Journal of the Endocrine Society, 2024, **8**, 1–11
https://doi.org/10.1210/jendso/bvae008
Advance access publication 24 January 2024
Clinical Research Article

Effect of a 6-Week Carbohydrate-Reduced High-Protein Diet on Levels of FGF21 and GDF15 in People With Type 2 Diabetes

Michael M. Richter,[1,2] Mads N. Thomsen,[3] Mads J. Skytte,[3,4] Sasha A. S. Kjeldsen,[1,2] Amirsalar Samkani,[3] Jan Frystyk,[5] Faidon Magkos,[6] Jens J. Holst,[7,8] Sten Madsbad,[9] Thure Krarup,[3,6] Steen B. Haugaard,[3,10] and Nicolai J. Wewer Albrechtsen[1,2]

[1]Department of Clinical Biochemistry, Copenhagen University Hospital—Bispebjerg and Frederiksberg, Copenhagen, 2400, Denmark
[2]Novo Nordisk Foundation Center for Protein Research, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, 2200, Denmark
[3]Department of Endocrinology, Copenhagen University Hospital—Bispebjerg and Frederiksberg, Copenhagen, 2400, Denmark
[4]Department of Forensic Medicine, University of Copenhagen, Copenhagen, 2100, Denmark
[5]Department of Endocrinology, Odense University Hospital, Odense, 5000, Denmark
[6]Department of Nutrition, Exercise and Sports, University of Copenhagen, Copenhagen, 2200, Denmark
[7]Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, 2200, Denmark
[8]Novo Nordisk Foundation Center for Basic Metabolic Research, University of Copenhagen, Copenhagen, 2200, Denmark
[9]Department of Endocrinology, Copenhagen University Hospital—Hvidovre, Hvidovre, 2650, Denmark
[10]Institute of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, 2200, Denmark

Correspondence: Michael M. Richter, MD, PhD, Department of Clinical Biochemistry, Copenhagen University Hospital—Bispebjerg and Frederiksberg, Nielsine Nielsens Vej 4B, Copenhagen, 2400, Denmark. E-mail: michael.martin.richter.02@regionh.dk; or Nicolai J. Wewer Albrechtsen, MD, PhD, Department of Clinical Biochemistry, Copenhagen University Hospital—Bispebjerg and Frederiksberg, Nielsine Nielsens Vej 4B, Copenhagen, 2400, Denmark. E-mail: nicolai.albrechtsen@regionh.dk.

Abstract

Context: Fibroblast growth factor 21 (FGF21) and growth differentiation factor 15 (GDF15) are increased in type 2 diabetes and are potential regulators of metabolism. The effect of changes in caloric intake and macronutrient composition on their circulating levels in patients with type 2 diabetes are unknown.

Objective: To explore the effects of a carbohydrate-reduced high-protein diet with and without a clinically significant weight loss on circulating levels of FGF21 and GDF15 in patients with type 2 diabetes.

Methods: We measured circulating FGF21 and GDF15 in patients with type 2 diabetes who completed 2 previously published diet interventions. Study 1 randomized 28 subjects to an isocaloric diet in a 6 + 6-week crossover trial consisting of, in random order, a carbohydrate-reduced high-protein (CRHP) or a conventional diabetes (CD) diet. Study 2 randomized 72 subjects to a 6-week hypocaloric diet aiming at a ∼6% weight loss induced by either a CRHP or a CD diet. Fasting plasma FGF21 and GDF15 were measured before and after the interventions in a subset of samples (n = 24 in study 1, n = 66 in study 2).

Results: Plasma levels of FGF21 were reduced by 54% in the isocaloric study ($P < .05$) and 18% in the hypocaloric study ($P < .05$) in CRHP-treated individuals only. Circulating GDF15 levels increased by 18% ($P < .05$) following weight loss in combination with a CRHP diet but only in those treated with metformin.

Conclusion: The CRHP diet significantly reduced FGF21 in people with type 2 diabetes independent of weight loss, supporting the role of FGF21 as a "nutrient sensor." Combining metformin treatment with carbohydrate restriction and weight loss may provide additional metabolic improvements due to the rise in circulating GDF15.

Key Words: hepatokines, weight loss, weight maintenance, metformin, nutrients

Abbreviations: BMI, body mass index; CD, conventional diabetes diet; CRHP, carbohydrate-reduced high-protein diet; E%, energy percent; FGF21, fibroblast growth factor 21; GDF15, growth and differentiation factor 15; GFRAL, glial cell line–derived neurotrophic factor family receptor alpha like; HbA1c, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment for insulin resistance.

Fibroblast growth factor 21 (FGF21) is a member of the fibroblast growth factor family and is expressed in multiple tissues including liver, adipose tissue, and pancreas [1]. It is secreted to the circulation primarily from the liver [2, 3] and acts on other tissues by binding to a cell surface receptor complex consisting of an FGF receptor and the co-receptor β-klotho [4, 5]. In rodents, FGF21 acts on the brain and shifts dietary preferences by suppressing sweet intake and stimulating protein

Received: 27 November 2023. **Editorial Decision:** 18 January 2024. **Corrected and Typeset:** 19 February 2024

intake [6-8]. Exogenous FGF21 administration to obese rodents decreases body weight, improves insulin sensitivity, and increases energy expenditure [9-11]. In humans, FGF21 analogues have shown promising results in the treatment of metabolic diseases by reducing body weight and hepatic fat fraction and improving lipoprotein profile in individuals with obesity and type 2 diabetes [12-14].

Growth differentiation factor 15 (GDF15) is a member of the TGF-β family expressed in several tissues including liver, kidney, and adipose tissue and its circulating levels increase in response to cellular stress [15]. In humans, circulating GDF15 is thought to originate from either the liver or the intestine [16-18]. GDF15 is a ligand for the glial cell line–derived neurotrophic factor family receptor alpha like (GFRAL), located in the hindbrain. Activation of the GFRAL receptor results in reduced appetite and weight loss in rodents [19-21], potentially via activation of the autonomic nervous system [22, 23]. In humans, bariatric surgery increases circulating GDF15 and may drive some of the metabolic benefits [24]. Metformin therapy increases circulating GDF15, and it has been proposed that weight loss in response to metformin therapy may depend on an increase in circulating GDF15 [16, 17]; however, this is still to be confirmed [25]. GDF15 analogues are currently undergoing examination in clinical trials for treatment of obesity [26].

