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Brief Communication

Circulating FGF21 in humans is potently induced by short term overfeeding of carbohydrates

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ABSTRACT

Objective: Fibroblast-growth factor 21 (FGF21) is thought to be important in metabolic regulation. Recently, low protein diets have been shown to increase circulating FGF21 levels. However, when energy contribution from dietary protein is lowered, other macronutrients, such as carbohydrates, must be increased to meet eucaloric balance. This raises the possibility that intake of a diet rich in carbohydrates may induce an increase in plasma FGF21 levels per se. Here we studied the role of dietary carbohydrates on the levels of circulating FGF21 and concomitant physiologic effects by feeding healthy men a carbohydrate rich diet without reducing protein intake.

Methods: A diet enriched in carbohydrates (80 E% carbohydrate; CHO) and a eucaloric control diet (CON) were provided to nine healthy men for three days. The energy intake during the CHO diet was increased (+75% energy) to ensure similar dietary protein intake in CHO and CON. To control for the effect of caloric surplus, we similarly overfed (+75% energy) the same subjects for three days with a fat-rich diet (78 E% fat; FAT), consisting of primarily unsaturated fatty acids. The three diets were provided in random order.

Results: After CHO, plasma FGF21 concentration increased 8-fold compared to CON (329 ± 99 vs. 39 ± 9 pg ml−1, p < 0.05). In contrast, after FAT only a non-significant tendency (p = 0.073) to an increase in plasma FGF21 concentration was found. The increase in FGF21 concentration after CHO correlated closely (r = 0.88, p < 0.01) with increased leg glucose uptake (62%, p < 0.05) and increased hepatic glucose production (17%, p < 0.01), indicating increased glucose turnover. Plasma fatty acid (FA) concentration was decreased by 68% (p < 0.01), supported by reduced subcutaneous adipose tissue HSL Ser160 phosphorylation (p < 0.01) and perilipin 1 protein content (p < 0.01), pointing to a suppression of adipose tissue lipolysis. Concomitantly, a 146% increase in the plasma marker of hepatic de novo lipogenesis C16:1 n−7 FA (p < 0.01) was observed together with 101% increased plasma TG concentration (p < 0.001) in association with CHO intake and increased plasma FGF21 concentration.

Conclusion: Excess dietary carbohydrate, but not fat, led to markedly increased FGF21 secretion in humans, notably without protein restriction, and affected glucose and lipid homeostasis.

Keywords FGF21; Diet; Carbohydrates; Lipolysis; Liver

1. INTRODUCTION

The hormone fibroblast growth factor 21 (FGF21) was early reported to improve glucose homeostasis and to lower circulating triglycerides in obese and insulin resistant mice [1] and later observed to be important in the metabolic adaptations to fasting and ketosis [2,3]. Now, FGF21 has received increasing interest due to its role in the regulation of energy homeostasis and its potential antidiabetic and lipid-lowering properties [4,5]. Therefore, FGF21 holds promise as a therapeutic target, although intriguingly being elevated in obesity and type 2 diabetes [6,7]. In mice, restriction of essential amino acids [8–10] as well as non-essential amino acids [11] has been shown to increase plasma FGF21 levels. In humans, 28 days restriction of dietary protein content to 6 E%, compared to 15 E% in the baseline diet, was shown to increase plasma FGF21 concentration by 171% [12]. This was, however, under hypercaloric (+39 E%) conditions resulting in a weight gain (+3.16 kg on average). Furthermore, the reduced protein was compensated by increasing the fat intake from 25 to 52 E% and decreasing the carbohydrate intake from 60 to 42 E% [13]. Thus, it is not clear whether the increase in circulating FGF21 obtained in the

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Abbreviations: ATGL, adipose triglyceride lipase; AMPK, AMP-activated kinase; BCA, bicinchoninic acid; BM, body mass; BMI, body mass index; CHO, carbohydrate-rich diet; ChREBP, carbohydrate-responsive element binding protein; CON, control diet; FA, fatty acid; FAT, fat-rich diet; FGF21, fibroblast growth factor 21; GLUT4, glucose transporter 4; HSL, hormone sensitive lipase; LM, leg mass; VO2peak, maximal oxygen consumption; PKA, protein kinase A; R0, rate of appearance; TG, triacylglycerol; VLDL, very low density lipoprotein

