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RESEARCH ARTICLE

# Protein-carbohydrate interaction effects on energy balance, FGF21, IGF-1, and hypothalamic gene expression in rats

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## Abstract

Amino acids are involved in energy homeostasis, just as are carbohydrates and lipids. Therefore, mechanisms controlling protein intake should operate independently and in combination with systems controlling overall energy intake to coordinate appropriate metabolic and behavioral responses. The objective of this study was to quantify the respective roles of dietary protein and carbohydrate levels on energy balance, plasma fibroblast growth factor 21 (FGF21) and insulin growth factor 1 (IGF-1) concentrations, and hypothalamic neurotransmitters (POMC, NPY, AgRP, and CART). In a simplified geometric framework, 7-wk-old male Wistar rats were fed 12 diets containing 3%–30% protein for 3 wk, in which carbohydrates accounted for 30%–75% of the carbohydrate and fat part of the diet. As a result of this study, most of the studied parameters (body composition, energy expenditure, plasma FGF21 and IGF-1 concentrations, and *Pomc/AgRP* ratio) responded mainly to the protein content and to a lesser extent to the carbohydrate content in the diet.

**NEW & NOTEWORTHY** As mechanisms controlling protein intake can operate independently and in combination with those controlling energy intakes, we investigated the metabolic and behavioral effects of the protein-carbohydrate interaction. With a simplified geometric framework, we showed that body composition, energy balance, plasma FGF21 and IGF-1 concentrations, and hypothalamic *Pomc/AgRP* ratio were primarily responsive to protein content and, to a lesser extent, to carbohydrate content of the diet.

*energy balance; FGF21; IGF-1; low-protein diet; protein-carbohydrate interaction*

## INTRODUCTION

An adequate qualitative and quantitative supply of amino acids is essential for growth and health. Regulatory systems dedicated specifically to the detection of insufficient protein intake operate both independently and in combination with the systems controlling energy intake to coordinate appropriate metabolic and behavioral responses (1). Thus, these systems are able to sense a defect in protein intake and to respond by triggering changes in appetite for protein (2, 3), in energy expenditure, in substrate metabolism, and in regulation of various metabolic processes as well as in simultaneously reducing growth to reduce protein requirements.

It appeared recently that fibroblast growth factor 21 (FGF21) is an essential mediator of these responses (1, 3–8). FGF21 is expressed in multiple tissues (3, 9), but its circulating concentrations have been primarily linked to FGF21 synthesis in the liver (5, 10, 11) that is well positioned to sense alterations in amino acid intake due to its direct sensing of absorbed amino acids via the portal circulation (7). FGF21 also mediates brain-specific responses to fasting (12) and protein deprivation (3) and therefore coordinates the hepatic response through a liver-brain signaling axis. It is now clear that the chronic

metabolic effects of FGF21 are mediated through actions on the central nervous system (CNS), as deletion of the  $\beta$ -klotho receptors in the CNS, but not in hepatocytes or adipocytes, impairs FGF21-mediated effects on energy homeostasis (13–17). Other parameters than low-protein diets, such as fasting, ketogenic diets, cold (18), high blood glucose or insulin concentrations, and carbohydrate intake (13, 19, 20) have been shown to increase plasma FGF21 concentrations. Several studies reported that ingestion of carbohydrate stimulated the release of FGF21, at least in the short term. In mice, it has been shown that ingestion of a high-carbohydrate low-protein diet stimulates FGF21 synthesis and secretion through the high blood glucose delivery but also the low amino acid concentration (21). The same study demonstrated that FGF21 synthesis and secretion in the liver are controlled by hepatic glucose levels by ex vivo analysis of FGF21 mRNA expression in hepatocytes. In agreement with these results, other studies have shown that this carbohydrate-induced increase in FGF21 gene expression in mouse hepatocytes and liver is mediated by the transcription factor ChREBP (22, 23). In humans, it has also been shown that a single monosaccharide (fructose) intake is a powerful but brief activator of FGF21 secretion (24), as is an excess of dietary carbohydrates (25).

As in most studies, low-protein diets were formulated by an exchange between protein and carbohydrate or inversely to maintain a fixed fat content, all these results do not lead to a clear conclusion regarding the possible interaction between protein and carbohydrate to control the FGF21 response. To our knowledge, there is only one study in which variations in dietary protein and carbohydrate did not systematically evolve in opposite directions (26). This study involved, in a “nutritional framework,” more than 800 mice consuming a wide range of diets and is thus difficult to replicate using a rat model which we consider a better model than the mouse to study human nutritional requirements (27, 28). The authors concluded that the dietary protein level was by far the main parameter affecting plasma FGF21 concentrations, but noticed also that the largest FGF21 responses occurred in the context of a low-protein high-carbohydrate diet and therefore that carbohydrate also affected FGF21 to some extent.

Furthermore, the interest in the role of FGF21 in protein and energy homeostasis has led to neglecting somewhat the important role played by insulin-like growth factor-1 (IGF-1). IGF-1 is very much implicated in the regulation of body composition and therefore protein synthesis. As for FGF21, nutritional status and protein intake are critical regulators of IGF-1 production (29, 30). IGF-1 is a low-molecular weight peptide that mediates the growth-promoting effects of growth hormone (GH). It circulates bound to specific carrier proteins and possess, to a different extent, sulfation, mitogenic, and insulin-like activity (31–33). The principal source of circulating IGF-1 is the liver, and it is secreted as it is produced. Plasma IGF-1 concentrations are decreased in patients with poor nutritional conditions (31) and rise significantly when the nutritional status is improved and is considered as a reliable nutritional parameter (32, 34). The decrease in plasma IGF-1 also appears to be involved in the metabolic adaptation to starvation, as treatment with IGF-1 to prevent the starvation-induced decrease in IGF-1 reduces weight loss (35). Studies in laboratory animals and volunteer human subjects revealed a strong relationship between caloric intake, dietary protein, and circulating IGF-1 (36–38). However, that protein rather than energy balance may be particularly involved in the regulation of IGF-1 can be suspected from the observation that patients with protein or protein-calorie malnutrition have lower IGF-1 than have patients with calorie-only malnutrition and that changes in IGF-1 are correlated with nitrogen balance (39). It appears that IGF-1 responses to changes in nutrition status is much quicker than that of other protein like albumin or transferrin but require several hours (29, 39), whereas FGF21 responds on a meal to meal basis (24). In a previous study conducted in mice, we observed also that the increase in FGF21 and the decrease in IGF-1 that occurred in response to a decrease in dietary protein content were closely correlated (11).

There is still debate as to whether FGF21 is regulated solely by the protein content of the diet or whether the carbohydrates content of the diet also plays a significant role, due to the reported effects of carbohydrates on the plasma and liver FGF21 levels at least in the short term. The aim of this paper was to quantify the respective roles of carbohydrates and protein in the long-term regulation of FGF21 and IGF-1 using various diets in which protein and carbohydrates did not systematically evolve in opposite directions.

## MATERIALS AND METHODS

### Animals and Housing

Male rats (7-wk-old; 215–240 g) of the Wistar RccHan strain were used (ENVIGO, Gannat, France). The rats were delivered weekly by groups of 10 and assigned randomly to one of the experimental diets, for a total of 70 rats. After 1 wk of adaptation to the laboratory conditions (temperature 22°C ± 1°C, humidity 60%, 12/12 L/D cycle lights on at 08:00), the rats were singly housed for 3 wk in Plexiglas cages bedded with wood shavings. The experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609 EEC), regarding the care and use of laboratory animals and were approved by the area Ethics Committee (APAFIS#16996).

### Composition of Diets and Pellet Preparation

Twelve diets in which the protein-to-energy ratios (PE) varied from 3% to 30% and the carbohydrate-to-carbohydrate + fat ratio, subsequently named the carbohydrate-to-nonprotein-energy ratios (CnpE) varied from 30% to 75% were used (Table 1). We established a fixed value of CnpE ratios rather than a fixed value of carbohydrates-to-energy ratios in the diet, to better control the effects of the fat/carbohydrates content of the diet that is well known to affect energy intake. The diets were named according to their PE and CnpE ratios in the form “xxPxxCnp.” The food pellets were prepared twice per week from dry powders manufactured by the research *Sciences de l'Animal et de l'Aliment de Jouy-en-Josas - Equipe Régimes à Façon* (Jouy-en-Josas, France). Proteins were whole milk proteins LR 85 F (Armor Protein, Nantes, France), carbohydrates were corn starch (Roquette, Lestrem, France) and sucrose (Eurosucré, Paris, France), and fat was soy oil (Bailly SA, Aulnay-sous-bois, France). The LR 85 F milk protein is produced by ultrafiltration, and was initially designed for infant nutrition, sports nutrition, slimming diets, and hospital nutrition. We did not directly measure the nitrogen content of the batch used during this study, but according to the manufacturer analyses and quality control periodically performed in the laboratory, protein content amounts 77% of powder weight assuming 6.25 g protein per gram of nitrogen. Its digestibility after accounting for nitrogen losses in urine and feces was previously measured in rats of the same strain and age, as those used in this experiment and amounts 85%. Therefore, digestible protein intake was computed as milk powder intake × 77% × 85% (Table 1). All the diets were complemented with minerals and vitamins, according to the AIN-93 requirements (40). The powders were mixed with the necessary amount of water to make a consistent dough and then formed into pellets sufficient for 3 days of feeding. The pellets were left to dry at room temperature for 3 days before being given to the rats. The diets and water were provided ad libitum.

### Body Weight, Body Composition, and Tissues Sampling

Body weight (g) was measured twice per week throughout the experiments. At the end of the studies, the rats were anesthetized with isoflurane, blood was collected from the vena cava until cardiac arrest, and the rats were immediately

**Table 1.** Composition and energy content of tested diets

	3P45Cnp	3P75Cnp	5P30Cnp	5P60Cnp	8P45Cnp	8P75Cnp	15P30Cnp	15P60Cnp	15P75Cnp	30P30Cnp	30P45Cnp	30P60Cnp
Whole milk protein, g/kg	38.8	30.5	73.8	56.7	101	80.9	208	166	151	380	346	319
Corn starch, g/kg	509	667	379	582	472	629	318	507	575	239	328	402
Sucrose, g/kg	81.9	108	61.0	93.7	76.0	101	51.9	81.7	92.7	38.5	52.8	64.7
Soy oil, g/kg	274	98.0	389	171	254	92.3	327	149	84.6	246	176	117
Mineral compound												
AIN-93M, g/kg	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0
Vitamin compound												
AIN-93M, g/kg	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Cellulose, g/kg	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Choline chloride, g/kg	2.30	2.30	2.30	2.30	2.30	2.30	2.30	2.30	2.30	2.30	2.30	2.30
Energy, kJ/g	20.3	16.3	22.9	17.9	19.8	16.1	21.5	17.4	16.0	19.7	18.1	16.7
PE ratio, %	3.00	3.00	5.00	4.90	8.00	7.90	15.2	15.0	14.8	30.3	30.1	30.0
CnpE ratio, %	46.7	76.2	46.7	76.2	46.7	76.2	31.5	61.6	76.2	31.4	46.7	61.7
Carbohydrate-to-energy ratio, %	45.3	74.0	29.8	58.5	42.9	70.2	26.7	52.4	64.9	21.9	32.6	43.2
Fat-to-energy ratio, %	51.7	23.1	65.1	36.5	49.0	21.9	58.1	32.7	20.3	47.8	37.3	26.8

Whole milk protein was provided by Armor protein. According to the manufacturer, control quality measurements regularly performed by mass spectrometry in our laboratory, protein amounted 77% of the wet weight of the powder. In addition, from studies performed in collaboration with Pr. Vermorel at unit of Human Nutrition at Clermont-Ferrand, we previously measured that digestibility after nitrogen losses in urine and feces was 85%. Accordingly, the metabolizable energy content of the milk powder was computed as  $5.725 \times 0.77 \times 0.85 = 3.75$  kcal/g (15.7 kJ/g). Corn starch was provided by Roquette, dry matter amounted 88%, digestibility 1, and metabolizable energy was thus  $4.18 \times 0.88 = 3.4$  kcal/g (15.7 kJ/g). Saccharose was provided by Eurosucre, dry matter amounted 99.5% digestibility was 1, and metabolizable energy was thus  $3.94 \times 0.995 = 3.92$  kcal/g (16.41 kJ/g). Soy oil was provided by Bailly SA, dry matter was 100% and digestibility 1, so that metabolizable energy was 9 kcal/g (37.7 kJ/g). The carbohydrate-to-nonprotein-energy (CnpE) ratio was computed as the carbohydrates-to-carbohydrates + lipid content of the diet. We controlled this parameter rather than the carbohydrates content only of the diet to prevent to numerous different values of the fat content of the diet, as this parameter is known to affect energy intake. Fixing a limited value of CnpE ratios allowed to vary the carbohydrates content of the diet and simultaneously to limit to two to three different levels the carbohydrates/fat content in the diet. CnpE, carbohydrate-to-nonprotein-energy; PE, protein-to-energy.

decapitated to remove the brain to collect the hypothalamus and the nucleus accumbens (NAcc). The liver and the interscapular brown adipose tissue (IBAT) were also collected. All samples were dissected quickly, deep frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for subsequent analysis. Body composition was measured by dissection and weighing of all the fat depots (visceral, subcutaneous, retroperitoneal, and epididymal), the main tissues and organs. Lean body mass (LBM) was computed as body weight minus fat mass and adiposity as fat mass divided by body weight. Active metabolic mass (AMM) (g) was computed as the weight of lean body mass plus 20% of the weight of fat mass (41, 42). AMM was used to normalize energy intake and expenditure. In previous studies, we have confirmed the validity of this adjustment mode for body composition (11, 42–45). Initial body composition of rats was estimated from a database in which body composition was measured by MRI at the “Small Animal Imaging Platform” of Paris-Descartes University (Paris, France), and in which we selected of a reference group of nine rats of the same strain delivered by the same provider (ENVIGO, Gannat, France). Mean body weight of this reference group was  $267.9 \pm 2.2$  g for  $269.4 \pm 1.4$  g in the rats of this study.

