ± 3.3	14.2 ± 0.4^{ab}	**<0.001**

Data are presented as means ± SD, n = 12 healthy men. HE-HFD, hyper-energetic, high-fat diet; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

^{a}Significantly different from baseline assessment, $P < 0.05$.

^{b}Significantly different from control diet, $P < 0.05$

Table 2. Anthropometric variables, metabolic rate and substrate oxidation before and after seven days of the control and hyper-energetic, high-fat diets.

	Control diet		**HE-HFD**		***P*-value** (Diet effect)	***P*-value** (Interaction effect)
	Pre-diet	**7 d**	**Pre-diet**	**7 d**		
Body mass (kg)	77.1 ± 4.3	77.1 ± 4.3	76.8 ± 3.7	78.0 ± 4.1[#]	0.20	**0.009**
BMI ($kg \cdot m^{-2}$)	24.2 ± 1.6	24.2 ± 1.6	24.1 ± 1.5	24.5 ± 1.5[#]	0.42	**0.001**
SBP (mmHg)	127 ± 8	128 ± 8	128 ± 8	128 ± 5	0.33	0.33
DBP (mmHg)	71 ± 8	71 ± 7	72 ± 5	74 ± 4	0.31	0.66
RMR ($MJ \cdot d^{-1}$)[a]	4.71 (2.09)	5.05 (1.66)	4.81 (1.61)	4.64 (1.25)	0.22	0.98
Fat oxidation (%)	12.5 ± 10.3	13.1 ± 9.3	10.7 ± 8.4	16.7 ± 9.8	0.60	0.19
CHO oxidation (%)	87.5 ± 10.3	86.9 ± 9.3	89.3 ± 8.4	83.3 ± 9.8	0.60	0.19

Data are means ± SD or medians (IQR), n = 12 healthy men. CHO, carbohydrate; DBP, diastolic blood pressure; HE-HFD, hyper-energetic, high-fat diet; RMR, resting metabolic rate; SBP, systolic blood pressure.

[a]Indicates data log transformed prior to analysis and therefore presented as medians (IQR).

[#]Tended to differ from control diet at the same time point, $P < 0.06$.

Table 3. Fasting plasma metabolite and liver enzyme concentrations, and indices of insulin resistance during the seven-day control and hyper-energetic, high-fat diets.

	Intervention	**Pre-diet**	**1 d**	**3 d**	**7 d**	***P*-value** (Diet effect)	***P*-value** (Interaction effect)
Glucose	Control diet	4.9 ± 0.4	4.8 ± 0.4	4.6 ± 0.4	4.8 ± 0.4	**0.003**	**<0.001**
($mmol \cdot L^{-1}$)	HE-HFD	4.8 ± 0.4	5.0 ± 0.3**	5.0 ± 0.5**	5.0 ± 0.3*		
Insulin	Control diet	25 ± 12	28 ± 13	22 ± 9	23 ± 7	0.06	0.21
($pmol \cdot L^{-1}$)	HE-HFD	27 ± 11	30 ± 8	30 ± 8	31 ± 11		
NEFA	Control diet	0.37 ± 0.13	0.30 ± 0.12	0.33 ± 0.16	0.32 ± 0.13	0.12	0.94
($mmol \cdot L^{-1}$)	HE-HFD	0.31 ± 0.12	0.26 ± 0.14	0.30 ± 0.09	0.25 ± 0.09		
TAG	Control diet	0.75 ± 0.19	0.76 ± 0.19	0.74 ± 0.20	0.86 ± 0.29	**0.031**	**0.001**
($mmol \cdot L^{-1}$)	HE-HFD	0.82 ± 0.16	0.63 ± 0.20	0.57 ± 0.16**	0.57 ± 0.16**		
HOMA-IR	Control diet	0.8 ± 0.4	0.9 ± 0.5	0.7 ± 0.3	0.7 ± 0.3	**0.049**	0.09
(AU)	HE-HFD	0.8 ± 0.4	1.0 ± 0.3	1.0 ± 0.3*	1.0 ± 0.4*		
Adipo-IR[a]	Control diet	8.8 (8.5)	6.7 (5.6)	5.2 (3.7)	6.5 (5.9)	0.82	0.36
(AU)	HE-HFD	8.6 (4.7)	6.2 (8.4)	8.9 (5.2)	6.8 (6.4)		
ALT	Control diet	21.5 ± 7.9	21.7 ± 7.3	22.2 ± 6.8	21.9 ± 9.3	0.11	0.11
($U \cdot L^{-1}$)	HE-HFD	20.1 ± 6.4	24.5 ± 9.6	31.2 ± 16.9	53.7 ± 63.6		
AST	Control diet	28.7 (11.8)	27.8 (7.2)	27.9 (12.4)	27.3 (6.7)	0.14	0.18
($U \cdot L^{-1}$)[a]	HE-HFD	28.2 (9.7)	27.6 (12.3)	30.3 (14.6)	36.8 (32.5)		
GGT	Control diet	20.1 (7.3)	21.5 (7.8)	22.0 (12.2)	21.3 (12.3)	0.23	0.22
($U \cdot L^{-1}$)[a]	HE-HFD	20.0 (12.8)	19.2 (9.6)	19.9 (14.5)	22.2 (12.3)		

Data are means ± SD or medians (IQR), n = 12 healthy men. Adipo-IR, adipose tissue insulin resistance index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AU, arbitrary units; GGT, gamma-glutamyl transferase; HE-HFD, hyper-energetic, high-fat diet; HOMA-IR, homeostatic model assessment of insulin resistance; NEFA, non-esterified fatty acids; TAG, triacylglycerol.

[a]Indicates data log transformed prior to analysis and therefore presented as medians (IQR).

*Significantly different from control diet at the same time point, $P < 0.05$.

**Significantly different from control diet at the same time point, $P < 0.01$.

Figure legends

Figure 1. Schematic illustration of the study design and trial procedures. HE-HFD = 7-day hyper-energetic, high-fat diet; control intervention = 7-day habitual diet. Food intake was measured during the control intervention by a 3-day food diary, whilst all food was provided during the HE-HFD intervention. Participant trial order was randomised. OGTT, oral glucose tolerance test.

Figure 2. Fasting plasma concentrations of (A) fibroblast growth factor 21 (FGF21), (B) leukocyte cell-derived chemotaxin 2 (LECT2), (C) fetuin-A, (D) fetuin-B and (E) selenoprotein P (SeP) during the seven-day control diet and hyper-energetic, high-fat diet (HE-HFD). Data are presented as means ± SEM, n = 12 healthy men**.**

*Significantly different from control diet at the same time point, $P < 0.05$.

**Significantly different from control diet at the same time point, $P < 0.01$.

***Significantly different from control diet at the same time point, $P < 0.001$.

Figure 3. Plasma (A) glucose and (B) insulin area under the curve; and (C) the Matsuda Insulin Sensitivity Index calculated during an oral glucose tolerance test before (pre-diet) and after (post-diet) seven days of the control diet and hyper-energetic, high-fat diet (HE-HFD). Data are presented as means ± SEM or medians (IQR), n = 12 healthy men.

[a]Matsuda Insulin Sensitivity Index data were log transformed prior to analysis and therefore presented as medians (IQR).

*Significantly different from control diet at the same time point, $P < 0.05$.