Circulating FGF21 is highly stimulated by macronutrient consumption, especially carbohydrate intake [27-29], but not after ingestion of protein or fat [29]. Protein restriction is also a potent stimulator of circulating FGF21 [30-33]. In contrast, circulating GDF15 seems unaffected by dietary shifts or type of macronutrients composition [34-37]. While both FGF21 and GDF15 are increased in dysregulated metabolic conditions, including obesity [38, 39], type 2 diabetes [39-42], and liver disease [38, 43], it is unclear how a clinically significant weight loss affects their plasma concentrations, especially in combination with changes in macronutrient composition. To further improve our understanding of the regulation of plasma levels of FGF21 and GDF15, we explored the effects of a carbohydrate-reduced high-protein diet with and without a clinically significant weight loss in patients with type 2 diabetes. Outline of the interventions are illustrated in Fig. 1. This study is important as it enables the independent evaluation of the effects of macronutrient composition and weight loss on FGF21 and GDF15. This allows for a greater understanding of the primary factors driving the elevated levels of FGF21 and GDF15 in patients with type 2 diabetes. We hypothesized that circulating levels of FGF21 would decrease following carbohydrate restriction and weight loss, while GDF15 would decrease after weight loss independent of diet.

Research Design and Methods

We analyzed a subset of subjects who participated in 2 previously published randomized controlled trials [44-47]. The 2 studies were designed to investigate the effect of carbohydrate restriction on glucose and lipid metabolism in patients with type 2 diabetes with or without a clinically relevant weight loss. For both studies, inclusion criteria included men and women from 18 years of age diagnosed with type 2 diabetes with glycated hemoglobin A_{1c} (HbA1c) of 48 to 97 mmol/mol. Exclusion criteria included injectable diabetes medication and treatment with systemic corticosteroids.

Study 1 Design: An Isocaloric Study

Study 1 included 28 individuals with type 2 diabetes. The protocol was approved by the Health Ethics Committee of Copenhagen and the Danish Data Protection Agency. The study was registered at ClinicalTrials.gov (NCT02764021) and conducted in accordance with the Helsinki declaration. All subjects gave written informed consent before inclusion. Details of the study protocol have been described elsewhere [44, 46].

In short, 28 individuals participated in a 6 + 6-week open-label, randomized, crossover trial. They consumed, in random order, either an iso-energetic conventional diabetes diet (CD, 50 energy percent [E%] carbohydrate, 17 E% protein, and 33 E% fat) or an energy-matched carbohydrate-reduced high-protein diet (CRHP, 30 E% carbohydrate, 30 E% protein, and 40 E% fat) for 6 weeks followed by 6 weeks of the alternate diet. Fasting blood samples were obtained before (at baseline) and after each diet intervention.

For the current study, only samples collected before and after the first 6 weeks intervention were included, thereby excluding samples following the crossover. In total, 14 individuals consumed an iso-energetic CD diet, and 14 individuals consumed an iso-energetic CRHP diet.

Study 2 Design: A Hypocaloric Study

Study 2 included 72 individuals with type 2 diabetes. The protocol was approved by the Health Ethics Committee of Copenhagen and the Danish Data Protection Agency and was registered at ClinicalTrials.gov (NCT03814694) and conducted in accordance with the Helsinki declaration. All subjects gave written informed consent before inclusion. Details of the study protocol have been described elsewhere [45, 47].

In short, 72 individuals participated in an open-label, parallel, controlled trial; they were randomized in a 1:1 ratio to either a hypo-energetic CD diet or a hypo-energetic CRHP diet (with same macronutrient compositions as in study 1) for 6 weeks. The 2 diets were energy-matched, and both aimed at a 6% weight loss. Five participants did not complete the study and 1 participant had missing values for most parameters used in this study. Therefore, only 66 participants are included in the data analysis. In total, 32 individuals consumed a hypo-energetic CD diet, and 34 individuals consumed a hypo-energetic CRHP diet. Fasting blood samples were obtained before (at baseline) and after the diet intervention.

Diet Interventions and Weight Management

In both studies, all meals were prepared and distributed to the participants. Participants were instructed to only consume the provided meals and beverages. Adherence to the diet was evaluated twice weekly using food records, and adjustments were made on an individual basis if participants were unable to consume all provided food. Daily total energy expenditure for each participant were calculated as previously described [44, 45] to ensure weight stability in the isocaloric study and to achieve a ~6% weight loss in the hypocaloric study. Caloric intake was adjusted based on body weight monitored twice weekly. Participants were instructed to maintain their habitual physical activity level.

Plasma Analysis

In both studies, blood was collected following an overnight fast. Plasma levels of FGF21 were analyzed using a sandwich

