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ORIGINAL CONTRIBUTION

Postprandial ghrelin and PYY responses of male subjects on low carbohydrate meals to varied balancing proportions of proteins and fats

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Abstract
Purpose This study was conducted to investigate whether a higher proportion of protein or fat is more favorable for optimal ghrelin and peptide YY (PYY) release in subjects consuming low carbohydrate meals.
Methods Eight normal weight men received, on three separate occasions, high protein low fat (HPLF) (40% protein, 25% fat), low protein high fat (LPHF) (10% protein, 55% fat) or medium protein medium fat (MPMF) (25% protein, 40% fat) meals, with equal low carbohydrates content in all three meals (35% of energy). Postprandial blood samples were collected before and 15, 30, 60, 120, 180 and 240 min following the ingestion of each meal. Plasma acylated ghrelin and PYY_{3-36} as well as serum insulin, glucose and triglycerides were measured.
Results Comparing meals and considering each time point separately, a trend for a statistically significant difference in acylated ghrelin was observed between HPLF and LPHF meals and a statistically significant change of PYY from baseline was noted between HPLF and LPHF meals as compared to the MPMF meal at certain time points. When data were pooled together, a statistically significant difference in acylated ghrelin change from baseline was observed between HPLF and LPHF meals, while both HPLF and LPHF meals resulted in a significantly higher PYY_{3-36} release in comparison to MPMF meal. AUC data analysis for PYY_{3-36} revealed significantly higher values following HPLF in comparison to MPMF meal. Correlation analysis revealed a significant negative correlation between acylated ghrelin and insulin only with the HPLF meal. Postprandial glucose and triglyceride levels were not significantly different between the three meals.
Conclusions In subjects consuming low carbohydrate meals, higher concentrations of proteins to fat seem to have more favorable effects on postprandial appetite hormones.

Keywords Proteins · Fats · Peptide YY · Ghrelin · Males

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Introduction

Understanding the effects of different macronutrient proportions in a diet is essential for providing sound advice to individuals attempting to lose weight [1]. High-carbohydrate (HC) low-fat (LF) diets have been previously recommended as the macronutrient composition of choice for both health-related outcomes and weight loss responses [2]. However, the long-term compliance with this dietary pattern was found to be challenging [3], and a worsening of the metabolic profile was frequently described following HC diets [4]. Thus, a considerable public interest emerged for low-carbohydrate (LC) diets, as several prospective trials demonstrated equivalent weight loss on LC and LF diets but with significantly more favorable effects on the metabolic risk factors for cardiovascular diseases following the LC diet [1].

In LC diets, it is not clear what proportion of dietary protein to fat should accompany such diets for more favorable metabolic outcomes and weight loss. High

protein (HP) diets have been frequently promoted for their effectiveness in weight loss and for having a greater satiating effect in comparison to dietary fats [5, 6]. This effect was attributed to their thermic property [7] and/or to alterations in the plasma or central concentrations of satiety factors that can subsequently affect appetite and decrease spontaneous food intake and body weight [8].

Several gastrointestinal hormones are implicated in satiety and appetite regulation, including ghrelin and peptide YY (PYY). Ghrelin is a 28-amino-acid hormone, isolated from the enteroendocrine cells of the stomach [9], whose levels increase preprandially and decrease postprandially [10]. Ghrelin has been suggested to have a physiological role in meal initiation in humans and to stimulate hunger and food intake [9]. Both total and acylated ghrelin levels were found to be decreased in obesity, probably as a compensatory response to a sustained positive energy balance [11]. Peptide YY is a 36-amino-acid intestinal polypeptide, released mainly from the ileum, colon, and rectum [12]. It exists in two molecular forms, $PYY_{1\text{-}36}$ and $PYY_{3\text{-}36}$, with the latter being the dominant form in the fed state [13]. PYY levels decrease following 2–3 days of fasting [14], increase after meal ingestion and induce satiety when peripherally injected [15], suggesting a role for PYY in meal termination in humans. Reduced PYY concentrations were described in obese adults [15, 16] and children [17], suggesting a deficiency rather than a resistance condition.