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shown in humans that single monosaccharide intake brie
additive effect of high-carbohydrate feeding to the induction of circu-
providing 25 diets with varying macronutrient composition, showed an
% protein, 9 % fat, 24 E% fat) and a eucaloric control diet (CON) (62 E% car-
volunteers (unpublished). In the present study, these subjects ingested
the effects of different types of diets on metabolism in nine male
were healthy, moderately physically active, and with no family history
was received from each participant prior to study inclusion. All subjects
accordance with the Declaration of Helsinki. Informed written consent
Nine men were recruited for the study, which was approved by the
2.1. Subjects and diets

2. METHODS

2.1. Subjects and diets
Nine men were recruited for the study, which was approved by the
Copenhagen Ethics Committee (KF 01 261127) and performed in
accordance with the Declaration of Helsinki. Informed written consent
was received from each participant prior to study inclusion. All subjects
were healthy, moderately physically active, and with no family history
of diabetes. This study is part of a larger project aiming at elucidating
the effects of different types of diets on metabolism in nine male
volunteers (unpublished). In the present study, these subjects ingested
a hypercaloric carbohydrate-rich diet (CHO) (80 E% carbohydrate, 11 E
% protein, 9 E% fat) and a eucaloric control diet (CON) (62 E% car-
bohydrates, 14 E% protein, 24 E% fat), reflecting their habitual diet. A
hypercaloric high-fat diet, consisting primarily of unsaturated FA (FAT)
(10 E% carbohydrate, 12 E% protein, 78 E% fat) was also provided in
order to serve as control for the caloric excess. The macronutrient
composition of CHO, FAT, and CON are summarized in Supplemental
Table S1. All diets were provided for three days in a randomized
cross-over design, with each intervention separated by at least three
weeks. Before each three-day intervention, all subjects consumed the
eucaloric control diet for 5 days to ensure the same conditions before
each intervention. All food items were weighed and prepared in the
metabolic kitchen. The daily menus were delivered to the subject, who
ingested them at home. The CHO diet consisted of carbohydrate-rich
food items as bread, pasta, cereals, corn, jam, and juice, with high
to moderate glycemic index. The diet was mainly comprised of poly-
saccharides, with 19% refined sugar and the ratio between glucose
and fructose was 1:1 (Supplemental Table S3 and S4). In the FAT diet,
mono- and polysaturated fatty acids both comprised 34 E%
(Supplemental Table S1).

To evaluate the training status of the participants, maximal oxygen
uptake was measured by an incremental exercise test on a Monark
Ergomedic 839E bicycle ergometer (Monark, Sweden). Body
composition and leg mass (LM) were determined using dual-energy
X-ray absorptiometry (Lunar DPX-iQ DEXA Scanner, Lunar Corpora-
tion, USA) after 4 h of fasting. The nine male subjects were 23 ± 3
(mean ± SD) years old, with a body mass index (BMI) of
23.7 ± 1.7 kg m⁻² and maximal oxygen uptake (VO₂peak) of
52 ± 4 ml kg⁻¹ min⁻¹. The daily energy requirements were individually determined from
weighed dietary registrations and calculations of energy requirements
(WHO/FAO/UNU). The three days CHO and FAT diets were provided in
excess of eucaloric energy requirements, with a +75% increase in
daily energy intake. Hence, the subjects ingested 13.7 ± 0.2 MJ
during the CON diet and according to the hypercaloric energy provision
24.0 ± 0.4 MJ during the experimental diets (Table S1).

2.2. Experimental protocol
After three days on the experimental diets or the control diet, subjects
ingested a light standardized breakfast (1.6 MJ) at 5 A.M at home
(Supplemental Table S5). Subjects arrived at the institute at 7.30 A.M,
and, after a period of supine rest, teflon catheters were inserted in the
femoral artery and vein under local anesthesia. Then, a [6,6-²H₂]
glucose tracer infusion (0.055 mg kg⁻¹ min⁻¹) was administered in
order to measure hepatic glucose production. The infusion was initi-
ated by a bolus injection of [6,6-²H₂] glucose (3.203 mg kg⁻¹). After
120 min, blood samples were obtained from the femoral artery and
vein. Femoral arterial blood flow was determined simultaneously with
the arterio-venous blood sampling by a laser ultra-sound Doppler
technique (Philips IU22, ViCare Medical, Denmark) [25]. In order to
study molecular metabolism in adipose tissue, biopsies were obtained
from the subcutaneous periumbilical adipose tissue, immediately
frozen in liquid nitrogen and stored at −80 °C. All blood and tissue
samples were collected 6 h after the breakfast meal, when subjects
were in the post-absorptive state.