## Energy and Water Intakes

Food intake (g/day) was measured twice per week by weighing the pellets and was then converted to kJ/day according to the energy content of the diet and after correction for the pellet water content (Table 1). The water content at the time of weighing was calculated based on the water evaporation curve determined on pellets produced at the same time as those given to the rats. Water intake was also measured during the study and added to the amount of

water consumed in the pellets to estimate the total amount of water consumed (mL/day).

## Energy Expenditure, Food Efficiency, and Protein Oxidation

Mean total energy expenditure (TEE) throughout the study was computed by subtracting energy retrained in the body from energy intake. Energy intake was not corrected for the percent of energy lost in feces, on the average 10%–15% of the ingested calories (46, 47). Therefore, TEE was overestimated by 10%–15%. We also assumed that digestibility was not affected by the dietary protein level (but see discussion) and therefore that the overestimation was similar in all groups.

Food efficiency (kJ/kJ) was measured from energy intake and the gain in fat and LBM assuming 32.2 kJ for 1 g of fat and 7.6 kJ for 1 g of LBM (48) and computed as energy retained in the body divided by energy ingested. Protein intake computed from total energy intake times the percentage of protein in the diet, and evolution of body composition during the study were used to compute protein oxidation (Pox) as the difference between protein intake (kJ/day) and the amount of protein fixed in the body (kJ/day) assuming that protein amounted to 25% of LBM.

## Biochemistry

Blood samples (0.5 mL) were collected from the tail vein in EDTA tubes in the morning (10:00–12:00) from rats that had not been previously fasted. A drop was immediately used to measure blood glucose with an automatic analyzer (Life-Scan, One Touch Vita). Then the blood was centrifuged (5,000 g, 15 min,  $4^{\circ}\text{C}$ ) and the plasma stored at  $-20^{\circ}\text{C}$  until assayed. Plasma concentrations of nonesterified fatty acids



(NEFA), albumin, cholesterol, HDL cholesterol, and triglyceride (TG) were determined on an Olympus AU400 robot (CEFI Bichat-Paris). Plasma insulin, FGF21, and IGF-1 were assayed by ELISA tests using commercial kits from Mercodia (Rat Insulin ELISA 10–1250–10), BioVendor (Mouse/Rat FGF-21 ELISA RD291108200R), and Alpco (Mouse/Rat IGF-1 ELISA 22-IG1MS-E01). The Homeostatic Model Assessment of the Insulin Resistance (HOMA-IR) index was calculated as  $[\text{insulinemia (pmol/L)} \times \text{blood glucose (mmol/L)}]/405$  (49). Liver samples collected during dissection (80–100 mg) were homogenized in 1 mL of buffer (150 mM NaCl, 10 mM Tris HCL, and 0.1% Triton X-100) (50) and their TG content was determined using a commercial kit (Randox).

### Molecular Biology

RNAs were extracted from liver, hypothalamus and NAcc samples in 1 mL of TRIzol reagent (Invitrogen, Breda, The Netherlands), and in IBAT in 1.5 mL of TRIzol reagent (Invitrogen, Breda, the Netherlands). The RNA concentrations were measured (NanoDrop One spectrophotometer), and after dilution, 0.4  $\mu\text{g}$  of total RNA was reverse-transcribed using a high-capacity cDNA archive kit (Applied Biosystems). Real-time PCR was performed using a StepOne Real-time PCR (Applied Biosystems) and the Power SYBR Green PCR Master Mix (Applied Biosystems), as described previously (51). For each run, the efficiency and possible contaminations were checked. Gene expression was calculated as  $2^{-\Delta\text{CT}}$  relative to 18 s RNA (liver and IBAT) or RPL13 RNA (hypothalamus and NAcc), used as the housekeeping gene. Target genes and primers used are detailed in Supplemental Table S1 (all Supplemental material is available at <https://doi.org/10.6084/m9.figshare.14892405>).

### Statistical Analysis

Statistical tests were performed using RStudio software (2015; Integrated Development for R, RStudio, Inc.; Boston) and a threshold of  $P \leq 0.05$  was chosen as significant. Values in the tables are means  $\pm$  standard error of the mean (SEM) and values in figures are presented in the form of boxplot (1st quartile, median, 3rd quartile). Normality of the parameter's distribution was previously verified by a Shapiro test and corrected by the Box and Cox transformation before statistical comparisons. Dietary PE and CnpE ratios were converted in nonnumeric factors and differences between groups were analyzed by two-way ANOVA test (parameter  $\sim$  PE ratio  $\times$  CnpE ratio) and a post hoc Bonferroni test. All groups were presented in the figures when we observed an effect of both PE and CnpE ratios. However, in most cases, we observed an effect of only the PE ratio, and for clarity, the data have been plotted by PE ratios only. When ANOVA tests revealed significant effects of both PE and CnpE ratios on a given parameter, the analysis was continued by performing a stepwise regression analysis to quantify the respective roles of PE and CnpE ratios on that parameter. Correlation analyses between various parameters and the PE ratio were also performed in Excel and the significance of the correlation was assessed using the Pearson correlation coefficient.

## RESULTS

### Body Weight and Body Composition

Body weight gain was negative in the 3 P fed rats, and positive but significantly lower in 5 P and 8 P fed rats than in 15 P and 30 P fed rats (Table 2). The weight of LBM (Table 2), and of all measured organs and skin (Supplemental Table S2), was reduced when the PE ratio was reduced at or below 8%, whereas the CnpE ratio induced no significant effects. The significant decrease in the weights of tail, head, and limbs (Supplemental Table S2) in which bone mass amounts to a significant part of the weight, with the decrease in the dietary PE ratio, suggests that together with the LBM decrease, bone mass was also decreased in rats fed diets with less than 15% of protein. Total fat mass, IBAT mass, and to a lesser extent adiposity and visceral fat mass were affected only by the PE ratio (Table 2). LBM gain was negative in the P3 fed mice and fat mass gain was also lower than in all the other groups. As a result, energy retained in the body was extremely low, even when compared with P5 fed rats. The parameter that was significantly affected by PE ratio and to a lower extent by CnpE ratio was the subcutaneous fat mass that tended to be lower in 15 P and 30 P fed rats than in low-protein fed ones (Table 2), indicating that fat tended to deposit more in subcutaneous tissues in low-protein fed rats.

In summary, body composition shows that LBM decreased in rats fed diets with less than 15% protein, but that the changes in fat mass were less systematic. On the other hand, the CnpE ratio had no significant effects on most of the parameters of body composition.

### Energy, Protein, and Water Intake

Energy intake increased as the PE ratio in the diet decreased, but also increased as the CnpE ratio decreased except for 3 P and 30 P diets (Fig. 1A). Regression analysis showed also that the PE ratio alone ( $R^2 = 0.42$ ), but not the CnpE ratio alone ( $R^2 = 0.04$ ), allowed for a significant prediction of mean energy intake, but stepwise regression analysis showed that the combination of the PE and CnpE ratios improved the prediction of mean energy intake ( $R^2 = 0.56$ ; Fig. 1B). Protein intake was primarily affected by the PE ratio, the CnpE ratio having only a marginal effect in 5 P and 8 P fed rats (Fig. 1C). In a stepwise regression analysis, the model including only the PE ratio as predictors of protein intake ( $R^2 = 0.97$ ; Fig. 1D) was not improved by the addition of the CnpE ratio ( $R^2 = 0.98$ ). Water intake was affected by both the PE and CnpE ratios (Fig. 1E), being larger with high PE and high CnpE ratios, i.e., when the protein and carbohydrates contents of the diets were higher. Stepwise regression analysis also showed that the combination of protein and carbohydrate intakes best predicted water intake ( $R^2 = 0.62$ ; Fig. 1F) and that catabolism of protein required 15 times more water than that of carbohydrates (0.266 mL/kJ vs. 0.017 mL/kJ).

### Energy Expenditure, Food Efficiency, and Protein Oxidation

TEE was larger in rats fed the low-protein diets, especially in the groups fed the 3 P and 5 P diets, and was also affected by the CnpE ratio (Fig. 2A). Stepwise regression analysis

**Table 2.** Body weight and composition in response to PE and CnpE ratios

	P (1)												PE Ratio *	
	3P45Cnp (n = 6)	3P75Cnp (n = 6)	5P30Cnp (n = 6)	5P60Cnp (n = 6)	8P45Cnp (n = 6)	8P75Cnp (n = 6)	15P30Cnp (n = 6)	15P60Cnp (n = 6)	15P75Cnp (n = 5)	30P30Cnp (n = 6)	30P45Cnp (n = 6)	30P60Cnp (n = 5)	PE Ratio	CnpE Ratio
Initial body weight, g	261 ± 5.37	266 ± 3.04	269 ± 4.32	275 ± 4.55	267 ± 5.81	276 ± 3.33	274 ± 3.49	271 ± 8.29	272 ± 2.83	273 ± 3.39	263 ± 6.85	266 ± 4.52	0.510	0.380
Final body weight, g	254 <sup>a</sup> ± 5.41	257 <sup>a</sup> ± 4.59	300 <sup>b</sup> ± 9.03	297 <sup>b</sup> ± 4.51	319 <sup>b</sup> ± 6.37	314 <sup>b</sup> ± 8.39	365 <sup>c</sup> ± 6.31	359 <sup>c</sup> ± 10.7	353 <sup>c</sup> ± 3.29	356 <sup>c</sup> ± 5.65	355 <sup>c</sup> ± 5.46	344 <sup>c</sup> ± 7.85	<10 <sup>-15</sup>	0.630
Body weight gain, g	-9.70 <sup>a</sup> ± 4.90	-8.38 <sup>a</sup> ± 3.39	311 <sup>b</sup> ± 5.27	22.0 <sup>b</sup> ± 3.97	52.1 <sup>c</sup> ± 3.70	38.1 <sup>c</sup> ± 6.61	90.6 <sup>d</sup> ± 5.03	87.3 <sup>d</sup> ± 3.39	81.8 <sup>d</sup> ± 3.36	83.6 <sup>d</sup> ± 5.00	91.8 <sup>d</sup> ± 5.20	77.8 <sup>d</sup> ± 4.18	<10 <sup>-15</sup>	0.133
Lean body mass, g	219 <sup>a</sup> ± 3.22	225 <sup>a</sup> ± 4.76	253 <sup>b</sup> ± 7.11	255 <sup>b</sup> ± 3.73	276 <sup>c</sup> ± 5.65	273 <sup>c</sup> ± 5.52	308 <sup>d</sup> ± 6.98	306 <sup>d</sup> ± 8.83	307 <sup>d</sup> ± 2.88	314 <sup>d</sup> ± 5.67	311 <sup>d</sup> ± 5.11	300 <sup>d</sup> ± 6.52	<10 <sup>-15</sup>	0.750
Lean body mass gain, g	-23.4 <sup>a</sup> ± 3.57	-18.8 <sup>a</sup> ± 5.42	11.8 <sup>b</sup> ± 8.02	14.2 <sup>b</sup> ± 4.29	36.5 <sup>bc</sup> ± 6.23	32.7 <sup>bc</sup> ± 6.27	68.4 <sup>d</sup> ± 7.62	66.5 <sup>d</sup> ± 10.1	67.4 <sup>d</sup> ± 3.00	73.7 <sup>d</sup> ± 6.73	72.0 <sup>d</sup> ± 6.51	59.6 <sup>cd</sup> ± 6.52	<10 <sup>-15</sup>	0.807
Total white adipose tissue, g	211 <sup>a</sup> ± 2.52	215 <sup>a</sup> ± 1.59	35.3 <sup>bc</sup> ± 2.97	29.7 <sup>b</sup> ± 1.71	29.5 <sup>ba</sup> ± 2.06	28.2 <sup>ba</sup> ± 4.03	43.1 <sup>c</sup> ± 4.95	38.8 <sup>c</sup> ± 3.15	32.6 <sup>c</sup> ± 1.76	29.2 <sup>b</sup> ± 1.31	30.4 <sup>b</sup> ± 2.12	31.5 <sup>b</sup> ± 1.93	1.11E-4	0.302
Adipose tissue gain, g	6.45 <sup>a</sup> ± 2.77	8.05 <sup>ab</sup> ± 1.13	20.6 <sup>bcd</sup> ± 3.26	15.0 <sup>bc</sup> ± 1.87	14.8 <sup>a</sup> ± 2.26	13.5 <sup>ac</sup> ± 4.42	28.4 <sup>d</sup> ± 5.43	24.0 <sup>cd</sup> ± 3.46	17.9 <sup>cd</sup> ± 1.77	14.5 <sup>bc</sup> ± 1.44	15.0 <sup>cd</sup> ± 2.46	16.8 <sup>cd</sup> ± 1.94	1.60E-4	0.189
Energy retained in the body, kJ	30.1 <sup>a</sup> ± 1.11	117 <sup>ab</sup> ± 4.16	752 <sup>c</sup> ± 143	590 <sup>bc</sup> ± 73.1	752 <sup>c</sup> ± 93.8	685 <sup>c</sup> ± 170	1,435 <sup>d</sup> ± 156	1,280 <sup>d</sup> ± 156	1,090 <sup>cd</sup> ± 58.7	1,016 <sup>cd</sup> ± 50.8	1,065 <sup>cd</sup> ± 88.4	993 <sup>cd</sup> ± 101	<10 <sup>-13</sup>	0.276
Adiposity (% of body weight)	8.35 <sup>a</sup> ± 0.833	8.37 <sup>a</sup> ± 0.651	11.7 <sup>b</sup> ± 0.809	9.98 <sup>b</sup> ± 0.509	9.21 <sup>ab</sup> ± 0.569	8.88 <sup>ab</sup> ± 1.12	11.8 <sup>b</sup> ± 1.30	10.8 <sup>b</sup> ± 0.688	9.23 <sup>b</sup> ± 0.463	8.2 <sup>a</sup> ± 0.425	8.57 <sup>a</sup> ± 0.592	9.12 <sup>a</sup> ± 0.434	0.016	0.249
Subcutaneous fat mass (% of total fat mass)	47.0 <sup>ab</sup> ± 1.50	53.4 <sup>ab</sup> ± 4.58	47.9 <sup>ab</sup> ± 1.08	49.8 <sup>ab</sup> ± 0.776	50.6 <sup>a</sup> ± 1.04	52.4 <sup>a</sup> ± 3.42	42.9 <sup>b</sup> ± 1.19	44.8 <sup>b</sup> ± 1.38	50.5 <sup>b</sup> ± 2.02	46.8 <sup>b</sup> ± 1.66	48.3 <sup>b</sup> ± 1.80	45.2 <sup>b</sup> ± 1.57	0.025	0.027
Visceral fat mass (% of total fat mass)	18.2 <sup>a</sup> ± 0.792	16.9 <sup>a</sup> ± 0.640	16.7 <sup>ab</sup> ± 0.680	16.7 <sup>ab</sup> ± 0.413	15.9 <sup>b</sup> ± 0.524	15.9 <sup>b</sup> ± 0.603	15.6 <sup>b</sup> ± 0.761	14.2 <sup>b</sup> ± 0.677	15.3 <sup>b</sup> ± 0.502	16.0 <sup>ab</sup> ± 1.68	16.2 <sup>ab</sup> ± 1.15	15.1 <sup>ab</sup> ± 1.24	0.019	0.226
Total brown adipose tissue, g	0.647 <sup>a</sup> ± 0.072	0.672 <sup>a</sup> ± 0.059	0.870 <sup>b</sup> ± 0.102	0.798 <sup>b</sup> ± 0.063	0.692 <sup>a</sup> ± 0.064	0.421 <sup>a</sup> ± 0.056	0.640 <sup>b</sup> ± 0.060	0.564 <sup>a</sup> ± 0.040	0.542 <sup>a</sup> ± 0.029	0.544 <sup>a</sup> ± 0.023	0.550 <sup>a</sup> ± 0.037	0.494 <sup>a</sup> ± 0.055	5.57E-6	0.073