Controversy remains regarding the dietary macronutrient composition for optimal appetite control in terms of release of the satiety hormones ghrelin and PYY. Dietary carbohydrates were found to induce greater postprandial ghrelin suppression than dietary fat or protein [18–20], whereas dietary protein had a longer-lasting suppressive effect on circulating acylated ghrelin levels than carbohydrates and fat in healthy volunteers [20, 21]. As for PYY, dietary fat was reported to be the most powerful stimulant of PYY secretion when investigating single nutrients [12, 22]. When mixed meals were used, the HP meal resulted in the greatest increase in plasma total PYY and $PYY_{3\text{-}36}$ levels as compared with HC and high-fat (HF) meals, both in obese and normal-weight male subjects [23]. However, it is still not clear whether, subjects on LC diets, would benefit more from a higher proportion of protein to fat for appetite control, as measured by a decrease in ghrelin release and/or an increase in PYY secretions.

Thus, the present study was conducted to examine the effect of three low carbohydrate isocaloric meals, with varied protein to fat ratio, on the postprandial levels of acylated ghrelin and $PYY_{3\text{-}36}$ under normal physiological conditions in normal weight male subjects. Serum concentrations of insulin, glucose and triglycerides were also measured. The purpose is to shed light on the optimal meal composition recommended for subjects following a LC diet, for better appetite hormones' responses and weight loss. It was hypothesized that the LC meal with the highest protein to fat ratio will result in a more favorable profile of appetite hormones.

Subjects and methods

Subjects

Eight normal weight adult male subjects, with a mean age of 23.9 ± 1.76 years and a mean body mass index (BMI) of 22.5 ± 0.92 kg/m^2, were recruited for the study. All subjects were ethnic Lebanese, non-smokers, had normal exercise and alcohol drinking habits (<1 drink per day) and a stable body weight during the last 3 months, as ascertained by a clinical interview prior to the study. None of the subjects had previous history of substance abuse, medical or psychological illness or gastrointestinal surgery. All subjects were fully informed about the nature and procedures of the experiment and gave their written consent form. The study was conducted according to the principles expressed in the Helsinki Declaration and was approved by the Institutional Review Board of the American University of Beirut, Lebanon.

Experimental protocol

Subjects' heights and weights were measured at fasting and after voiding, prior to the beginning of the experiment. Resting Energy Expenditure (REE) was also measured after an overnight fast using indirect calorimetry (Vmax Spectra 29 series; SensorMedics, Yorba Linda, CA, USA).

Before each experiment, subjects received a 3-day pre-experiment weight-maintenance diet, consisting of 50% carbohydrates, 20% protein and 30% fat, based on the calculated energy needs of each subject.

The study used a within-subject repeated-measure design, in which each subject served as his own control. Each of the eight subjects randomly received three test meals on three separate occasions, with a 2 week interval period. The experimental meals consisted of different protein and fat concentrations, while having the same carbohydrates content set at a concentration of 35%. On the day of the experiment, the subjects arrived at 08.00 h to the Department of Nutrition and Food Sciences after a 12 h fast. An intravenous catheter was then inserted into an antecubital vein. At 08.30 h, the subjects received either a high protein, low fat (HPLF; 40% protein, 25% fat), a low protein, high fat (LPHF; 10% protein, 55% fat) or a medium protein, medium fat (MPMF; 25% protein, 40% fat) liquid meal. The meals provided 30% of the subjects' REE

and were consumed within 15 min. The caloric content of the meals ranged from 414 to 618 kcal/meal between individuals, with a mean of 548 kcal/meal. Water was added in order to maintain a caloric density of 1 kcal/mL in all meals. The same food products were used throughout the whole experiment for the preparation of meals: Pro-Performance Soy and Whey Protein (Vanilla flavor), Ensure (Complete Balanced Formula, Vanilla flavor), sugar and sunflower oil. Table 1 details the composition of the three experimental meals.

Collection and preparation of blood samples

Blood samples were collected before meals (time 0), and then postprandially after 15, 30, 60, 120, 180, and 240 min. Around 12 mL of blood were collected, at each withdrawal, into three tubes, two tubes with ethylenediamine tetra-acetate (EDTA) as the anticoagulant substance for plasma separation and one serum separator tube with clot activator. Tubes were kept on ice and then centrifuged at 3,500 rounds per min for 10–15 min at 3 °C, for the separation of plasma and serum. One of the EDTA tubes contained 100 μL (500-kIU) of aprotinin and 10 μL of DiPeptidyl Peptidase IV (DPP-IV) inhibitor per 1 ml of blood to prevent the breakdown of PYY_{1-36} into PYY_{3-36}. To protect the ghrelin octanoyl group from being cleaved off, plasma samples for ghrelin analysis were immediately acidified with 50 μL 1 N HCl and 10 μL phenylmethylsulfonyl-fluoride (PMSF; 10 mg/ml of methanol) per 1 ml of plasma. All serum and plasma samples were then stored at −80 °C until analyses.