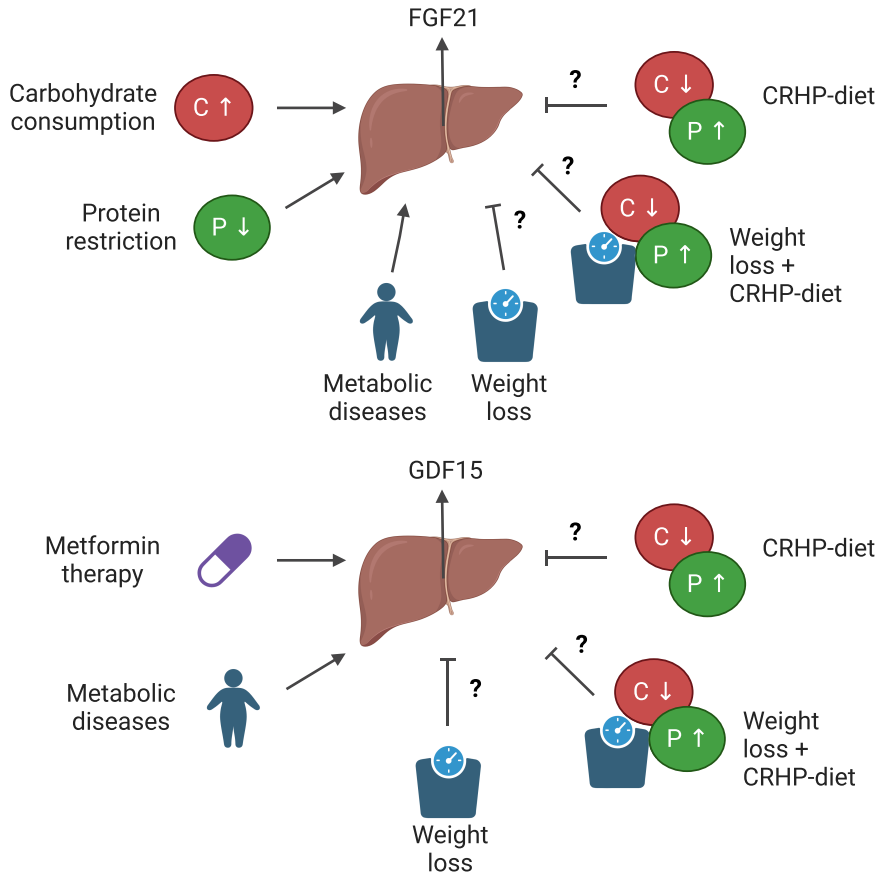

Figure 1. Outline of the interventions in the present study. Abbreviations: C, Carbohydrate; P, Protein; CRHP diet, carbohydrate-reduced high-protein diet. Created with BioRender.com.

ELISA kit (R&D Systems Inc., Minneapolis, Cat# DF2100, RRID:AB_2783729) and plasma levels of GDF15 were analyzed using a sandwich ELISA kit (R&D Systems Inc., Minneapolis, Cat# DGD150, RRID:AB_2877710) according to the manufacturer instructions.

Due to insufficient amount of plasma, samples from only 24 of the 28 subjects in study 1 (isocaloric intervention) were analyzed for FGF21 and GDF15, and only samples from 60 out of 66 subjects were analyzed for GDF15 in study 2 (hypocaloric intervention). The FGF21 level of 1 subject in the hypocaloric study was extremely nonphysiologically elevated compared with the other subjects and that FGF21 result was therefore excluded.

Measurements of plasma glucose, serum insulin, and HbA1c in the isocaloric and hypocaloric study were described previously [44-47]. The homeostatic model assessment for insulin resistance (HOMA-IR) was calculated as fasting glucose (mmol/L) × fasting insulin (μU/mL)/22.5.

Statistical Analysis

Data distribution and homoscedasticity were evaluated using histograms, residual plots, and Q-Q plots. For comparisons between studies (isocaloric vs hypocaloric) or diet interventions within studies (CD vs CRHP) over time (week 0 vs week 6), a mixed-effects analysis with repeated measurements was used, with diet and time as fixed effects and subjects as random effect. Post hoc testing (isocaloric vs hypocaloric at week 6 or CD vs CRHP at week 6) was applied in case of significant diet × time effects. Sidak's post hoc test was applied for multiple comparisons. Unpaired *t* tests were used to compare baseline characteristics and differences between absolute changes (baseline-subtracted) or relative changes between studies (isocaloric vs hypocaloric) or diets (CD vs CRHP). $P < .05$ was considered statistically significant. Data are presented as mean ± SD in tables and as mean ± SEM in figures. Statistical analyses and graphical presentation were made in GraphPad Prism 9.4.1 (https://www.graphpad.com/).

Results

Subject Characteristics

Baseline characteristics for the isocaloric study 1 [44, 46] and hypocaloric study 2 [45, 47] are presented in Table 1. All study participants were diagnosed with type 2 diabetes prior to inclusion in the present studies and had elevated HbA1c levels as expected. Fasting plasma levels of FGF21 and GDF15 did not differ between the 2 studies at baseline. Subjects in the 2 studies had similar characteristics, except that those in the hypocaloric study had higher body mass index (BMI) and HOMA-IR compared with subjects in the isocaloric study ($P < .05$). There were no baseline differences between the CD and CRHP diet groups within each study.

The Effect of Weight Loss on FGF21 and GDF15

First, we evaluated differences in plasma levels of FGF21 and GDF15 between the isocaloric and hypocaloric study

Table 1. Baseline characteristics

	Isocaloric study			Hypocaloric study		
	Total (n = 24)	CD diet (*n* = 12)	CRHP diet (*n* = 12)	Total (*n* = 66)	CD diet (*n* = 32)	CRHP diet (*n* = 34)
Age (years)	64 ± 8	62 ± 9	66 ± 7	67 ± 8.0	67 ± 9	66 ± 7
Sex (% male)	67	58	75	53	47	59
Body weight (kg)	88 ± 20*	86 ± 24	89 ± 17	98 ± 20*	98 ± 25	98 ± 14
BMI (kg/m^2)	29.7 ± 5.3**	29.5 ± 5.8	30.0 ± 4.9	33.4 ± 4.9**	33.2 ± 5.2	33.6 ± 4.6
Metformin (% prescribed)	92	92	92	68	63	74
DPP4 inhibitor (% prescribed)	0	0	0	21	9	32
Plasma glucose (mmol/L)	9.2 ± 1.4	9.5 ± 1.4	8.9 ± 1.4	8.9 ± 2.1	9.0 ± 2.1	8.9 ± 2.1
HbA1c (mmol/mol)	59.6 ± 8.9	60.6 ± 9.7	58.7 ± 8.4	57.4 ± 8.0	57.1 ± 7.6	57.6 ± 8.4
HOMA-IR	5.2 ± 4.4**	4.2 ± 2.5	6.3 ± 5.7	8.7 ± 4.1**	9.2 ± 4.6	8.2 + 3.5
FGF21 (ng/L)	352 ± 235	388 ± 288	316 ± 173	324 ± 203	343 ± 181	306 ± 222
GDF15 (ng/L)	1225 ± 665	1101 ± 458	1349 ± 826	1130 ± 583	1182 ± 628	1081 ± 544

Abbreviations: BMI, body mass index; CD, conventional diabetes diet; CRHP, carbohydrate-reduced high-protein diet; DPP4, dipeptidyl peptidase 4; FGF21, fibroblast growth factor 21; GDF15, growth and differentiation factor 15; HbA1c, glycated hemoglobin A1c; HOMA-IR, homeostatic model assessment for insulin resistance.