Values are means ± SE, 3 wks. (1) P for ANOVA test. Means with different letters within a line are statistically different ( $P \leq 0.05$ ). CnpE ratio: Carbohydrate:non-protein-energy ratio; PE ratio: protein-to-energy ratio.

showed that use of both the PE and CnpE ratios more accurately predicted TEE ( $R^2 = 0.70$ ; Fig. 2B) than the PE ratio alone ( $R^2 = 0.60$ ). Food efficiency was affected by the PE ratio only and was significantly lower in 5 P and 8 P fed rats than in 15 P and 30 P fed ones and further reduced to zero, in 3 P fed rats (Fig. 2C). Pox amounted 94%–96% of protein intake in 5 P, 8 P, 15 P, and 30 P fed rats and was larger than protein intake in 3 P fed rats (Fig. 2D), meaning that, as testified by the negative gain in LBM, the 3 P fed rats had to mobilize their protein stores to survive. In the 5 P and 8 P fed rats, the high proportion of dietary protein that was oxidized (~95%) occurred at the expense of protein deposition as in these rats, the amount of protein retained in the body was significantly lower than in 15 P and 30 P fed rats. All groups taken together, Pox increased as a linear function of protein intake (Fig. 2E), and protein retained in the body as a log function of protein intake (Fig. 2F).

## FGF21 and IGF-1

Plasma FGF21 and IGF-1 concentrations appeared fairly tightly correlated (Fig. 3A). Plasma FGF21 concentrations responded to the PE ratio but not to the CnpE ratio (Fig. 3B). Accordingly, plasma FGF21 correlated with dietary protein intake. The relation was best described by a power function indicating that FGF21 increased slowly when dietary protein intake decreased from 100 to 40 kJ, then sharply when the protein intake decreased below 40 kJ (Fig. 3C). Plasma IGF-1 concentrations responded also only to the PE ratio (Fig. 3D). The relation between plasma IGF-1 concentrations and dietary protein intake was best described by a Log function (Fig. 3E) indicating that IGF-1 decreased abruptly when protein intake decreased below 20 kJ, i.e., in the P3 group.

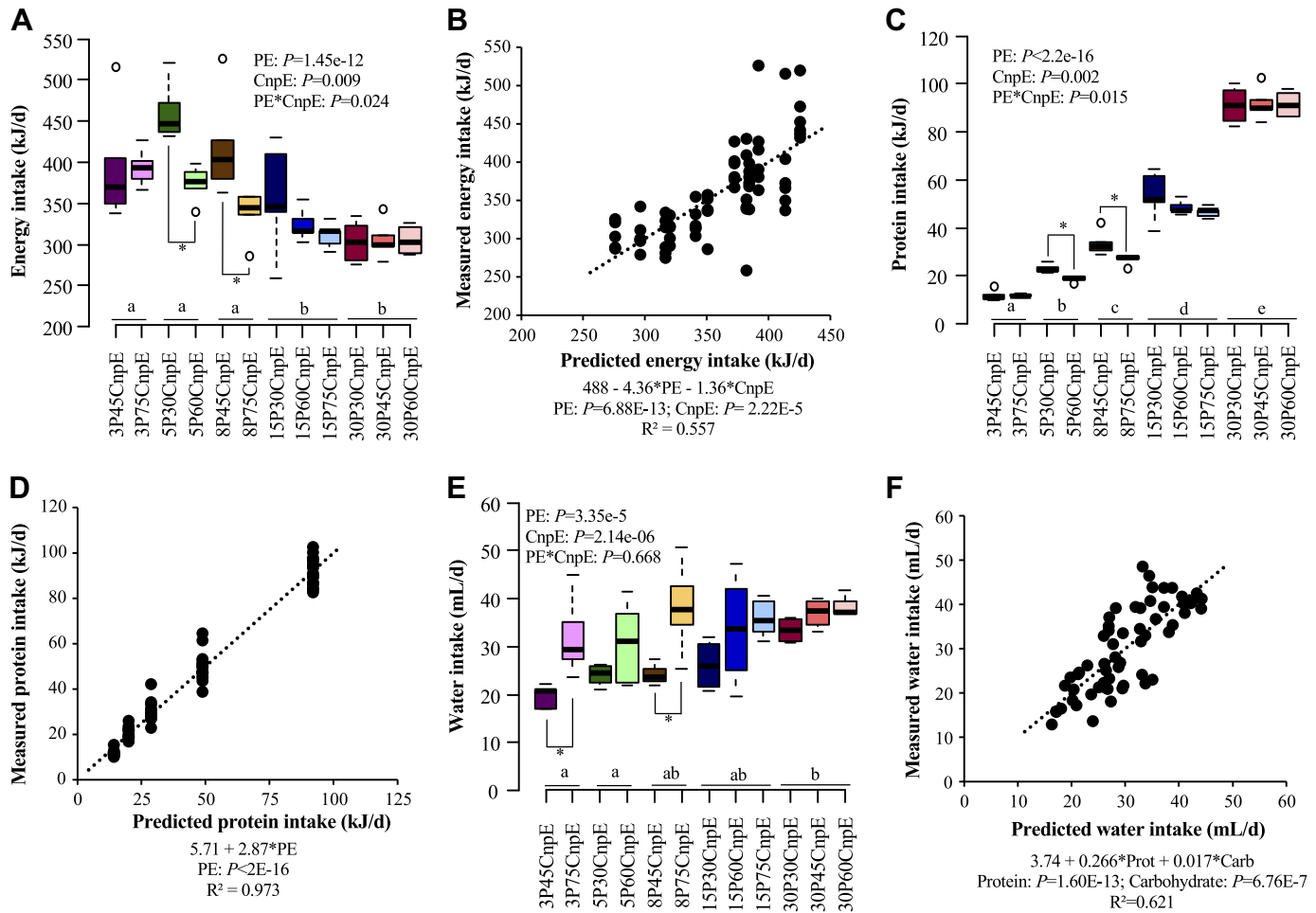
Plasma FGF21 and IGF-1 correlated with energy (Fig. 4, A and B), protein balance (Fig. 4, C and D), body weight gain (Fig. 4, E and F), and LBM (Supplemental Fig. S1). They did not correlate with total fat mass and adiposity (Supplemental Fig. S1).

## Plasma Parameters

Blood glucose and plasma HDL concentrations were not affected by the various diets, but plasma NEFA, albumin, insulin, and the HOMA index decreased as the PE ratio decreased (Table 3). Plasma cholesterol was mildly affected by the CnpE ratio (Table 3), but no clear relation between the CnpE ratio and plasma cholesterol emerged. Plasma TG concentrations were the only parameters that were affected by both PE and CnpE ratios (Table 3), the effect of the PE ratio being, however, much stronger than the effect of the CnpE ratio: TG increased as the PE ratio increased but in the 30 P and 15 P fed rats, the increase was reduced when the CnpE ratio was low. Plasma TG correlated also positively with FGF21 ( $R^2 = 0.16$ ,  $P = 0.001$ ) and negatively but poorly with IGF-1 ( $R^2 = -0.07$ ,  $P = 0.040$ ).

## Liver Triglycerides

In contrast to plasma TGs, which were mainly affected by the PE ratio, liver TGs were affected almost exclusively by the CnpE ratio and increased when the CnpE ratio decreased (Table 3). In opposition to FGF21, liver TGs correlated negatively with plasma FGF21 ( $R^2 = 0.10$ ,  $P = 0.007$ ) and positively



**Figure 1.** Energy, protein, and water intake in response to protein-to-energy (PE) and carbohydrate-nonprotein-energy (CnpE) ratios. A, C, and E: PE and CnpE ratios effects tested by ANOVA ( $P \leq 0.05$ ) and post hoc tests (values with different letter are significantly different). B, D, and F: predictive values determined by stepwise regression and prediction quality tested by Pearson correlation test.  $n$  per group = 5 or 6 rats, 3 wk.

correlated with plasma IGF-1 ( $R^2 = 0.15$ ,  $P = 0.001$ ), but liver and plasma TGs did not correlate ( $R^2 = -0.01$ ,  $P = 0.480$ ).