2.3. Plasma analyses
Plasma glucose concentration was measured on an ABL615 (Radi-
ometer Medical A/S, Denmark), and insulin concentration was
measured by an enzyme-linked immunosorbent assay (ELISA)
(ALPCO, USA). The plasma concentrations of FA (NEFA C kit, Wako
Chemicals GmbH, Germany) and triacylglycerol (TG) (GPO-PAP kit,
Roche Diagnostics, Germany) were measured using enzymatic
colorimetric methods (Hitachi 912 automatic analyzer, Boehringer,
Germany). Plasma cholesterol concentration was measured by a
fluorometric method (Roche Diagnostics, Germany). Plasma
epinephrine and norepinephrine concentrations were determined by
radioimmunoassay (2-CAT 125I RIA kit, Labor Diagnostika,
Germany).
Plasma adiponectin concentration was measured on an AutoDELFIA (Perkin Elmer, USA) automated analyzer. Plasma FGF21 concentration was measured by ELISA (FGF21 Quantikine ELISA kit, R&D Systems, USA), in which the standard curve range for the assay was 10–2000 pg ml\(^{-1}\). FGF21 was analyzed in both arterial and venous plasma, with no difference obtained in FGF21 concentration. Plasma enrichments of the stable glucose isotope were measured using liquid chromatography mass spectrometry (ThermoQuest Finnegan AQA, USA) as previously described [26]. For analysis of palmitoleic acid concentration in plasma, lipids were extracted using chloroform/methanol (2:1, v/v) and filtered prior to saponification and methylation using 12% BF\(_3\) in methanol. Methylated FA were separated as described in detail elsewhere [27]. The respective FA species were identified by retention time using standard mixtures of methyl esters (Nu-Chek, USA).

2.4. Western blotting
Samples of subcutaneous adipose tissue were homogenized (Tissue Lyzer II, Qiagen, Germany) in ice-cold buffer as described previously [28]. Homogenates were rotated end-over-end for one hour, and lysate supernatants were collected by centrifuging 20 min at 16,000 g at 4 °C. The upper fat layer was carefully removed before the lysate supernatant was collected. Protein concentrations were determined in triplicates using the bichinchoninic acid (BCA) method (Pierce Biotechnology BCA, USA). Samples were heated (96 °C) in Laemmli buffer before subjected to SDS-PAGE and semi-dry blotting. The primary antibodies used are described in Supplemental Table S2. Secondary antibodies were from Dako Cymation (Denmark). Membranes were probed with enhanced chemiluminescence (ECL \(^{+}\); Amersham Biosciences, USA), and immune complexes were visualized using BioRad ChemiDoc™ MP Imaging System (Hercules, USA). Signals were quantified (Image Lab, Life Sciences, USA) and expressed as arbitrary units.

2.5. Calculations
Leg glucose uptake was calculated as the arterial-venous difference multiplied by blood flow in accordance with Fick’s principle and related to leg mass (LM). Glucose rate of appearance (Ra) was calculated from the last 30 min of the 120 min tracer infusion period using Steele’s steady state equations [29].

2.6. Statistical analyses
All data are expressed as means ± SEM, except characteristics of the subjects (means ± SD). Data from each experimental trial were compared to the control trial by paired t-tests, to test for effect of the respective experimental diets. The strength of association between parameters was analyzed using Pearson correlation analysis.