Table 2. Effect of a 6-week isocaloric or hypocaloric diet

	Isocaloric study		Hypocaloric study		Difference between effect of treatments
	Baseline	Effect of treatment	Baseline	Effect of treatment	*P* value
Body weight (kg)	87.6 ± 20.1	−1.7 ± 1.2***	98.2 ± 20.2	−5.8 ± 2.1***	<.001
Plasma glucose (mmol/L)	9.2 ± 1.4	−0.82 ± 1.1*	8.9 ± 2.1	−2.0 ± 1.7***	<.01
Serum insulin (pmol/L)	78.8 ± 61.0###	−2.5 ± 19.8	133.8 ± 61.4###	−30.1 ± 38.6***	<.001
HbA1c (mmol/mol)	59.6 ± 8.9	−0.67 ± 2.1***	57.4 ± 8.0	−3.3 ± 2.9***	.20
FGF21 (ng/L)	352 ± 235	−125 ± 137**	324 ± 203	−37 ± 190	<.05
GDF15 (ng/L)	1225 ± 665	41 ± 289	1130 ± 583	71 ± 310	.68

Abbreviations: FGF21, fibroblast growth factor 21; GDF15, growth and differentiation factor 15; HbA1c, glycated hemoglobin A1c.

independent of diet (CD and CRHP pooled together in each study). Data are shown in Table 2.

Both studies resulted in reduction of body weight, and as expected, the hypocaloric study resulted in a greater weight loss (−5.9%, $P < .001$) compared with the isocaloric study (−1.9%, $P < .001$).

In addition, both studies resulted in statistically significant improvements of several markers of metabolism, including plasma glucose and HbA1c, with a larger reduction in insulin and plasma glucose in the hypocaloric study compared with the isocaloric study ($P < .05$).

For FGF21, the isocaloric study resulted in a ∼30% decrease at week 6 ($P < .05$), whereas no significant change was observed in the hypocaloric study. No changes in GDF15 were observed following either study intervention.

The Effect of Macronutrient Composition on FGF21 and GDF15

Next, we evaluated the effect of macronutrient composition (CD vs CRHP) in each study (isocaloric and hypocaloric study).

A 6-week CD diet had no significant effect on FGF21 levels in either the isocaloric study (Fig. 2A) or the hypocaloric study (Fig. 2B). In both studies, the CRHP diet resulted in a decrease in FGF21 compared with baseline (54% decrease in the isocaloric study and 18% decrease in the hypocaloric study, $P < .05$). The CRHP diet also reduced or tended to reduce FGF21 levels when compared with the CD diet ($P = .07$ in the isocaloric study, $P < .05$ in the hypocaloric study). Both the absolute and relative changes of FGF21 were significantly greater following the CRHP diet as compared with the CD diet in both studies ($P < .05$).

Circulating levels of GDF15 were unaffected by diets in the isocaloric study (Fig. 3A). Unexpectedly, the GDF15 levels in the hypocaloric study (Fig. 3B) increased by 14% compared with baseline, but only after the CRHP diet ($P < .05$). The absolute and relative changes of GDF15 in the hypocaloric study were greater for the CRHP diet compared with the CD diet ($P < .05$).

Both the isocaloric and hypocaloric study improved several metabolic markers (Table 3). Independent of caloric intake, the CRHP diet further improved several markers of metabolism compared with the CD diet ($P < .05$).