### Gene Expression

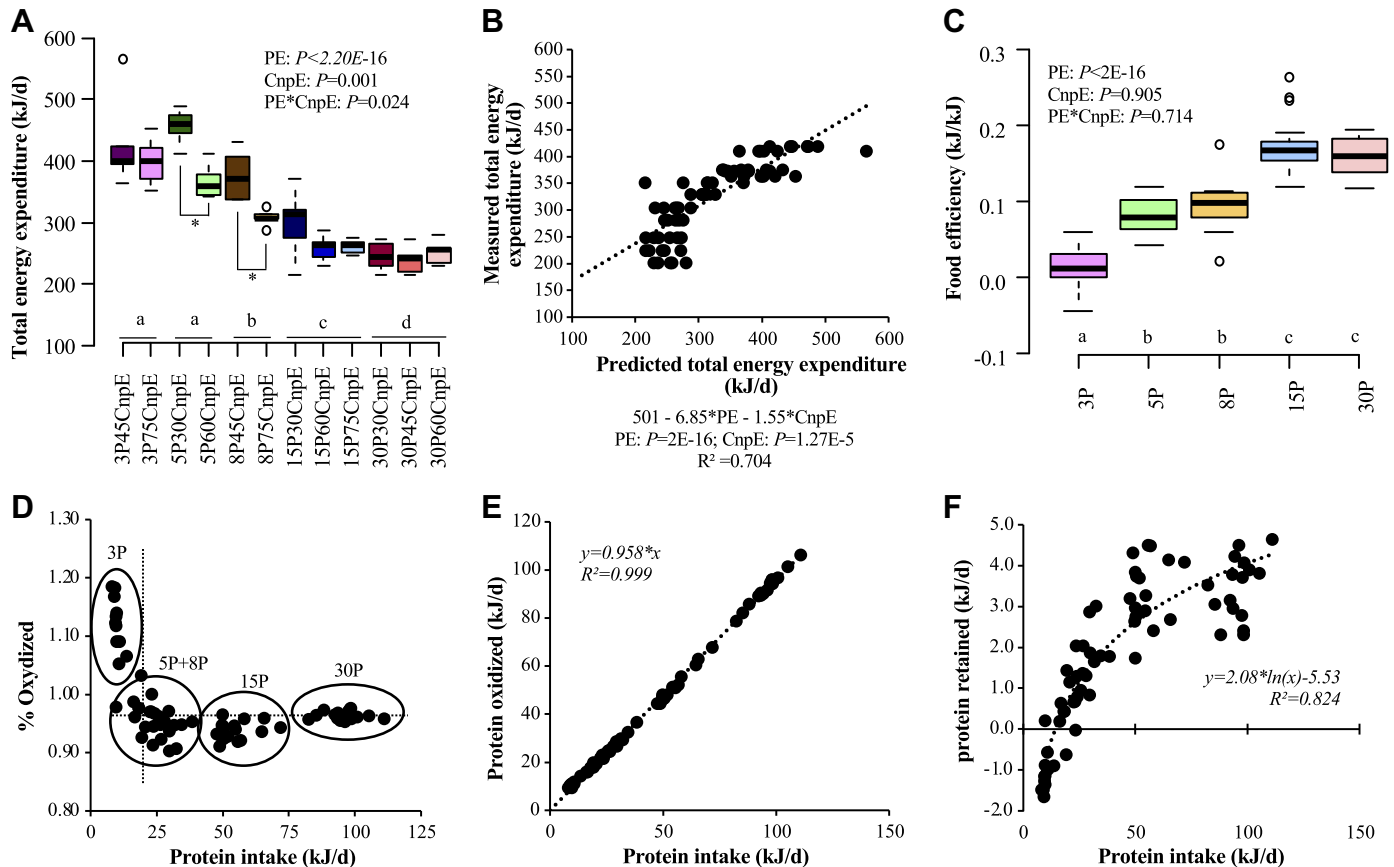
In the liver, *Fgf21* and *Igf-1* expressions were affected only by the dietary PE ratio (Table 4) and correlated with their plasma values (*Fgf21*:  $R^2 = 0.35$ ,  $P = 8.84 \times 10^{-6}$ ; *Igf-1*:  $R^2 = 0.21$ ,  $P = 1.39 \times 10^{-4}$ ). *Fas* expression tended to be higher in low-protein fed rats. *Cpt1* expression was affected by the PE ratio, and to a lesser extent by the interaction between the PE and CnpE ratios (Table 4). Finally, the expression of *Acca*, *Lpk*, and *Gk* were insensitive to the dietary PE and CnpE ratios (data not shown).

In the hypothalamus, it was notable that the expression of *Fgf21*, and of the FGF21 receptor genes, (*Fgf21r1*, *Fgf21r2b*, *Fgf21r2c*, and *Fgf21r3*) was not modified by the diets (data not shown). Only *Pomc* expression was affected significantly by the diet, and especially by the PE ratio (Fig. 5A). *Cart*, *Agrp*, and *Npy* expressions were unaffected by the PE and CnpE ratios (data not shown). Because *Pomc* and *Cart* expressions on the one hand, and *Agrp* and *Npy* expressions on the other hand, are considered closely related, we also

investigated whether various combinations of the expression of these neurotransmitters were affected by the diet. We observed that although *Agrp* expression was not at all correlated with the PE ratio ( $R^2 = 0.002$ ), the *Pomc/Agrp* ratio was much better correlated with the PE ratio ( $R^2 = 0.64$ ; Fig. 5, C and D) than *Pomc* expression alone ( $R^2 = 0.35$ , Fig. 5B) suggesting that the *Pomc/Agrp* ratio conveyed to the hypothalamus a more precise information on the dietary PE ratio than *Pomc* alone.

We investigated also whether *Npy*, *Agrp*, *Pomc*, and *Cart* expressions were correlated with plasma FGF21 and IGF-1 concentrations. Only *Pomc* was correlated to both FGF21 and IGF-1 (Fig. 6, A and B), and we also observed that the *Pomc/Agrp* ratio was well correlated with FGF21 and IGF-1 (Fig. 6, C and D). These results suggested that FGF21 and IGF-1 in plasma and *Pomc* and *Pomc/Agrp* ratio in the brain conveyed the information of the dietary PE ratio.

Finally, in the NAcc, there was no difference between groups regarding the expression of *Dr1*, *Dr2*, *Dor*, and *Kor* (Data not shown). In the IBAT, there was no difference between groups regarding the expressions of *Ucp1*, *Ucp2*, and *Ucp3* (data not shown).



**Figure 2.** Energy expenditure, food efficiency, and protein balance in response to protein-to-energy ratio (PE) and carbohydrate-nonprotein-energy ratio (CnpE ratios). A and C: PE and CnpE ratios effects tested by ANOVA ( $P \leq 0.05$ ) and post hoc tests (values with different letter are significantly different). B: predictive values determined by stepwise regression and prediction quality tested by Pearson correlation test. D, E, and F: correlation by Pearson test.  $n$  per group = 5 or 6 rats, 3 wk.

## DISCUSSION

The main result of this study was that long-term regulation of plasma FGF21 concentration depends exclusively on the dietary protein level and that this is also true for the regulation of plasma IGF-1 concentration. Most of the parameters of body composition and energy expenditure were affected also by the dietary protein content only, except for energy and water intake which were affected by a combination of dietary protein and carbohydrate content. In the hypothalamus, *Pomc* expression and with more significance the *Pomc/AgRP* ratio were the parameters most responsive to dietary protein content.

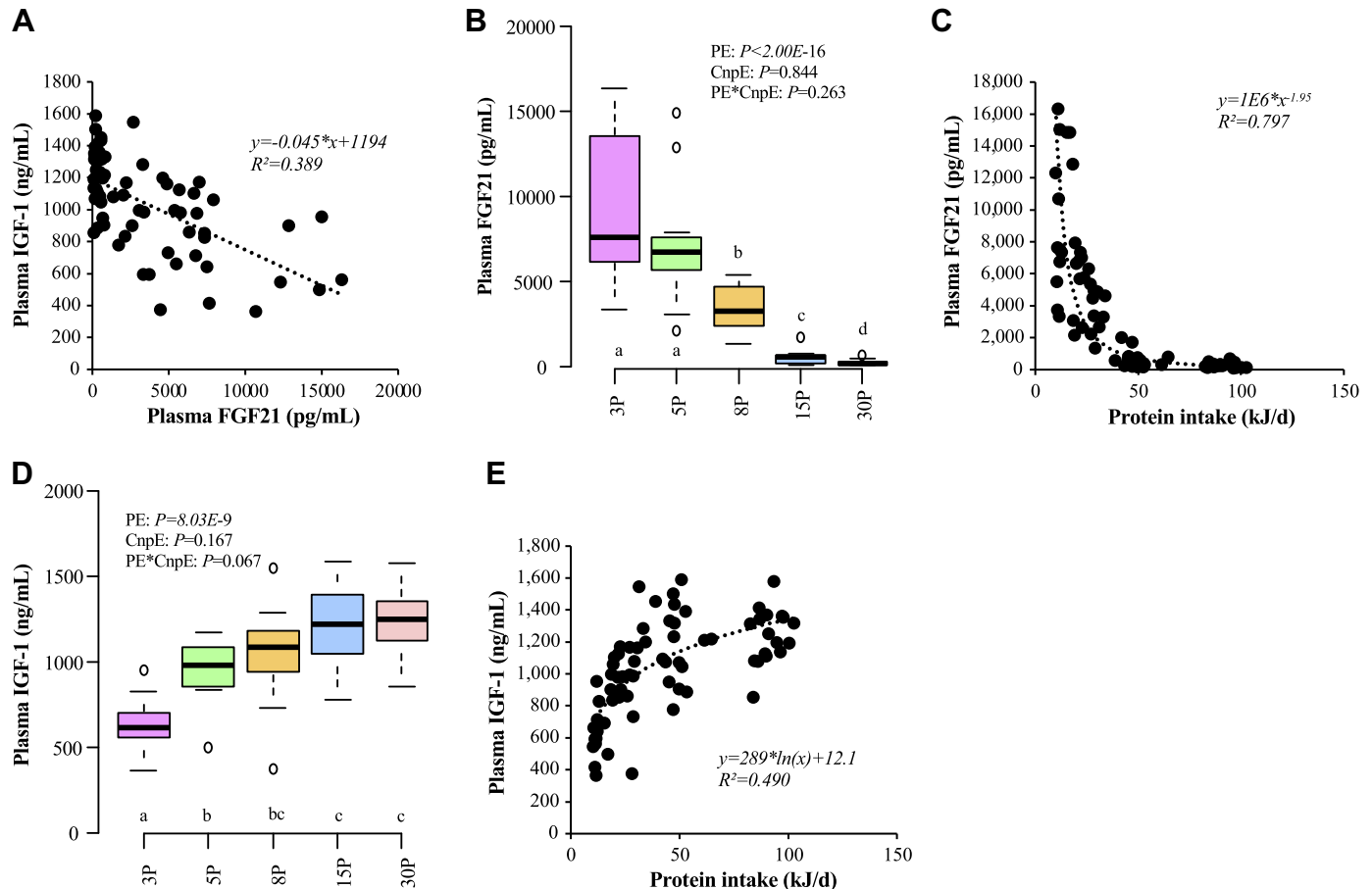
### Effects of the Low-Protein Diets on Body Composition, Protein Oxidation, Energy Expenditure, and Food Efficiency

Analysis of body composition showed that all organs and tissues were affected by the dietary protein level, and none of them, including fat mass, was affected by the CnpE ratio, with the exception of subcutaneous fat mass, which was however only slightly impacted by the CnpE ratio. Body weight, weight of most of the lean tissues, and overall LBM were reduced significantly in rats fed the 3 P, 5 P, and 8 P diets and were similar in 15 P and 30 P fed rats. Fat mass did

not exhibit a progressive decrease in relation to the decrease in the dietary protein content as observed for the other organs, but did not change significantly from 5 P to 30 P diets and was noticeably decreased only in rats fed the 3 P. The evolution of fat mass and LBM led to the fact that the differences in adiposity between the groups finally proved to be limited and were not correlated with the dietary PE ratio. Weight of IBAT was increased also in 3 P and 5 P fed rats. Taken together, these results showed that the weight of the lean tissues decreased rapidly, as the dietary protein level was decreased below the requirement that in this study appeared to be between 15% and 8%. The lack of effect of the CnpE ratio on the majority of body composition parameters was probably due to it being hidden by the stronger effects of protein levels.

Surprisingly, we observed that the 8 P and 5 P fed rats continued to oxidize up to 95% of their dietary protein intake rather than preserving the dietary proteins to sustain growth. This result suggests strongly that protein oxidation plays a very important regulatory function in the metabolic fate of energy substrates, so strong that the rats apparently preferred to sacrifice growth of lean tissues rather than to reduce the participation of amino acids in energy production. This result raises an important question, because it shows that, behind the apparent capacity of rats and other



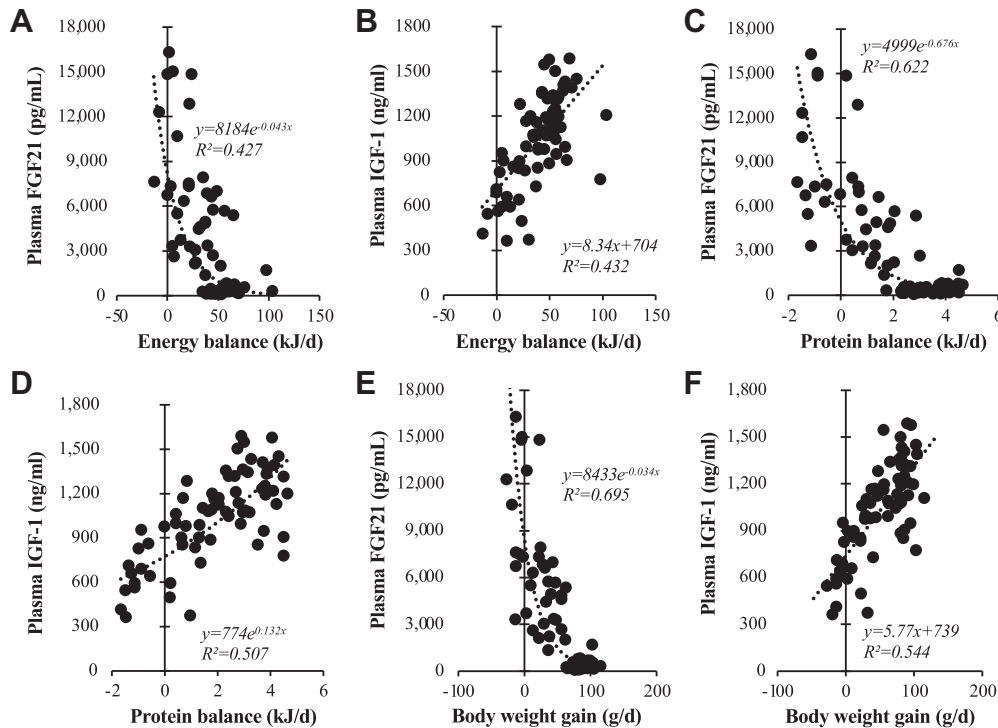


**Figure 3.** Plasma FGF21 and IGF-1 concentrations in response to protein-to-energy ratio (PE) and carbohydrate-nonprotein-energy ratio (CnpE ratios). A, C, and E: correlation by Pearson test. B and D: PE and CnpE ratios effects tested by ANOVA ( $P \leq 0.05$ ) and post hoc tests (values with different letter are significantly different).  $n$  per group = 5 or 6 rats, 3 wk.

living organisms to deal with a large variability in dietary protein levels, the role of protein in the regulation of substrate fluxes to provide energy is very important and is preserved even at the cost of growth. The optimal protein intake has been suggested to be around 15%, but in this study, even if the 15 P fed rats had the highest growth rate, they also tended to be fatter than the 30 P fed ones, suggesting that 30% protein would be a better level than 15% to sustain growth with an optimal ratio of LBM to fat deposition. The lower adiposity level of the 30 P fed rats may have resulted from the fact that high dietary protein levels increase postprandial lipid oxidation (52) and therefore may reduce the retention of lipids in adipose tissues. Accordingly, in self-selecting rats, many studies have reported that rats prefer to select a diet in which proteins amount for around 30% of energy, and sometimes more (28). Therefore, this study has shown that decreasing dietary protein levels below 15% rapidly slows growth rate, indicating that 15% dietary protein is close to the lowest acceptable level. Finally, the decrease in tail weight in proportion to the dietary protein level also testified to the essentiality of protein intake to sustain bone growth (53).