3. RESULTS AND DISCUSSION

3.1. Dietary carbohydrate but not caloric excess increases circulating FGF21
Here we demonstrate in healthy male volunteers that three days consumption of a hypercaloric carbohydrate-rich diet (80 %) increased the plasma FGF21 concentration 803% compared with CON (p < 0.05) (Figure 1). In contrast, the concentration of FGF21 in plasma only tended (p = 0.073) to be increased after three days consumption of a hypercaloric unsaturated high-fat diet (78 %) (Figure 1). These data underscore that dietary carbohydrates potently induce FGF21 during conditions of non-restricted protein intake, highlighting a novel and important effect of dietary carbohydrates in the regulation of circulating FGF21 in humans. The fact that the FAT diet did not appreciably increase plasma FGF21 concentration indicates that caloric excess per se does not induce FGF21 secretion. In mice, FGF21 mRNA is reported to be predominantly expressed in pancreas and liver [30], with markedly lower FGF21 mRNA levels present in human skeletal muscle [31,32] and subcutaneous and visceral adipose tissue [6,33]. Elevated circulating levels of FGF21 in mice and humans have mainly been linked to increased FGF21 synthesis in the liver [34,35]. Thus, the observed increase in plasma FGF21 concentration in the present human study was likely related to induction of FGF21 in the liver.

3.2. Lipid and glucose homeostasis in the context of excess carbohydrate intake and FGF21 induction
Next we aimed to investigate the metabolic effects of the CHO diet and whether the dietary upregulation of circulating FGF21 was associated with changes in glucose and lipid homeostasis. After CHO, the plasma TG concentration was increased by 101% compared with CON (p < 0.001) (Figure 2A) at the same time as the plasma FA concentration was decreased by 68% (Figure 2B). Hence, the increase in plasma TG concentration does not seem to be ascribed to increased hepatic FA supply from plasma. It has been shown in another study in healthy men that 4 days hypercaloric provision of carbohydrates (enteraly) increases VLDL-TG secretion rate markedly, in part due to increased secretion of de novo synthesized FA in the liver [36]. Extending these observations to the present study, the obtained increase in plasma TG concentration likely reflected increased VLDL-TG secretion after the several days of high carbohydrate intake. Along this line, we observed a 146% increase in plasma C16:1 FA concentration after CHO (Figure 2C), a marker of de novo lipogenesis [37], indicating an increased hepatic de novo lipogenesis. Interestingly, positive correlations were observed between the increase in plasma FGF21 concentration after
CHO and the increase in C16:1 n-7 (r = 0.79, p < 0.05) and the increase in plasma TG (r = 0.70, p < 0.05). Together these findings suggest that the carbohydrate load from the CHO diet caused substantial de novo lipogenesis in the liver. This may contribute to the observed increase in FGF21 secretion, as cross-sectional studies have demonstrated that plasma FGF21 concentration is positively associated with intrahepatic lipid content [24,38,39], which is further associated with hepatic lipid synthesis and FGF21 secretion. Notably, we observed that total cholesterol (p < 0.01) and LDL-cholesterol (p < 0.01) concentrations in plasma were reduced (Table 1), indicating an improved lipoprotein profile even in the face of the increase in plasma TG concentrations approaching mild hypertriglyceridemia. This is in line with the observation of reduced plasma LDL-cholesterol concentrations in obese humans after FGF21 administration [41].

After CHO, hepatic glucose production was increased by 17% compared with CON (p < 0.01) (Figure 2D), despite slightly increased plasma insulin levels (p < 0.05) (Table 1). The increase in plasma FGF21 after CHO was positively correlated to the increase in hepatic glucose production (r = 0.71, p < 0.05), suggesting a link between FGF21 and hepatic glucose regulation. Our data are supported by findings in mice, in which 8 days administration of FGF21 increased basal hepatic glucose production [42].

Notably, after CHO the increase in circulating FGF21 was accompanied by a 62% increase in leg glucose uptake compared to CON (p < 0.05) (Figure 2E). This increase in leg glucose uptake was strongly correlated to the increase in plasma FGF21 concentration (r = 0.88, p < 0.01) (Figure 2F). This may imply that FGF21 has direct effects on muscle glucose uptake, in agreement with FGF21 treatment increasing basal glucose uptake in human myotubes [43]. Concomitantly, a small increase in plasma insulin concentration after CHO compared to CON

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### Table 1 – Arterial plasma parameters

Data are means ± SEM and obtained in the late post-absorptive state 6 h after a small 1.6 MJ breakfast. *p < 0.05, **p < 0.01 compared to CON. n = 9 in CON and n = 8 in CHO. Paired t-tests were applied to test for effect of the CHO diet.