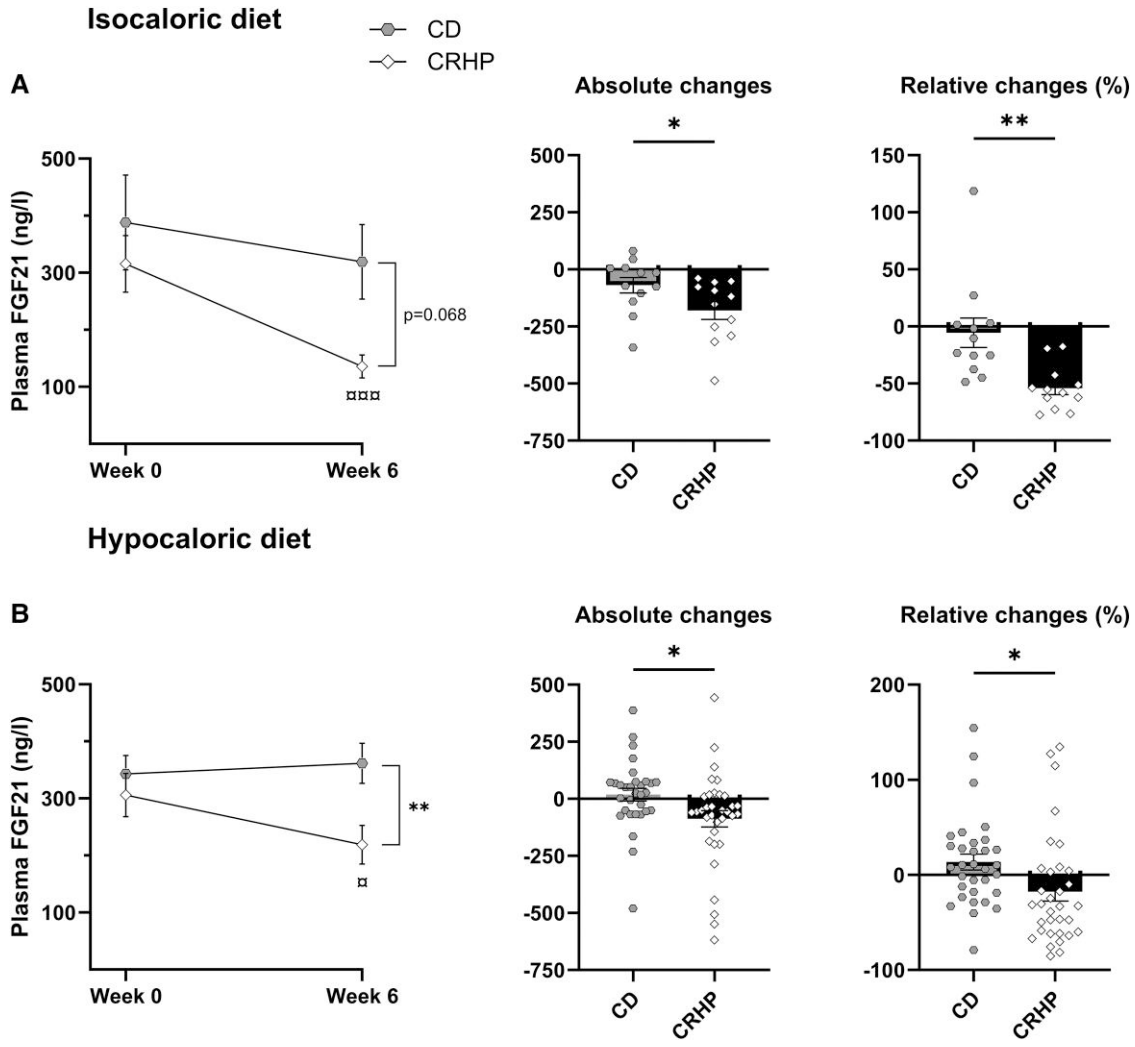

Figure 2. Effect of a 6-week conventional diabetes diet (CD, ⬡) or a carbohydrate-reduced high-protein diet (CRHP, ◊) on plasma levels of FGF21 following an isocaloric diet (a, n = 12 for CD and n = 12 for CRHP) or a hypocaloric diet (b, n = 31 for CD and n = 34 for CRHP) in patients with type 2 diabetes. Data are presented as means ± SEM or individually for each subject. Statistical significance is marked by: ¤; effect of time for the CRHP diet; *; for comparison between the CD and CRHP diet. One symbol (*/¤) indicates $P < .05$, 2 symbols (**/¤¤) indicates $P < .01$ and 3 symbols (***/¤¤¤) indicates $P < .001$.

The Effect of Metformin on GDF15

We conducted additional analyses to explore the unexpected increase in GDF15 following the CRHP diet in the hypocaloric study. Since metformin has been reported to increase circulating GDF15 [16, 17] and as 68% of subjects in the hypocaloric study were prescribed metformin, we stratified the hypocaloric groups according to metformin therapy. Only 16 out of the 61 (~26%) participants did not receive metformin (9 in CD and 7 in CRHP).

First, we looked at GDF15 levels in subjects with or without treatment with metformin independent of diet (CD and CRHP diet combined). Plasma levels of GDF15 (Fig. 4A) were significantly higher at baseline in the metformin group, but unaffected by 6 weeks of intervention in both the metformin group and the non-metformin group.

Next, we evaluated GDF15 levels in subjects without (Fig. 4B) and with metformin (Fig. 3C) in the hypocaloric study. Baseline levels were similar between diets but increased significantly (by ~18% compared with baseline) only in the CRHP group treated with metformin.

Discussion

The aim of the present study was to investigate the effects of carbohydrate restriction together with increased intake of protein and fat on circulating levels of FGF21 and GDF15 in the presence and absence of a clinically significant weight loss. Here, we show in people with type 2 diabetes that circulating levels of FGF21 were reduced after a 6-week CRHP diet independent of weight loss, providing supporting evidence that FGF21 is a hormonal sensor of the relative intake of carbohydrate and protein intake. Contradicting our hypothesis, plasma levels of GDF15 increased following a weight-reducing CRHP diet in people with type 2 diabetes who were prescribed metformin, suggesting that GDF15 may contribute to the metabolic benefits of weight loss induced by a CRHP diet.

FGF21 levels at fasting are increased in obesity [38] and type 2 diabetes [40]. When results from the groups were pooled, to investigate the effect of weight loss independent of diet, baseline levels of FGF21 were comparable between the isocaloric and hypocaloric study. Following the interventions,