In this study, we observed a significant increase in TEE and a decrease in food efficiency in low-protein fed rats, which is considered as a metabolic response induced by the

increase in plasma FGF21 concentrations (14, 54–56). We report here also that food efficiency was reduced by a half in 8 P and 5 P fed rats, by 80% in 3 P fed ones, and was similar in 15 P and 30 P fed rats. As described in MATERIALS AND METHODS, these calculations assumed that digestible energy was not affected by the protein level of the diet. However, one author, Pezeshki et al. (57) who, to our knowledge, was the only one to compare as large differences in dietary protein levels as those used in this study, reported that, compared with 15 P fed rats, digestible energy was unaffected in 10 P fed rats, but reduced by ~10% in 5 P fed rats and up to 40% in rats fed a protein-free diet. Therefore, it is possible that in this study, feed efficiency was larger than estimated, in particular in P3-fed rats, but given the very low amount of energy retained in the body by the 3 P fed rats [on the average less than 11% of the energy retained by the 5 P fed rats and 6% of the energy retained by the 15 P fed ones (Table 2)], this limitation does not negate the fact that feed efficiency was reduced in proportion to the reduction in protein intake. Food efficiency was not affected by the dietary CnpE ratio in all groups, which contradicts the repeated observation that food efficiency is increased by high-fat diets, a mechanism largely suspected to be responsible for the increase in fat mass and adiposity in high-fat fed subjects (58, 59). This difference remains to be understood, but it is possible that the



**Figure 4.** Plasma FGF21 and IGF-1 concentrations as a function of energy balance, protein balance, and body weight gain. Analysis done by Pearson correlation test. Predictive values determined by stepwise regression and prediction quality tested by Pearson correlation test. *n* per group = 5 or 6 rats, 3 wk.

reduced food efficiency induced already by the decrease in the protein content prevailed over a possible CnpE ratio effect and was part of a general adaptation that allowed these rats to increase energy intake without gaining in adiposity. In the rats of this study, TEE and food efficiency were also measured by indirect calorimetry, but in conditions of thermal neutrality (27°C) to assess the role played by the cost of thermal regulation in the increase in energy expenditure observed with the decrease in dietary protein. Accordingly, energy intake and TEE values measured during indirect calorimetry were on the average ~25% lower than computed from energy balance but confirmed the results by energy balance that TEE was increased and feed efficiency decreased in the P3 and P5 fed rats. These results will be the subject of a specific paper being prepared describing which component (s) of energy expenditure (thermal regulation, intensity and cost of activity, thermogenic response to feeding) sustain the increase in energy expenditure in low-protein fed rats.

#### Roles of Dietary Protein and Carbohydrate on FGF21 and IGF-1

One important question to which this study wanted to answer was to decipher the specific roles of the dietary protein and carbohydrate levels on the long-term regulation of plasma concentrations of FGF21 and IGF-1. For both hormones, the response is clearly that their plasma concentrations respond exclusively to changes in the dietary PE ratio and protein intake. Plasma FGF21 concentrations increased abruptly when the PE ratio and protein intake decreased below a threshold level somewhere between 15% and 8%, whereas IGF-1 concentrations increased from 3% to 15% dietary protein, then leveled from 15% to 30%. As a result of

these symmetrical changes, it appeared that plasma FGF21 and IGF-1 concentrations were fairly closely correlated, suggesting that the mechanisms controlling the secretion of these two hormones are linked. One possibility suggested by Inagaki et al. (60) may be that FGF21 inhibits growth hormone signaling by increasing resistance to growth hormone (GH) and thus that FGF21 reduces serum IGF-1 by reducing IGF-1 mRNA expression in the liver. As a result, when the evolution of one or another of the studied parameters was correlated with one of the hormones, it was also correlated with the other, and therefore it was not possible to decide, which was the hormone that could be suspected to affect this parameter.

That FGF21 is the signal of protein deprivation now widely accepted, but FGF21 was shown also to respond to various other metabolic signals such as food restriction (61), cold (61), lipolysis (62), exercise, and others (9). FGF21 increases energy expenditure, stimulates food intake and a specific appetite for protein, and improves glucose tolerance and insulin sensitivity in the liver and adipose tissue. In this study, plasma FGF21 concentrations were increased impressively in 8 P, 5 P, and 3 P fed rats, but none of these groups were influenced by the CnpE ratio. This result enters into a conflict with previous reports that showed that FGF21 responds to glucose or fructose ingestion, and also to conclusions of the study by Solon-Biet et al. (26) that FGF21 responds more strongly to a low dietary protein intake when it is coupled with a high-carbohydrate intake. One main reason for the lack of significant effects of carbohydrate in this study is certainly related to fact that, if carbohydrate intake can indeed increase FGF21 concentrations, the FGF21 response to carbohydrate intake is an order magnitude much smaller than the

**Table 3.** Plasma and liver parameters in response to PE and CnpE ratios

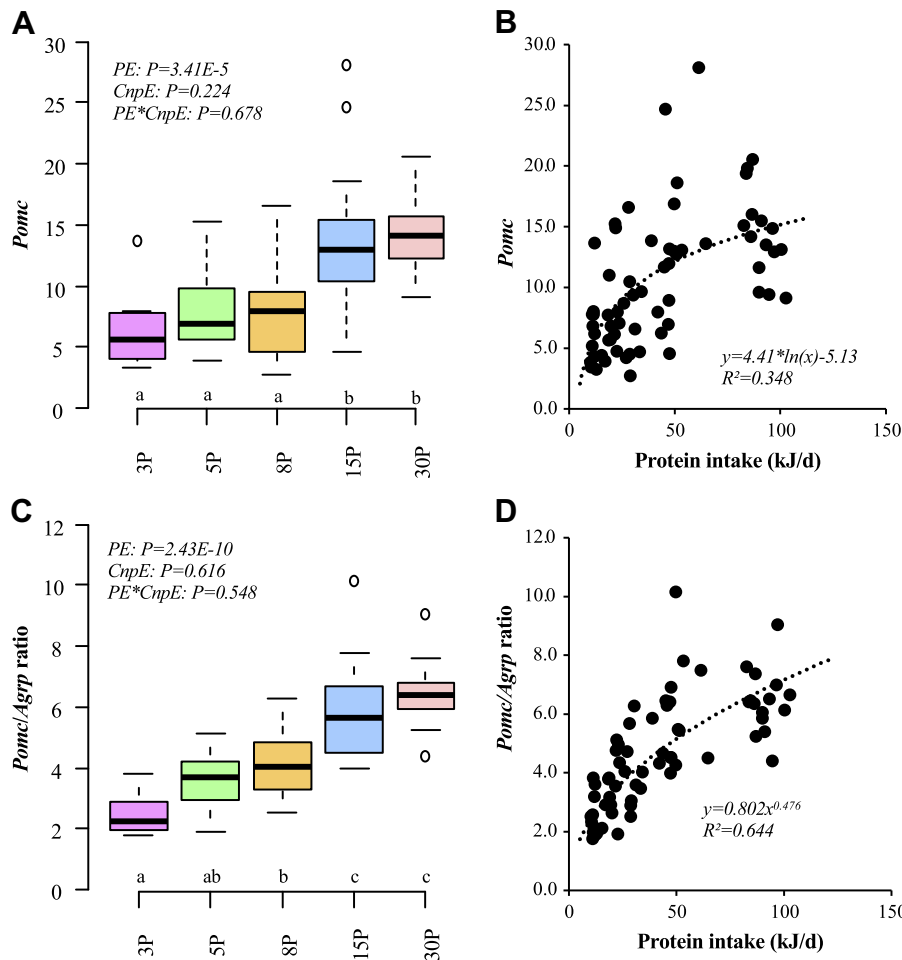
	3P45Cnp (n = 6)	3P75Cnp (n = 6)	5P30Cnp (n = 6)	5P60Cnp (n = 6)	8P45Cnp (n = 6)	8P75Cnp (n = 6)	15P30Cnp (n = 6)	15P60Cnp (n = 6)	15P75Cnp (n = 5)	30P30Cnp (n = 6)	30P45Cnp (n = 6)	30P60Cnp (n = 5)	P (1)		
													PE Ratio	Ratio	CnpE Ratio
<b>Plasma</b>															
Insulin, pmol/L	0.393 <sup>a</sup> ± 0.117	0.298 <sup>a</sup> ± 0.063	0.493 <sup>a</sup> ± 0.087	0.382 <sup>a</sup> ± 0.070	0.538 <sup>ab</sup> ± 0.069	0.552 <sup>ab</sup> ± 0.126	1.05b <sup>c</sup> ± 0.154	0.945b <sup>c</sup> ± 0.117	0.926b <sup>c</sup> ± 0.282	0.636 <sup>b,c</sup> ± 0.104	0.846 <sup>b,c</sup> ± 0.074	0.873 <sup>b,c</sup> ± 0.138	6.56E-7	0.801	0.589
Glucose, mmol/L	5.68 ± 0.366	5.46 ± 0.176	6.98 ± 0.365	6.57 ± 0.285	6.35 ± 0.269	6.48 ± 0.289	6.85 ± 0.286	6.88 ± 0.414	5.93 ± 0.360	6.39 ± 0.246	6.48 ± 0.343	6.41 ± 0.139	0.687	0.332	0.495
HOMA-IR index	1.18 <sup>a</sup> ± 0.375	0.805 <sup>a</sup> ± 0.153	1.46 <sup>ab</sup> ± 0.286	1.06 <sup>ab</sup> ± 0.196	1.46 <sup>ab</sup> ± 0.199	1.54 <sup>ab</sup> ± 0.385	3.05 <sup>c</sup> ± 0.492	2.91 <sup>c</sup> ± 0.548	2.40 <sup>c</sup> ± 0.863	1.76 <sup>c</sup> ± 0.321	2.28 <sup>b,c</sup> ± 0.258	2.37 <sup>b,c</sup> ± 0.360	9.37E-6	0.664	0.528
Cholesterol, mmol/L	1.80 <sup>b</sup> ± 0.156	2.16 <sup>ab</sup> ± 0.096	2.10 <sup>ab</sup> ± 0.064	2.31 <sup>ab</sup> ± 0.064	2.00 <sup>b</sup> ± 0.111	1.99 <sup>ab</sup> ± 0.130	2.11 <sup>ab</sup> ± 0.129	2.21 <sup>ab</sup> ± 0.107	2.27 <sup>ab</sup> ± 0.124	2.04 <sup>ab</sup> ± 0.142	1.96 <sup>b</sup> ± 0.128	1.97 <sup>b</sup> ± 0.248	0.866	0.031	0.298
HDL, mmol/L	1.04 ± 0.147	1.24 ± 0.089	1.26 ± 0.050	1.43 ± 0.059	1.29 ± 0.068	1.20 ± 0.122	1.40 ± 0.079	1.32 ± 0.039	1.30 ± 0.069	1.36 ± 0.094	1.32 ± 0.110	1.51 ± 0.127	0.459	0.475	0.286
NEFA, mmol/L	0.395 <sup>a</sup> ± 0.088	0.448 <sup>a</sup> ± 0.077	0.562 <sup>a</sup> ± 0.037	0.423 <sup>a</sup> ± 0.081	0.493 <sup>ab</sup> ± 0.056	0.650 <sup>ab</sup> ± 0.076	0.660 <sup>b</sup> ± 0.063	0.710 <sup>b</sup> ± 0.142	0.774 <sup>b</sup> ± 0.142	0.560 <sup>ab</sup> ± 0.092	0.484 <sup>ab</sup> ± 0.046	0.700 <sup>ab</sup> ± 0.107	0.013	0.277	0.410
Albumin, g/L	33.4 <sup>a</sup> ± 0.990	32.1 <sup>a</sup> ± 0.413	34.0 <sup>ab</sup> ± 0.563	33.6 <sup>ab</sup> ± 0.461	34.5 <sup>b,c</sup> ± 0.548	34.2 <sup>b,c</sup> ± 0.599	36.5 <sup>d</sup> ± 0.480	35.6 <sup>d</sup> ± 0.206	35.8 <sup>d</sup> ± 0.391	35.1 <sup>c,d</sup> ± 0.574	35.6 <sup>c,d</sup> ± 0.318	36.3 <sup>c,d</sup> ± 0.798	1.73E-7	0.598	0.331
TG, mmol/L	0.683 <sup>a</sup> ± 0.100	0.745 <sup>a</sup> ± 0.148	0.830 <sup>ab</sup> ± 0.080	0.850 <sup>ab</sup> ± 0.165	0.700 <sup>a</sup> ± 0.083	1.34 <sup>ab</sup> ± 0.357	1.08 <sup>ab</sup> ± 0.146	1.57 <sup>ab</sup> ± 0.310	1.94 <sup>a</sup> ± 0.301	0.797 <sup>ab</sup> ± 0.086	1.07 <sup>ab</sup> ± 0.170	1.63 <sup>ab</sup> ± 0.285	3.70E-4	0.028	0.150
<b>Liver</b>															
TG, mg/g	2.42 <sup>ab</sup> ± 0.331	0.979 <sup>cd</sup> ± 0.109	2.83 <sup>ab</sup> ± 0.480	1.13 <sup>cd</sup> ± 0.112	2.46 <sup>ab</sup> ± 0.264	1.01 <sup>cd</sup> ± 0.076	3.05 <sup>a</sup> ± 0.186	1.97 <sup>abc</sup> ± 0.232	1.43 <sup>b,cd</sup> ± 0.131	2.51 <sup>ab</sup> ± 0.259	2.42 <sup>ab</sup> ± 0.259	2.20 <sup>ab</sup> ± 0.366	0.041	6.56E-11	0.057