<table>
<thead>
<tr>
<th>Arterial plasma parameters</th>
<th>CON</th>
<th>CHO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mmol l⁻¹</td>
<td>5.7 ± 0.1</td>
<td>5.6 ± 0.0</td>
</tr>
<tr>
<td>Insulin, µU ml⁻¹</td>
<td>5.0 ± 0.9</td>
<td>7.9 ± 1.4*</td>
</tr>
<tr>
<td>Epinephrine, nmol l⁻¹</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Norepinephrine, nmol l⁻¹</td>
<td>1.3 ± 0.5</td>
<td>1.6 ± 0.6</td>
</tr>
<tr>
<td>Adiponectin, µg ml⁻¹</td>
<td>7.1 ± 1.5</td>
<td>7.4 ± 1.1</td>
</tr>
<tr>
<td>Total cholesterol, mmol l⁻¹</td>
<td>4.2 ± 0.3</td>
<td>3.6 ± 0.2**</td>
</tr>
<tr>
<td>HDL-cholesterol, mmol l⁻¹</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.1*</td>
</tr>
<tr>
<td>LDL-cholesterol, mmol l⁻¹</td>
<td>2.4 ± 0.3</td>
<td>1.8 ± 0.2**</td>
</tr>
</tbody>
</table>

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Figure 2: Systemic and peripheral lipid and glucose homeostasis after the control diet (CON) and the carbohydrate-rich diet (CHO). A. Arterial plasma triacylglycerol (TG) concentration. B. Arterial fatty acid (FA) concentration. C. Arterial palmitoleic acid concentration. D. Hepatic glucose rate of appearance (Ra). E. Leg glucose uptake expressed per kg leg mass (LM). F + G. Scatter plots illustrating the associations between the change in plasma FGF21 (F) or plasma insulin (G) after CHO and the change in leg glucose uptake. Data are means ± SEM. Paired t-tests were used in A–E. Pearsons correlation analysis was applied in F + G. *p < 0.05, **p < 0.01, ***p < 0.001 compared with CON. n = 9 in CON, one subject did not complete the CHO trial, hence n = 8 in CHO. Data were obtained in the post-absorptive state 6 h after a small 1.6 MJ breakfast (5 A.M).
was obtained (7.9 ± 1.4 versus 5.0 ± 0.9 μU ml⁻¹, Table 1), which was also correlated to the increase in leg glucose uptake (r = 0.82, p < 0.05) (Figure 2G). The plasma insulin concentration reported to half-maximally stimulate leg glucose uptake is ~ 50 μU ml⁻¹ [44,45]. Thus, it can be questioned to what extent the 2.9 μU ml⁻¹ increase in plasma insulin, with insulin levels on the lower flat part of the dose-response curve, was responsible for the significant increase in leg glucose uptake after the CHO diet. However, it is possible that the apparent effect of insulin on leg glucose uptake is due to potentiation of the insulin effect by FGF21, as FGF21 has been shown to potentiate the effect of insulin on glucose uptake in human myotubes and mouse muscle [43]. Notably, when the absolute plasma concentrations of FGF21 and insulin after the CHO diet were correlated with leg glucose uptake, in contrast to the delta-values when compared to CON in Figure 2F, G, the plasma FGF21 concentration correlated closely with leg glucose uptake (r = 0.9, p < 0.01), while plasma insulin did not (r = 0.45, p = 0.265) (Supplemental Figure S1). This may suggest that FGF21 rather than insulin is the major regulator of the increase in leg glucose uptake observed in the present study.

FGF21 has also been suggested to regulate basal glucose uptake in human adipocytes [1], 3T3-L1 adipocytes [46] and mouse adipose tissue [47] via an adiponectin-AMPK dependent pathway [48,49]. However, the present data provide no indications of an adiponectin-AMPK axis activated in human adipose tissue in response to increased circulating FGF21 (Table 1 and Figure 3E–F).