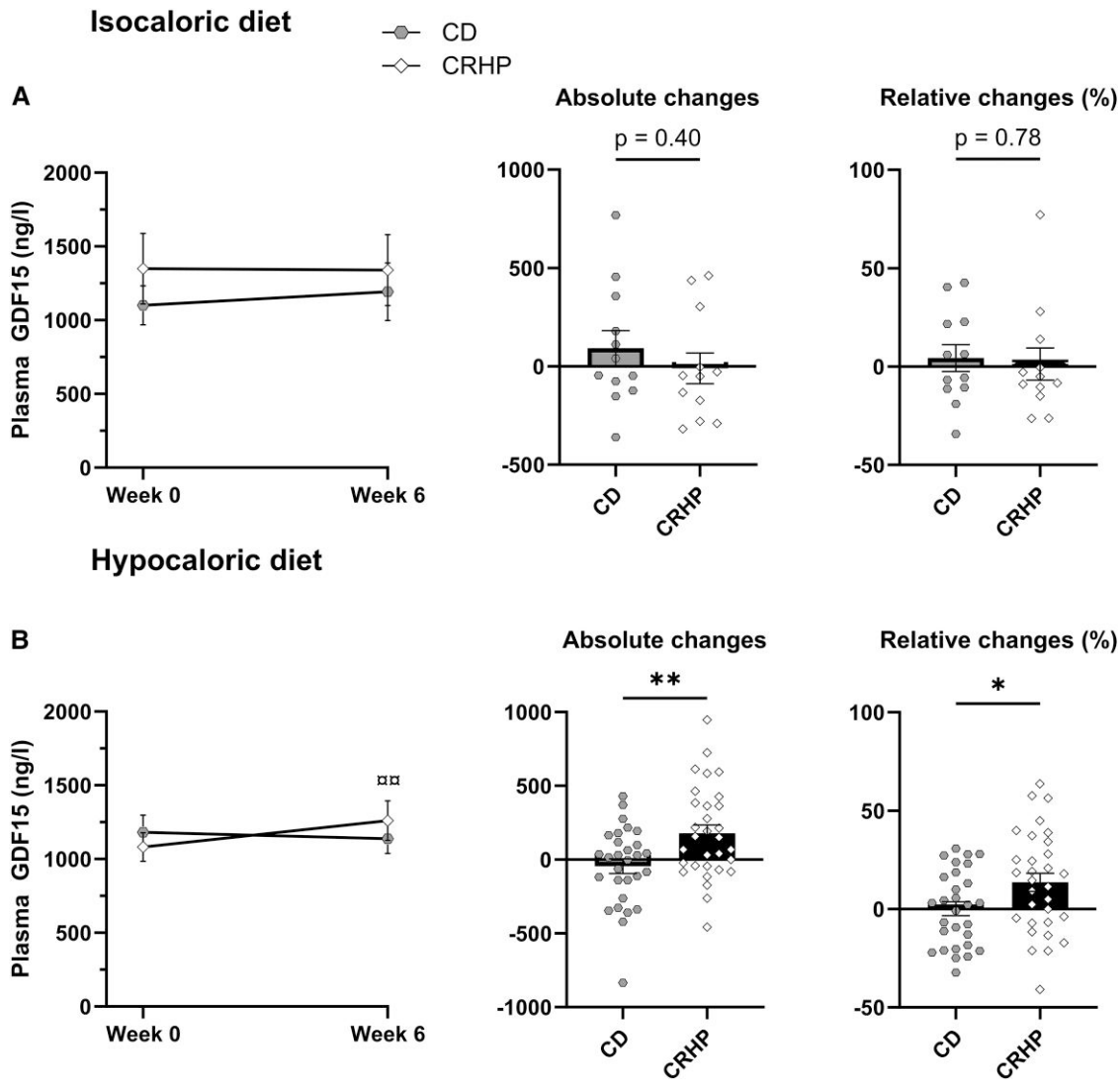

Figure 3. Effect of a 6-week conventional diabetes diet (CD, ⬡) or a carbohydrate-reduced high-protein diet (CRHP, ◊) on plasma levels of GDF15 following an isocaloric diet (a, n = 12 for CD and n = 12 for CRHP) or a hypocaloric diet (b, n = 29 for CD and n = 31 for CRHP) in patients with type 2 diabetes. Data are presented as means ± SEM or individually for each subject. Statistical significance is marked by: ¤; effect of time for the CRHP diet; *; for comparison between the CD and CRHP diet. One symbol (*/¤) indicates $P < .05$, 2 symbols (**/¤¤) indicates $P < .01$ and 3 symbols (***/¤¤¤) indicates $P < .001$.

the isocaloric study resulted in a decrease in FGF21 compared with the hypocaloric diet, suggesting that weight loss does not affect FGF21 levels. Previous reports have been contradictory, with studies reporting increased or decreased levels of FGF21 following weight loss [48-50]. However, some studies suggest that weight loss does not affect FGF21 levels in individuals with obesity [51, 52], which is in line with our findings. This is also in agreement with our finding that baseline FGF21 levels between the isocaloric and hypocaloric interventions were comparable, although subjects in the hypocaloric study had increased BMI compared to subjects in the isocaloric study.

We then looked at FGF21 levels in response to changes in macronutrient consumption and found that the CRHP diet decreased FGF21 levels in both the isocaloric and hypocaloric study, suggesting that the decrease in FGF21 is independent of total caloric intake and changes in body weight. Carbohydrates are known stimulators of FGF21 in humans and rodents. In humans, circulating FGF21 is increased after a single oral dose of carbohydrates [8, 27, 37] as well as after increased dietary carbohydrate consumption [28]. Several recent studies in rodents have shown that circulating levels and hepatic mRNA expression of FGF21 are increased following carbohydrate stimulation [6, 53, 54] and, conversely, FGF21 may suppress sweet intake via actions in the brain [6, 7], supporting the existence of a potential feedback mechanism for FGF21 serving to reduce carbohydrate intake [8].

Protein intake has also been described as a key regulator of FGF21 levels as it acutely decreases sucrose-induced FGF21 in humans [55]. Likewise, dietary protein restriction increases circulating levels of FGF21 in humans and rodents [30-33]. This has fueled the hypothesis that FGF21 signals to the brain to shift dietary preferences toward protein [56], which suggests another macronutrient feedback mechanism for FGF21. The combination of reduced dietary carbohydrates and increased dietary proteins in the CRHP diet may, therefore, both contribute to the decreased FGF21 levels observed in our study, consistent with a role for FGF21 as a regulator of macronutrient intake.

Table 3. Effect of a 6-week isocaloric or hypocaloric conventional diabetes or a carbohydrate-reduced high-protein diet

	Conventional diabetes (CD) diet		Carbohydrate-reduced high-protein (CRHP) diet		Difference between effect of treatments
	Baseline	Effect of treatment	Baseline	Effect of treatment	*P* value
Isocaloric study					
Body weight (kg)	86.2 ± 23.9	−1.5 ± 0.98***	89.1 ± 16.5	−1.9 ± 1.5***	.47
Plasma glucose (mmol/L)	9.5 ± 1.4	−0.38 ± 1.0	8.9 ± 1.4	−1.3 ± 1.1**	.05
Serum insulin (pmol/L)	63.2 ± 41.1	3.9 ± 15.9	94.5 ± 74.6	−9.0 ± 21.8	.11
HbA1c (mmol/mol)	60.6 ± 9.7	−4.8 ± 4.2**	58.7 ± 8.4	−8.8 ± 4.6***	.04
Hypocaloric study					
Body weight (kg)	98.4 ± 25.3	−5.8 ± 2.3***	98.0 ± 92.2	−5.8 ± 1.8***	.94
Plasma glucose (mmol/L)	9.0 ± 2.1	−2.0 ± 1.9***	8.9 ± 2.1	−2.0 ± 1.4***	.95
Serum insulin (pmol/L)	140.5 ± 67.2	−32.1 ± 42.9***	127.5 ± 55.7	−28.1 ± 34.5***	.68
HbA1c (mmol/mol)	57.1 ± 7.6	−7.0 ± 4.0***	57.6 ± 8.4	−9.1 ± 4.2***	.04

Abbreviation: HbA1c, glycated hemoglobin A1c.