Values are means  $\pm$  SE, 3 wk. (1) *P* for ANOVA test. Means with different letters within a line are statistically different ( $P \leq 0.05$ ). CnpE ratio: carbohydrate-nonprotein-energy ratio; HOMA-IR: homeostatic model assessment of insulin resistance; NEFA: nonesterified fatty acids; PE ratio: protein-to-energy ratio; TG: triglycerides.

**Table 4.** Gene expression vs. 18S in liver (Fgf21, Igf-1, Fas and Cpt1) in response to PE and CnpE ratios

[illegible]

Values are means  $\pm$  SE, 3 wk. (1) *P* for ANOVA test. Means with different letters within a line are statistically different ( $P \leq 0.05$ ). CnpE ratio: carbohydrate-nonprotein-energy ratio; PE ratio: protein-to-energy ratio.



**Figure 5.** Gene expression vs. RPL13 in hypothalamus (*Pomc* and *Pomc/AgRP* ratio) in response to PE and CnpE ratios. A and C: protein-to-energy ratio (PE) and carbohydrate-nonprotein-energy ratios (CnpE) effects tested by ANOVA ( $P \leq 0.05$ ) and post-hoc tests (values with different letter are significantly different). B and D: correlation with PE ratio tested by Pearson correlation test.  $n$  per group = 5 or 6 rats, 3 wk.

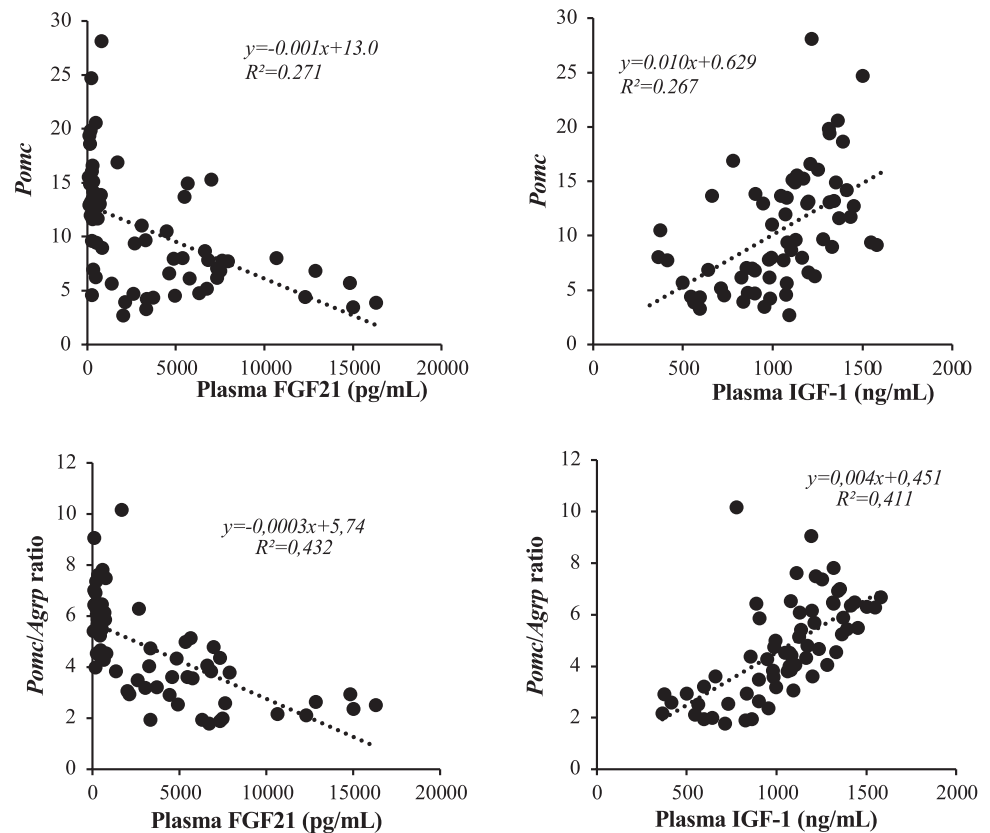
response to low-protein diets. Lundsgaard et al. (25) reported that excess carbohydrate intake induced an eightfold increase of plasma FGF21, whereas the response to low-protein diets observed in this study and others corresponded to at least a 100-fold increase of plasma FGF21. It must be noticed also that changes in plasma FGF21 in response to carbohydrate intake have always been measured in the short term, and therefore, that no study has reported long-term changes in plasma FGF21 specifically related to changes in the dietary carbohydrate content (17, 19, 24, 63–65). Therefore, it is not surprising that in conditions where both dietary protein and carbohydrate levels are modified, the long-term effects of carbohydrates do not appear significant because they are hidden by the much stronger effect of proteins. On the other hand, although it is clear that protein deprivation induces a strong increase in the appetite for protein, carbohydrate deprivation does not induce such a response (66) but at best, as described by Raubenheimer and Simpson (67), a mild attempt to eat carbohydrate when possible. Thus, there is little evidence that carbohydrate intake is necessary per se, or that carbohydrate restriction promotes a specific appetite for carbohydrate (66). In contrast to fat and carbohydrate, there is a large and growing literature that demonstrates clearly that animals selectively detect and adaptively respond to the restriction of

dietary protein and that FGF21 is strongly involved in this response (68–70).

FGF21 acts centrally to induce the expression of thermogenic genes via activation of the sympathetic nervous system, as reported by Owen et al. (14), and the FGF21 action requires corticotropin-releasing factor (CRF). They proposed that CRF stimulates sympathetic nerve activity which in turn induces *Ucp1* expression and lipolysis in IBAT. In this study, we found that IBAT weight was slightly larger in low-protein fed rats but observed no differences in *Ucp1* expression in IBAT between groups despite extremely large increases in plasma FGF21 in the low-protein fed rats. This result indicates that in this study, the increase in TEE was probably not resulting from an increased heat release in the IBAT. The differences between Owen's study and our results may be related to the species (rat vs. mouse), or to the fact that, in the Owen study, the increased expression of *Ucp1* in IBAT was observed after plasma FGF21 concentrations were increased by 2 wk of osmotic pump infusion, whereas in our study, plasma FGF21 concentrations were increased more physiologically by 2–3 wk of low-protein feeding.

Concerning IGF-1, we observed that the plasma concentrations of this hormone were inversely correlated to the plasma concentrations of FGF21 and therefore, logically increased exclusively in response to the decrease in protein





**Figure 6.** Gene expression versus RPL13 in hypothalamus (*Pomc* and *Pomc/Agrp* ratio) as a function of plasma FGF21 and IGF-1 concentrations. Analysis done by Pearson correlation test. Predictive values determined by stepwise regression and prediction quality tested by Pearson correlation test. *n* per group = 5 or 6 rats, 3 wk.

intake. Therefore, in the studies that reported that IGF-1 was reduced in food restricted rats (71–73), it is possible that the decrease in protein intake resulting from the decrease in energy intake may have been the factor primarily responsible for the decrease in plasma IGF-1 and that IGF-1 responds to the quantity and quality of the nutrient mix as a function of its capacity to promote growth.

Correlation analyses of FGF21 and IGF-1 with the PE ratios and protein intake showed that FGF21 was not much affected as long as protein intake remained above 40 kJ/day (PE ratio  $\geq$  8%) then it increased considerably. In contrast, IGF-1 concentrations increased continuously, whereas protein intake increased from 10 to 40 kJ/day (PE ratio = 3%–15%) and then leveled out from 40 to 80 kJ/day (PE ratio = 15%–30%), which fits with the global evolution of body weight, body composition, and bone growth. Therefore, if changes in plasma FGF21 and IGF-1 concentrations are correlated, it appears that the threshold at which significant changes in plasma levels are induced by protein intake are different, and higher for FGF21 than for IGF-1. Our interpretation of these results is that IGF-1 would respond to protein content by decrease significantly when protein intake was very low, while FGF21 would respond more early as soon as dietary protein content decreased below 10%–15% to induce behavioral changes, in particular the motivation to seek out and ingest protein-rich diets (63, 74, 75).

### Energy and Protein Intake Regulation in the Hypothalamus

Neurotransmitters analyzed in our study (POMC, CART, NPY, and AgRP) are important regulators of energy intake

(76, 77). Both POMC and CART are involved in the inhibition of food intake. NPY is considered one of the most potent neurotransmitters stimulating food intake, but with short-lived effects, in contrast to AgRP, which is considered the most robust chronically orexigenic molecule (78). We observed that *Cart*, *Npy*, and *Agrp* expressions were insensitive to the PE and CnpE ratios, a result that contrasted with previous reports (79), including from our group (74). In contrast, we observed that *Pomc* expression was lower in low-protein fed rats. However, it was the *Pomc/Agrp* ratio that best correlated with the dietary PE ratio. This result shows that the decrease in the dietary protein content, and by extension to protein intake, was translated into the hypothalamus by a decrease in the expression of *Pomc* and more finely in the *Pomc/Agrp* ratio. These results can be interpreted as a relaxation of signals inhibiting energy intake rather than a simulation of signals stimulating food intake. On the other hand, *Pomc* expression and the *Pomc/Cart* ratio were correlated also with the CnpE ratio, which may suggest that a subtle balance in *Pomc*, *Cart*, *Npy*, and *Agrp* expressions modulate the metabolic and behavioral responses to both protein and energy intake in the hypothalamus (70, 74).

Because FGF21 and IGF-1 cross the blood-brain barrier to activate neurons in the brainstem and hypothalamus, and to induce responses affecting energy balance and feeding behavior, we studied also the relation between plasma concentrations of FGF21 and IGF-1 with these brain neurotransmitters. It appeared that *Pomc* and *Pomc/Agrp* correlated with both FGF21 and IGF-1, but also correlated with very similar accuracy as testified by the fact that the coefficients of

determination were extremely close in all cases, which was not necessarily expected as the coefficients of determination linking FGF21 and IGF-1 were only equal to 0.39. Therefore, we can consider the hypothesis that FGF21 and IGF-1 acted to adjust the *Pomc*/*Agrp*/*Cart*/*Npy* balance in the hypothalamus in response to protein and energy intake. Consistent with these findings, some authors have shown that FGF21 administration (as a single administration in rats or by infusion for 2 wk in obese mice) increased food intake in parallel with increased expression of *Agrp* and *Npy* (80, 81), and decreased expression of *Cart* and *Pomc* (80) in the hypothalamus. In addition, one study showed that FGF21-KO mice had higher levels of *Pomc* expression and lower levels of *Agrp* expression in the hypothalamus compared with wild-type mice (82).

## Conclusions

This study used 12 different diets with various protein, carbohydrate and lipid levels, and created a dietary framework that has allowed us to analyze the effects of the dietary protein and carbohydrate contents on several parameters involved in the regulation of energy balance. Most of the organs and tissues and energy expenditure were affected only by the dietary protein content. Plasma FGF21, IGF-1, and hypothalamic *Pomc* expression and *Pomc*/*Agrp* ratios responded also primarily to the dietary protein content. This study has deciphered the specific roles of dietary protein and carbohydrate levels on the long-term regulation of plasma FGF21 concentration.