In the present study, the subjects consumed a small and identical breakfast meal after CON and CHO (Supplemental Table S5) 6 h before blood sampling to ensure a similar post-absorptive state. It has been shown in healthy humans that oral administration of monosaccharides in the form of 75 g fructose or glucose acutely increase plasma FGF21 [21]. 75 g fructose increased FGF21 levels by 3.4 fold at 2 h, whereas 75 g glucose induced a more modest 2-fold increase in plasma FGF21 after 3 h. However, in both situations plasma FGF21 levels were back to baseline after approximately 4 h [21]. Considering the transient character of the acute glucose- and fructose-induced increases in circulating FGF21 [21] and that the half-life of recombinant human FGF21 is 1–2 h [46,50], it is likely that the substantial 8-fold increase in plasma FGF21 concentration obtained in the post-absorptive state in

![Figure 3: Molecular metabolism in subcutaneous adipose tissue after the control (CON) and the carbohydrate-rich diet (CHO). A. Hormone-sensitive lipase (HSL) Ser660 phosphorylation. B. HSL protein content. C. Adipose triglyceride lipase (ATGL) protein content. D. Perilipin 1 protein content. E. AMP-activated protein kinase (AMPK) Thr172 phosphorylation. F. AMPKα1 protein content. G. Representative blots. Data are means ± SEM. Paired t-tests were used in A–E. **p < 0.01 compared with CON. n = 9 in CON, one subject did not complete the CHO trial, hence n = 8 in CHO. Data were obtained in the post absorptive state 6 h after a small 1.6 MJ breakfast (5 A.M).]
CHO originates from a more persistent secretory regulation. Thus, this study shows the impact of carbohydrate-rich food items, occurring in a normal habitual diet, on FGF21 regulation in humans. Interestingly, genome-wide association studies in human cohorts have shown an association between SNPs related to the FGF21 locus and carbohydrate intake [51,52]. A link between FGF21 and dietary carbohydrate consumption is further supported by studies in mice and monkeys, which show that FGF21 knockout increases preference for mono- and disaccharide intake [19,53]. Whether the observed higher plasma FGF21 levels after the CHO diet in the present study affected food preference is a possibility, but was not directly evaluated.

3.3. Inhibition of adipose tissue lipolysis
Three days of FGF21 treatment has been reported to inhibit lipolysis in human adipocytes [54]. Supporting these findings, the increased plasma FGF21 concentration was observed at the same time as plasma FA concentration was markedly decreased in the present study after CHO. We observed in subcutaneous adipose tissue that phosphorylation of the activating Ser660 site on hormone sensitive lipase (HSL) was decreased by 47% (p < 0.01) (Figure 3A) and that the protein content of perilipin 1 was decreased by 29% after CHO (p < 0.01) (Figure 3D). Perilipin 1 is a key regulator of both basal and protein kinase A (PKA)-stimulated lipolysis in adipose tissue, and since HSL translocation to the lipid droplets requires a functional perilipin 1 [55] and perilipin 1 activation is required to stimulate HSL-mediated lipolysis [56], these molecular data collectively support that adipose tissue lipolysis was lowered after CHO. Consistent with our observations, Arner et al. found that the FGF21-induced inhibition of lipolysis in the human primary adipocytes was accompanied by a reduced perilipin 1 protein content [54].

In summary, in humans we here employed a dietary strategy by which the effect of short term overfeeding of carbohydrates revealed a paramount and remarkable stimulating effect on FGF21 secretion, without reduction in the dietary protein content. In contrast, energy excess per se, induced by a hypercaloric fat-rich diet, did not induce a detectable increase in circulating FGF21. Dietary carbohydrate excess led to differential responses in the liver and peripheral tissues. The increase in circulating FGF21 after high carbohydrate intake was associated with indices of increased hepatic lipogenesis and glucose production, and this was observed concomitantly with increased peripheral glucose disposal, with a close correlation between the increase in plasma FGF21 concentration and increased leg glucose uptake. This suggests that FGF21 secretion provided a mechanism for peripheral disposal of the carbohydrate load. Furthermore, the FGF21-mediated suppression of adipose tissue lipolysis could be a mechanism to suppress hepatic FA supply under conditions of increased de novo lipogenesis. Accordingly, we here demonstrate an important role of dietary carbohydrates in the regulation of human FGF21 secretion, and our data further point to effects of FGF21 in human glucose and lipid homeostasis.

AUTHOR CONTRIBUTIONS
BK, EAR, JFPW, AML, and KAS designed the study and carried out the experiments. AML, AMF, KAS, LSM, and LM contributed to the results. AML, AMF, and BK wrote the manuscript. All authors contributed to the manuscript and approved the final version of the manuscript.

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CONFLICTS OF INTERESTS
The authors have declared that no conflicts of interests exist.

APPENDIX A. SUPPLEMENTARY DATA
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2016.11.001.

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