While both interventions resulted in beneficial changes in several metabolic parameters, the CRHP diet resulted in greater metabolic improvements compared with the CD diet independently of caloric intake. Therefore, the reduction in FGF21 after the CRHP diet could, at least in part, be attributed to the greater metabolic improvements, as several studies have described a tight link between increased FGF21, obesity, and metabolic disease. However, in both studies, the CD diet caused beneficial effects on several markers of metabolism but did not affect FGF21 levels. This suggests that the metabolic improvements following the CD diet, in the studies investigated here, were unable to decrease FGF21 levels. It is of interest that levels of FGF21, with its potential beneficial effects on metabolism, are reduced by the CRHP diet, especially since there are several studies describing positive effects of high protein intake on metabolism, including maintenance of weight loss [57, 58]. However, other studies have shown that low protein diets improve insulin sensitivity and extend lifespan in mice [59] and improve overall mortality in middle-aged adults [60], potentially by improving energy expenditure via an increase in FGF21 [31]. Nevertheless, our study found decreased levels of FGF21 following the CRHP diet, suggesting that the greater metabolic improvements of this diet appear to be independent of FGF21.

Together, our studies suggest that fasting FGF21 levels in people with type 2 diabetes are in large part driven by macronutrient composition. In contrast to our hypothesis, a clinically significant weight loss of 6% had no effect on FGF21 levels. Elevated fasting levels of FGF21 observed in patients with metabolic disease may therefore be due to an enhanced response to chronically elevated carbohydrate intake [27] rather than metabolic disease *per se*.

Circulating levels of GDF15 are increased in conditions of metabolic disease [39, 41, 42]. However, we observed no changes in GDF15 during either of the isocaloric interventions, or following weight loss induced by the CD diet, despite significant metabolic improvements. This is in line with a previous study, where a 2-week very low-calorie diet, which improved metabolism and reduced body weight, did not increase circulating GDF15 in subjects with diabetes [61]. The effect of BMI on fasting GDF15 levels is unclear, with some studies reporting strong positive correlations [39] between increased BMI and increased fasting GDF15, and other studies reporting low correlations [34, 62]. Here, we found that a clinically significant weight loss of 6% had no effect on GDF15 levels.

The influence on circulating GDF15 by a shift in dietary macronutrient consumption appears limited. Several studies have shown that carbohydrate, protein, or fat intakes do not affect GDF15 levels in humans [34-37], although a small increase in GDF15 was observed several hours after pure glucose administration. We observed no differences in GDF15 levels between the CD and CRHP diets in the isocaloric study, supporting that macronutrient composition alone does not affect GDF15 levels. We found that a clinically significant weight loss induced by a CRHP diet increased circulating GDF15 levels; a similar increase was not seen after the same weight loss during the CD diet. To further explore this difference, we compared baseline levels of GDF15 between subjects treated with or without metformin and found GDF15 to only increase following the CRHP diet in combination with metformin therapy. It is unclear why macronutrient composition in combination with weight loss affects GDF15 in metformin-treated individuals only. Likewise, it is unclear which macronutrient may drive the increase in GDF15; this will require further investigations. A study comparing the effects on GDF15 levels of a carbohydrate-reduced high-protein diet to a high-carbohydrate protein-reduced diet in metformin-treated individuals could provide valuable insights into the optimal dietary approach.

In mice, GDF15 administration increases glucose tolerance and improves insulin sensitivity in liver and adipose tissue [23, 63], and in humans, increased levels of GDF15 have been associated with metabolic improvements [64]. It may be speculated that the increase in GDF15 could mediate some of the metabolic improvements observed in the hypocaloric CRHP diet. However, as previously discussed [45], adherence to a carbohydrate-reduced high-protein diet in the real world may pose challenges.

The present study has limitations. The 2 original trials were not designed to evaluate the effects of shifting macronutrient consumption with and without weight loss on FGF21 and GDF15 levels. Consequently, subjects in the 2 studies were