## SUPPLEMENTAL DATA

Supplemental Tables S1 and S2 and Supplemental Figs. S1: <https://doi.org/10.6084/m9.figshare.14892405>.

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## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

## AUTHOR CONTRIBUTIONS

J.G., D.A.-M., C.G., and P.C.E. conceived and designed research; J.G., D.A.-M., C.C., J.P., and P.C.E. performed experiments; J.G. and P.C.E. analyzed data; J.G. and P.C.E. interpreted results of experiments; J.G. and P.C.E. prepared figures; J.G., D.A.-M., C.G., and P.C.E. drafted manuscript; J.G. and P.C.E. edited and revised manuscript; J.G., D.A.-M., C.C., C.G., and P.C.E. approved final version of manuscript.

## REFERENCES

- Morrison CD, Laeger T. Protein-dependent regulation of feeding and metabolism. *Trends Endocrinol Metab* 26: 256–262, 2015. doi:10.1016/j.tem.2015.02.008.
- Larson KR, Chaffin AT-B, Goodson ML, Fang Y, Ryan KK. Fibroblast growth factor-21 controls dietary protein intake in male mice. *Endocrinology* 160: 1069–1080, 2019. doi:10.1210/en.2018-01056.
- Hill CM, Laeger T, Dehner M, Albarado DC, Clarke B, Wanders D, Burke SJ, Collier JJ, Qualls-Creekmore E, Solon-Biet SM, Simpson SJ, Berthoud HR, Münzberg H, Morrison CD. FGF21 signals protein status to the brain and adaptively regulates food choice and metabolism. *Cell Rep* 27: 2934–2947.e3, 2019. doi:10.1016/j.celrep.2019.05.022.
- Huang X, Hancock DP, Gosby AK, McMahon AC, Solon SMC, Le Couteur DG, Conigrave AD, Raubenheimer D, Simpson SJ. Effects of dietary protein to carbohydrate balance on energy intake, fat storage, and heat production in mice. *Obesity (Silver Spring)* 21: 85–92, 2013. doi:10.1002/oby.20007.
- Laeger T, Henagan TM, Albarado DC, Redman LM, Bray GA, Noland RC, Münzberg H, Hutson SM, Gettys TW, Schwartz MW, Morrison CD. FGF21 is an endocrine signal of protein restriction. *J Clin Invest* 124: 3913–3922, 2014. doi:10.1172/JCI74915.
- Solon-Biet SM, Mitchell SJ, Coogan SCP, Cogger VC, Gokarn R, McMahon AC, Raubenheimer D, de Cabo R, Simpson SJ, Le Couteur DG. Dietary protein to carbohydrate ratio and caloric restriction: comparing metabolic outcomes in mice. *Cell Rep* 11: 1529–1534, 2015. doi:10.1016/j.celrep.2015.05.007.
- Maida A, Zota A, Sjøberg KA, Schumacher J, Sijmonsma TP, Pfenninger A, Christensen MM, Gantert T, Fuhrmeister J, Rothermel U, Schmoll D, Heikenwälder M, Iovanna JL, Stemmer K, Kiens B, Herzig S, Rose AJ. A liver stress-endocrine nexus promotes metabolic integrity during dietary protein dilution. *J Clin Invest* 126: 3263–3278, 2016. doi:10.1172/JCI85946.
- Hill CM, Laeger T, Albarado DC, McDougal DH, Berthoud HR, Münzberg H, Morrison CD. Low protein-induced increases in FGF21 drive UCP1-dependent metabolic but not thermoregulatory endpoints. *Sci Rep* 7: 8209, 2017. doi:10.1038/s41598-017-07498-w.
- Staiger H, Keuper M, Berti L, Hrabec de Angelis M, Häring HU. Fibroblast growth factor 21—metabolic role in mice and men. *Endocr Rev* 38: 468–488, 2017. doi:10.1210/er.2017-00016.
- Hill CM, Berthoud HR, Münzberg H, Morrison CD. Homeostatic sensing of dietary protein restriction: a case for FGF21. *Front Neuroendocrinol* 51: 125–131, 2018. doi:10.1016/j.yfrne.2018.06.002.
- Chaumontet C, Azzout-Marniche D, Blais A, Piedcoq J, Tomé D, Gaudichon C, Even PC. Low-protein and methionine, high-starch diets increase energy intake and expenditure, increase FGF21, decrease IGF-1, and have little effect on adiposity in mice. *Am J Physiol Regul Integr Comp Physiol* 316: R486–R501, 2019. doi:10.1152/ajpregu.00316.2018.
- Liang Q, Zhong L, Zhang J, Wang Y, Bornstein SR, Triggie CR, Ding H, Lam KSL, Xu A. FGF21 maintains glucose homeostasis by mediating the cross talk between liver and brain during prolonged fasting. *Diabetes* 63: 4064–4075, 2014. doi:10.2337/db14-0541.
- BonDurant LD, Potthoff MJ. Fibroblast growth factor 21: a versatile regulator of metabolic homeostasis. *Annu Rev Nutr* 38: 173–196, 2018. doi:10.1146/annurev-nutr-071816-064800.
- Owen BM, Ding X, Morgan DA, Coate KC, Bookout AL, Rahmouni K, Klier SA, Mangelsdorf DJ. FGF21 acts centrally to induce sympathetic nerve activity, energy expenditure and weight loss. *Cell Metab* 20: 670–677, 2014. doi:10.1016/j.cmet.2014.07.012.
- Talukdar S, Zhou Y, Li D, Rossulek M, Dong J, Somayaji V, Weng Y, Clark R, Lanba A, Owen BM, Brenner MB, Trimmer JK, Gropp KE, Chabot JR, Erion DM, Rolph TP, Goodwin B, Calle RA. A long-acting FGF21 molecule, PF-05231023, decreases body weight and improves lipid profile in non-human primates and type 2 diabetic subjects. *Cell Metab* 23: 427–440, 2016. doi:10.1016/j.cmet.2016.02.001.
- Lan T, Morgan DA, Rahmouni K, Sonoda J, Fu X, Burgess SC, Holland WL, Klier SA, Mangelsdorf DJ. FGF19, FGF21, and an FGF19/β-klotho-activating antibody act on the nervous system to regulate body weight and glycemia. *Cell Metab* 26: 709–718.e3, 2017. doi:10.1016/j.cmet.2017.09.005.

17. **BonDurant LD, Ameka M, Naber MC, Markan KR, Idiga SO, Acevedo MR, Walsh SA, Ornitz DM, Potthoff MJ.** FGF21 regulates metabolism through adipose-dependent and -independent mechanisms. *Cell Metab* 25: 935–944.e4, 2017. doi:10.1016/j.cmet.2017.03.005.
18. **Hondares E, Iglesias R, Giral A, Gonzalez FJ, Giral M, Mampel T, Villarroya F.** Thermogenic activation induces FGF21 expression and release in brown adipose tissue. *J Biol Chem* 286: 12983–12990, 2011. doi:10.1074/jbc.M110.215889.
19. **von Holstein-Rathlou S, BonDurant LD, Peltekian L, Naber MC, Yin TC, Claflin KE, Urizar AI, Madsen AN, Ratner C, Holst B, Karstoft K, Vandenbeuch A, Anderson CB, Cassell MD, Thompson AP, Solomon TP, Rahmouni K, Kinnamon SC, Pieper AA, Gillum MP, Potthoff MJ.** FGF21 mediates endocrine control of simple sugar intake and sweet taste preference by the liver. *Cell Metab* 23: 335–343, 2016. doi:10.1016/j.cmet.2015.12.003.
20. **Sánchez J, Palou A, Picó C.** Response to carbohydrate and fat refeeding in the expression of genes involved in nutrient partitioning and metabolism: striking effects on fibroblast growth factor-21 induction. *Endocrinology* 150: 5341–5350, 2009. doi:10.1210/en.2009-0466.
21. **Chalvon-Demersay T, Even PC, Tomé D, Chaumontet C, Piedcoq J, Gaudichon C, Azzout-Marniche D.** Low-protein diet induces, whereas high-protein diet reduces hepatic FGF21 production in mice, but glucose and not amino acids up-regulate FGF21 in cultured hepatocytes. *J Nutr Biochem* 36: 60–67, 2016. doi:10.1016/j.jnutbio.2016.07.002.
22. **Iizuka K, Takeda J, Horikawa Y.** Glucose induces FGF21 mRNA expression through ChREBP activation in rat hepatocytes. *FEBS Lett* 583: 2882–2886, 2009. doi:10.1016/j.febslet.2009.07.053.
23. **Benhamed F, Denechaud PD, Lemoine M, Robichon C, Moldes M, Bertrand-Michel J, Ratzliff V, Serfaty L, Housset C, Capeau J, Girard J, Guillou H, Postic C.** The lipogenic transcription factor ChREBP dissociates hepatic steatosis from insulin resistance in mice and humans. *J Clin Invest* 122: 2176–2194, 2012. doi:10.1172/JCI41636.
24. **Dushay JR, Toschi E, Mitten EK, Fisher FM, Herman MA, Maratos-Flier E.** Fructose ingestion acutely stimulates circulating FGF21 levels in humans. *Mol Metab* 4: 51–57, 2015. doi:10.1016/j.molmet.2014.09.008.
25. **Lundsgaard AM, Fritzen AM, Sjøberg KA, Myrmet LS, Madsen L, Wojtaszewski JFP, Richter EA, Kiens B.** Circulating FGF21 in humans is potentially induced by short term overfeeding of carbohydrates. *Mol Metab* 6: 22–29, 2017. doi:10.1016/j.molmet.2016.11.001.
26. **Solon-Biet SM, Cogger VC, Pulpitel T, Heblinski M, Wahl D, McMahon AC, Warren A, Durrant-Whyte J, Walters KA, Krycer JR, Ponton F, Gokarn R, Wali JA, Ruohonen K, Conigrave AD, James DE, Raubenheimer D, Morrison CD, Le Couteur DG, Simpson SJ.** Defining the nutritional and metabolic context of FGF21 using the geometric framework. *Cell Metab* 24: 555–565, 2016. doi:10.1016/j.cmet.2016.09.001.
27. **Blais A, Chaumontet C, Azzout-Marniche D, Piedcoq J, Fromentin G, Gaudichon C, Tomé D, Even PC.** Low-protein diet-induced hyperphagia and adiposity are modulated through interactions involving thermoregulation, motor activity, and protein quality in mice. *Am J Physiol Endocrinol Metab* 314: E139–E151, 2018. doi:10.1152/ajpendo.00318.2017.
28. **Even PC, Gehring J, Tomé D.** What does self-selection of dietary proteins in rats tell us about protein requirements and body weight control? *Obes Rev* 22: e13194, 2021. doi:10.1111/obr.13194.
29. **Thissen JP, Ketelslegers JM, Underwood LE.** Nutritional regulation of the insulin-like growth factors. *Endocr Rev* 15: 80–101, 1994. doi:10.1210/edrv-15-1-80.
30. **Thissen JP, Underwood LE, Maiter D, Maes M, Clemmons DR, Ketelslegers JM.** Failure of insulin-like growth factor-I (IGF-I) infusion to promote growth in protein-restricted rats despite normalization of serum IGF-I concentrations. *Endocrinology* 128: 885–890, 1991. doi:10.1210/endo-128-2-885.
31. **Grant DB, Hambley J, Becker D, Pimstone BL.** Reduced sulphation factor in undernourished children. *Arch Dis Child* 48: 596–600, 1973. doi:10.1136/adc.48.8.596.
32. **Clemmons DR, Underwood LE, Dickerson RN, Brown RO, Hak LJ, MacPhee RD, Heizer WD.** Use of plasma somatomedin-C/insulin-like growth factor I measurements to monitor the response to nutritional repletion in malnourished patients. *Am J Clin Nutr* 41: 191–198, 1985. doi:10.1093/ajcn/41.2.191.
33. **Copinschi G, Chatelain P.** Recent developments in the study of growth factors: GRF and somatomedins. *Horm Res* 24: 81–200, 1986. doi:10.1159/000180545.
34. **Minuto F, Barreca A, Adami GF, Fortini P, Del Monte P, Cella F, Scopinaro N, Giordano G.** Insulin-like growth factor-I in human malnutrition: relationship with some body composition and nutritional parameters. *JPEN J Parenter Enteral Nutr* 13: 392–396, 1989. doi:10.1177/0148607189013004392.
35. **O'Sullivan U, Gluckman PD, Breier BH, Woodall S, Siddiqui RA, McCutcheon SN.** Insulin-like growth factor-1 (IGF-1) in mice reduces weight loss during starvation. *Endocrinology* 125: 2793–2794, 1989. doi:10.1210/endo-125-5-2793.
36. **Reeves RD, Dickinson L, Lee J, Kilgore B, Branham B, Elders MJ.** Effects of dietary composition on somatomedin activity in growing rats. *J Nutr* 109: 613–620, 1979. doi:10.1093/jn/109.4.613.
37. **Takano K, Hizuka N, Shizume K, Hasumi Y, Tsushima T.** Effect of nutrition on growth and somatomedin A levels in the rat. *Acta Endocrinol (Copenh)* 94: 321–326, 1980. doi:10.1530/acta.0.0940321.
38. **Clemmons DR, Klibanski A, Underwood LE, McArthur JW, Ridgway EC, Beitins IZ, Van Wyk JJ.** Reduction of plasma immunoreactive somatomedin C during fasting in humans. *J Clin Endocrinol Metab* 53: 1247–1250, 1981. doi:10.1210/jcem-53-6-1247.
39. **Donahue SP, Phillips LS.** Response of IGF-1 to nutritional support in malnourished hospital patients: a possible indicator of short-term changes in nutritional status. *Am J Clin Nutr* 50: 962–969, 1989. doi:10.1093/ajcn/50.5.962.
40. **Reeves PG, Nielsen FH, Fahey GC Jr.** AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* 123: 1939–1951, 1993. doi:10.1093/jn/123.11.1939.
41. **Arch JRS, Hislop D, Wang SJY, Speakman JR.** Some mathematical and technical issues in the measurement and interpretation of open-circuit indirect calorimetry in small animals. *Int J Obes (Lond)* 30: 1322–1331, 2006. doi:10.1038/sj.ijo.0803280.
42. **Even PC, Nadkarni NA.** Indirect calorimetry in laboratory mice and rats: principles, practical considerations, interpretation and perspectives. *Am J Physiol Regul Integr Comp Physiol* 303: R459–R476, 2012 [Erratum in *Am J Physiol Regul Integr Comp Physiol* 309: R1460, 2015] doi:10.1152/ajpregu.00137.2012.
43. **Azzout-Marniche D, Chaumontet C, Nadkarni NA, Piedcoq J, Fromentin G, Tomé D, Even PC.** Food intake and energy expenditure are increased in high-fat-sensitive but not in high-carbohydrate-sensitive obesity-prone rats. *Am J Physiol Regul Integr Comp Physiol* 307: R299–R309, 2014. doi:10.1152/ajpregu.00065.2014.
44. **Nadkarni NA, Chaumontet C, Azzout-Marniche D, Piedcoq J, Fromentin G, Tomé D, Even PC.** The carbohydrate sensitive rat as a model of obesity. *PLoS One* 8: e68436, 2013. doi:10.1371/journal.pone.0068436.
45. **François JC, Aid S, Chaker Z, Lacube P, Xu J, Fayad R, Côté F, Even P, Holzenberger M.** Disrupting IGF signaling in adult mice conditions leanness, resilient energy metabolism, and high growth hormone pulses. *Endocrinology* 158: 2269–2283, 2017. doi:10.1210/en.2017-00261.
46. **Elvert R, Wille A, Wandschneider J, Werner U, Glombik H, Herling AW.** Energy loss via urine and faeces - a combustive analysis in diabetic rats and the impact of antidiabetic treatment on body weight. *Diabetes Obes Metab* 15: 324–334, 2013. doi:10.1111/dom.12030.
47. **Le Bellego L, van Milgen J, Dubois S, Noblet J.** Energy utilization of low-protein diets in growing pigs. *J Anim Sci* 79: 1259–1271, 2001. doi:10.2527/2001.7951259x.
48. **Hall KD.** What is the required energy deficit per unit weight loss? *Int J Obes (Lond)* 32: 573–576, 2008. doi:10.1038/sj.ijo.0803720.
49. **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* 28: 412–419, 1985. doi:10.1007/BF00280883.
50. **Arakawa M, Masaki T, Nishimura J, Seike M, Yoshimatsu H.** The effects of branched-chain amino acid granules on the accumulation of tissue triglycerides and uncoupling proteins in diet-induced obese mice. *Endocr J* 58: 161–170, 2011. doi:10.1507/endocrj.K10E-221.



51. Chaumontet C, Azzout-Marniche D, Blais A, Chalvon-Dermersay T, Nadkarni NA, Piedcoq J, Fromentin G, Tomé D, Even PC. Rats prone to obesity under a high-carbohydrate diet have increased post-meal CCK mRNA expression and characteristics of rats fed a high-glycemic index diet. *Front Nutr* 2: 22, 2015. doi:10.3389/fnut.2015.00022.
52. Petzke KJ, Riese C, Klaus S. Short-term, increasing dietary protein and fat moderately affect energy expenditure, substrate oxidation and uncoupling protein gene expression in rats. *J Nutr Biochem* 18: 400–407, 2007. doi:10.1016/j.jnutbio.2006.07.005.
53. McNulty MA, Goupil BA, Albarado DC, Castaño-Martínez T, Ambrosi TH, Puh S, Schulz TJ, Schürmann A, Morrison CD, Laeger T. FGF21, not GCN2, influences bone morphology due to dietary protein restrictions. *Bone Rep* 12: 100241, 2020. doi:10.1016/j.bonr.2019.100241.
54. Markan KR, Naber MC, Ameka MK, Anderegg MD, Mangelsdorf DJ, Klierer SA, Mohammadi M, Potthoff MJ. Circulating FGF21 is liver derived and enhances glucose uptake during refeeding and overfeeding. *Diabetes* 63: 4057–4063, 2014. doi:10.2337/db14-0595.
55. Laeger T, Albarado DC, Burke SJ, Trosclair L, Hedgepeth JW, Berthoud HR, Gettys TW, Collier JJ, Münzberg H, Morrison CD. Metabolic responses to dietary protein restriction require an increase in FGF21 that is delayed by the absence of GCN2. *Cell Rep* 16: 707–716, 2016. doi:10.1016/j.celrep.2016.06.044.
56. Cuevas-Ramos D, Aguilar-Salinas CA. Modulation of energy balance by fibroblast growth factor 21. *Horm Mol Biol Clin Invest* 30, 2017. doi:10.1515/hmbci-2016-0023.
57. Pezeshki A, Zapata RC, Singh A, Yee NJ, Chelikani PK. Low protein diets produce divergent effects on energy balance. *Sci Rep* 6: 25145, 2016. doi:10.1038/srep25145.
58. Dulloo AG, Mensi N, Seydoux J, Girardier L. Differential effects of high-fat diets varying in fatty acid composition on the efficiency of lean and fat tissue deposition during weight recovery after low food intake. *Metabolism* 44: 273–279, 1995. doi:10.1016/0026-0495(95)90277-5.
59. Héliers JM, Diane A, Langlois A, Larue-Achagiotis C, Fromentin G, Tomé D, Mormède P, Marissal-Arvy N. Comparison of fat storage between Fischer 344 and obesity-resistant Lou/C rats fed different diets. *Obes Res* 13: 3–10, 2005. doi:10.1038/oby.2005.3.
60. Inagaki T, Lin VY, Goetz R, Mohammadi M, Mangelsdorf DJ, Klierer SA. Inhibition of growth hormone signaling by the fasting-induced hormone FGF21. *Cell Metab* 8: 77–83, 2008. doi:10.1016/j.cmet.2008.05.006.
61. Hollstein T, Heinitz S, Ando T, Rodzevik TL, Basolo A, Walter M, Chang DC, Krakoff J, Piaggi P. Metabolic responses to 24-hour fasting and mild cold exposure in overweight individuals are correlated and accompanied by changes in FGF21 concentration. *Diabetes* 69: 1382–1388, 2020. doi:10.2337/db20-0153.
62. Lee P, Brychta RJ, Linderman J, Smith S, Chen KY, Celi FS. Mild cold exposure modulates fibroblast growth factor 21 (FGF21) diurnal rhythm in humans: relationship between FGF21 levels, lipolysis, and cold-induced thermogenesis. *J Clin Endocrinol Metab* 98: E98–E102, 2013. doi:10.1210/jc.2012-3107.
63. Adams AC, Gimeno RE. The sweetest thing: regulation of macronutrient preference by FGF21. *Cell Metab* 23: 227–228, 2016. doi:10.1016/j.cmet.2016.01.013.
64. Lin Z, Gong Q, Wu C, Yu J, Lu T, Pan X, Lin S, Li X. Dynamic change of serum FGF21 levels in response to glucose challenge in human. *J Clin Endocrinol Metab* 97: E1224–E1228, 2012. doi:10.1210/jc.2012-1132.
65. Vienberg SG, Jacobsen SH, Worm D, Hvolris LE, Naver L, Almdal T, Hansen DL, Wulff BS, Clausen TR, Madsbad S, Holst JJ, Andersen B. Increased glucose-stimulated FGF21 response to oral glucose in obese nondiabetic subjects after Roux-en-Y gastric bypass. *Clin Endocrinol (Oxf)* 86: 156–159, 2017. doi:10.1111/cen.13241.
66. DiBattista D. Effects of time-restricted access to protein and to carbohydrate in adult mice and rats. *Physiol Behav* 49: 263–269, 1991. doi:10.1016/0031-9384(91)90042-m.
67. Raubenheimer D, Simpson SJ. Integrative models of nutrient balancing: application to insects and vertebrates. *Nutr Res Rev* 10: 151–179, 1997. doi:10.1079/NRR19970009.
68. Raubenheimer D, Simpson SJ. Protein leverage: theoretical foundations and ten points of clarification. *Obesity (Silver Spring)* 27: 1225–1238, 2019. doi:10.1002/oby.22531.
69. Morrison CD, Reed SD, Henagan TM. Homeostatic regulation of protein intake: in search of a mechanism. *Am J Physiol Regul Integr Comp Physiol* 302: R917–R928, 2012. doi:10.1152/ajpregu.00609.2011.
70. Berthoud H-R, Münzberg H, Richards BK, Morrison CD. Neural and metabolic regulation of macronutrient intake and selection. *Proc Nutr Soc* 71: 390–400, 2012. doi:10.1017/S0029665112000559.
71. Rahmani J, Kord Varkaneh H, Clark C, Zand H, Bawadi H, Ryan PM, Fatahi S, Zhang Y. The influence of fasting and energy restricting diets on IGF-1 levels in humans: a systematic review and meta-analysis. *Ageing Res Rev* 53: 100910, 2019. doi:10.1016/j.arr.2019.100910.
72. Speakman JR, Mitchell SE. Caloric restriction. *Mol Aspects Med* 32: 159–221, 2011. doi:10.1016/j.mam.2011.07.001.
73. Breese CR, Ingram RL, Sonntag WE. Influence of age and long-term dietary restriction on plasma insulin-like growth factor-1 (IGF-1), IGF-1 gene expression, and IGF-1 binding proteins. *J Gerontol* 46: B180–B187, 1991. doi:10.1093/geronj/46.5.b180.
74. Chaumontet C, Recio I, Fromentin G, Benoit S, Piedcoq J, Darcel N, Tomé D. The protein status of rats affects the rewarding value of meals due to their protein content. *J Nutr* 148: 989–998, 2018. doi:10.1093/jn/nxy060.
75. Talukdar S, Owen BM, Song P, Hernandez G, Zhang Y, Zhou Y, Scott WT, Paratala B, Turner T, Smith A, Bernardo B, Müller CP, Tang H, Mangelsdorf DJ, Goodwin B, Klierer SA. FGF21 regulates sweet and alcohol preference. *Cell Metab* 23: 344–349, 2016. doi:10.1016/j.cmet.2015.12.008.
76. Davidenko O, Darcel N, Fromentin G, Tomé D. Control of protein and energy intake - brain mechanisms. *Eur J Clin Nutr* 67: 455–461, 2013. doi:10.1038/ejcn.2013.73.
77. Wen S, Wang C, Gong M, Zhou L. An overview of energy and metabolic regulation. *Sci China Life Sci* 62: 771–790, 2019. doi:10.1007/s11427-018-9371-4.
78. Schwartz MW, Woods SC, Porte D Jr, Seeley RJ, Baskin DG. Central nervous system control of food intake. *Nature* 404: 661–671, 2000. doi:10.1038/35007534.
79. White BD, He B, Dean RG, Martin RJ. Low protein diets increase neuropeptide Y gene expression in the basomedial hypothalamus of rats. *J Nutr* 124: 1152–1160, 1994. doi:10.1093/jn/124.8.1152.
80. Recinella L, Leone S, Ferrante C, Chiavaroli A, Di Nisio C, Martinotti S, Vacca M, Brunetti L, Orlando G. Effects of central fibroblast growth factor 21 (FGF21) in energy balance. *J Biol Regul Homeost Agents* 31: 603–613, 2017.
81. Coskun T, Bina HA, Schneider MA, Dunbar JD, Hu CC, Chen Y, Moller DE, Kharitonov A. Fibroblast growth factor 21 corrects obesity in mice. *Endocrinology* 149: 6018–6027, 2008. doi:10.1210/en.2008-0816.
82. Badman MK, Koester A, Flier JS, Kharitonov A, Maratos-Flier E. Fibroblast growth factor 21-deficient mice demonstrate impaired adaptation to ketosis. *Endocrinology* 150: 4931–4940, 2009. doi:10.1210/en.2009-0532.
83. Lundsgaard A-M, Sjøberg KA, Høeg LD, Jeppesen J, Jordy AB, Serup AK, Fritzen AM, Pilegaard H, Myrmet LS, Madsen L, Wojtaszewski JFP, Richter EA, Kiens B. Opposite regulation of insulin sensitivity by dietary lipid versus carbohydrate excess. *Diabetes* 66: 2583–2595, 2017. doi:10.2337/db17-0046.