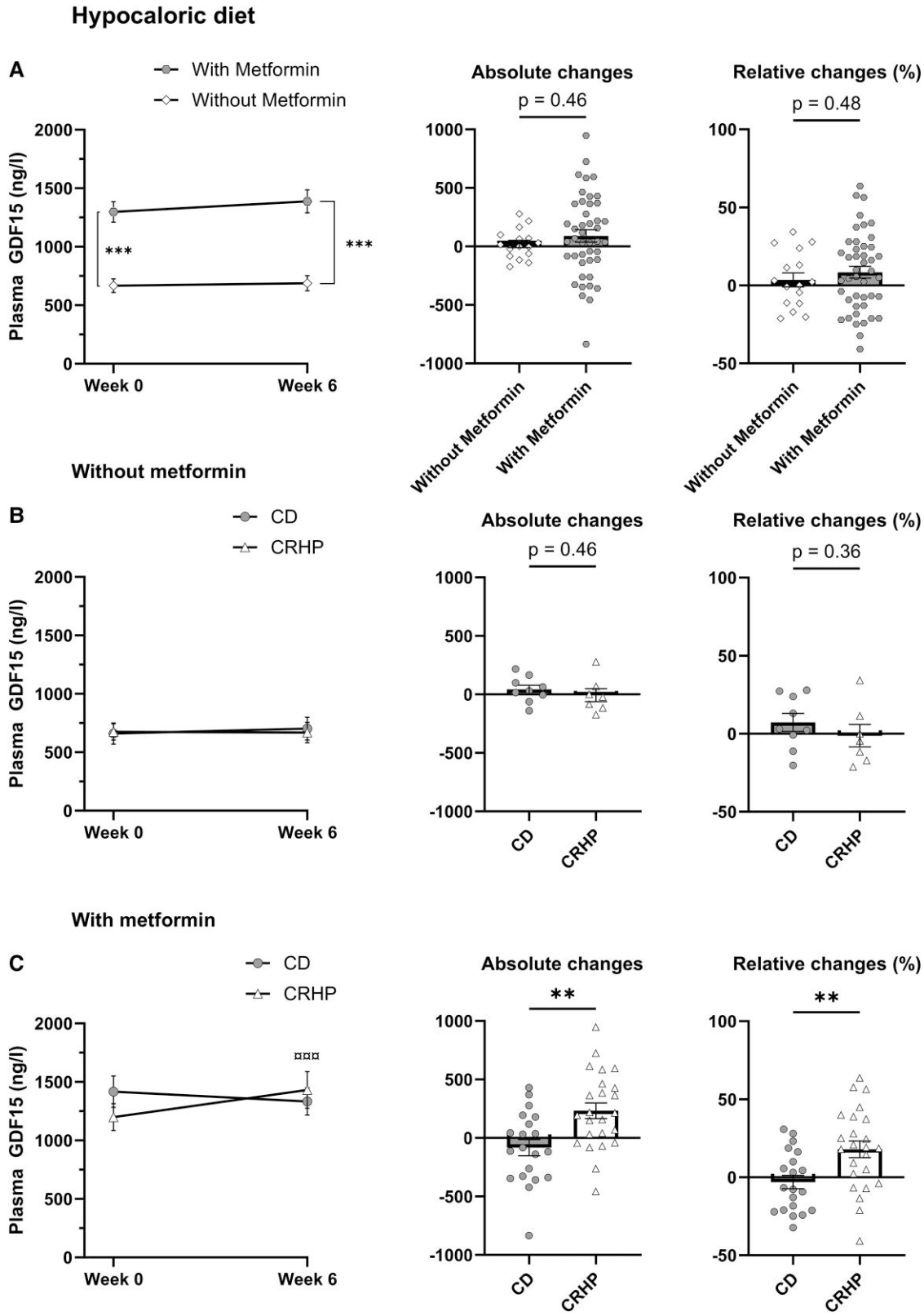

Figure 4. A, Effect of a 6-week hypocaloric diet with metformin (⬡, n = 44) and without metformin (◊, n = 16) on plasma levels of GDF15. B, Effect of a 6-week hypocaloric conventional diabetes diet (CD, ○, n = 9) or a carbohydrate-reduced high-protein diet (CRHP, Δ, n = 6) without metformin on plasma levels of GDF15. C, Effect of a 6-week hypocaloric conventional diabetes diet (CD, ○, n = 20) or a carbohydrate-reduced high-protein diet (CRHP, Δ, n = 24) with metformin on plasma levels of GDF15. Data are presented as means ± SEM or individually for each subject. Statistical significance is marked by: *; for comparison between the group with and without metformin. ¤; effect of time for the CRHP diet. One symbol indicates $P < .05$ (*/¤), 2 symbols (**/¤¤) indicates $P < .01$ and 3 symbols (***/¤¤¤) indicates $P < .001$.

not matched and baseline levels of BMI and HOMA-IR differed between the 2 studies, which may have affected our results. The effects of weight loss and changes in dietary composition on FGF21 and GDF15 levels in the hypocaloric study may therefore lead to an overestimation of their impact, given that the subjects in the hypocaloric study had greater metabolic impairment compared with the subjects in the isocaloric study.

In conclusion, we demonstrate that a CRHP diet significantly reduced FGF21 in patients with type 2 diabetes independent of weight loss, supporting that FGF21 may act as the liver's hormonal "nutrient sensor." Macronutrient composition, and not weight loss may thus be a main driver for changes in FGF21 levels in patients with type 2 diabetes. We also demonstrate that a CRHP diet combined with a clinically relevant weight loss increases GDF15 but only in metformin prescribed individuals. As the therapeutic benefits of metformin may depend on circulating GDF15, it is possible that the therapeutic effects of metformin may be enhanced by changing to a hypocaloric CRHP diet.

Acknowledgments

We thank Nicole J. Jensen for suggesting the collaboration between research groups.

Funding

Associate Prof. Nicolai J. Wewer Albrechtsen is supported by Novo Nordisk Foundation Excellence Emerging Investigator Grant—Endocrinology and Metabolism (Application No. NNF19OC0055001), EFSD Future Leader Award (NNF21SA0072746) and DFF Sapere Aude (1052-00003B). Novo Nordisk Foundation Center for Protein Research is supported financially by the Novo Nordisk Foundation (grant agreement NNF14CC0001). The isocaloric study (NCT02764021) was funded by grants from Arla Food for Health; the Novo Nordisk Foundation Center for Basic Metabolic Research, University of Copenhagen; the Department of Clinical Medicine, Aarhus University; the Department of Nutrition, Exercise and Sports, University of Copenhagen; and Copenhagen University Hospital—Bispebjerg and Frederiksberg. The hypocaloric study (NCT03814694) was funded by Arla Foods amba, The Danish Dairy Research Foundation, and Copenhagen University Hospital—Bispebjerg and Frederiksberg.

Author Contributions

N.J.W.A. conceived the idea of exploring the effects of diet and weight loss on FGF21 and GDF15 in patients with type 2 diabetes. M.N.T., M.J.S., A.S., S.B.H., and T.K. contributed with samples. M.M.R. measured FGF21 and GDF15 and wrote the first draft of the manuscript. All authors revised the manuscript and approved the final version for publication.

Disclosures

None of the authors have conflicts of interest to declare.

Data Availability

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Clinical Trial Information

ClinicalTrials.gov registration numbers NCT03814694 & NCT02764021.

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