Table 1 Amount, percent calories and type of food consumed during HPLF, LPHF and MPMF meals

Food products	Weight of ingredients (g/100 g)		
	HPLF	LPHF	MPMF
Ensure[a]	3.6	3.6	3.6
Soy/whey protein[b]	12.7	2.6	7.7
Sugar	4.1	6.1	5.1
Sunflower oil	1.4	5.4	3.4
Water	78.2	82.3	80.2
Energy density (kcal/mL)	1	1	1
% Calories			
CHO	35	35	35
Protein	40	10	25
Fat	25	55	40

[a] Ensure: complete balanced nutrition formula (containing 54.8% CHO; 14% protein; 31.2% fat)

[b] Soy and whey protein: Pro-performance hyperproteic hypercaloric formula; CHO, carbohydrates

Biochemical analysis

Plasma Octanoylated ghrelin was measured using a commercially available radioimmunoassay kit (Linco Research, Inc. St. Charles, MO, USA). The limit of sensitivity was 7.8 pg/mL (100 μL sample size); intra- and interassay coefficients of variation were less than 6.0 and 9.0, respectively. Plasma PYY_{3-36} was measured using a commercially available radioimmunoassay kit (Linco Research, Inc. St. Charles, MO, USA). The limit of sensitivity was 20 pg/mL (100 μL sample size); intra- and interassay coefficients of variation were less than 6.4 and 7.0, respectively. Serum insulin was measured using a commercially available radioimmunoassay kit (MP Biomedicals-Orangeburg, New York, NY, USA). The lower detection limit was 2 μU/mL; intra- and interassay coefficients of variation were less than 6.0 and 7.9, respectively. Serum glucose was analyzed using the UV testing, which utilizes the hexokinase/GGP-DH essay from Intermedics. Triglycerides were determined using commercial enzymatic colorimetric tests on Vitros analyzer (Ektachem DT60 II System; Johnson & Johnson Clinical Diagnostics, Rochester, NY, USA).

Statistical analysis

The data were analyzed using SPSS for Windows, version 16.0. Data were expressed as mean ± SE, and a P value of less than 0.05 was considered statistically significant. Differences in the variables' overall changes following the three meals were analyzed by two-way ANOVA with repeated measures, followed by least significant difference (LSD) test. One-way ANOVA followed by LSD tests were also performed in order to detect changes in variables over time after meal consumption, and to identify differences in the variables' responses to the three meals at every time point. Areas under the curve (AUCs) of variables were determined using the trapezoidal method, with identical time windows (0–240 min) for each of the three meals. The AUCs were determined as areas over the basal values. One-way ANOVA followed by LSD test were used in order to compare the AUCs of the three meals for each parameter. As measured variables were reportedly known to be interrelated, the Pearson product-moment correlation test was used in order to detect possible associations among our target variables.

Results

The study subjects had a mean REE of 1,644.5 ± 69.36 kcal/day. All three meals were well tolerated by the subjects, with no complaints regarding palatability or size

of meals. None of the eight subjects experienced any particular discomfort during blood withdrawal.

Plasma acylated ghrelin

Comparing meals and pooling all data together, a statistically significant difference in acylated ghrelin change from baseline was observed between HPLF (−4.4 ± 1.99) and LPHF (3.2 ± 2.53) meals ($P = 0.027$). However, when considering each time point separately, a trend for a statistically significant difference in acylated ghrelin change from baseline was only observed at 15 min between HPLF and LPHF meals ($P = 0.064$) (Fig. 1). The AUC data did not reveal any statistically significant difference between the three HPLF, LPHF and MPMF meals ($P = 0.285$). Within meals, postprandial mean plasma acylated ghrelin levels decreased below initial values only following the HPLF meal. This decline was maintained throughout the postprandial period, reaching significant levels at 60 min ($P = 0.044$) and 120 min ($P = 0.023$), to return thereafter to significantly higher than initial values at 240 min ($P = 0.004$).

Plasma $PYY_{3\text{-}36}$

Comparing meals and pooling all data together, a significantly greater $PYY_{3\text{-}36}$ increase above baseline was observed both following the HPLF (8.7 ± 1.75) and the LPHF (7.6 ± 1.44) meals in comparison to the MPMF meal (0.7 ± 1.42) ($P = 0.001$). Similarly, considering each time point separately, the change of $PYY_{3\text{-}36}$ from baseline was higher following the HPLF and LPHF meals as compared with the MPMF meal, with a tendency for a statistical significance at 180 min ($P = 0.062$) and with a statistical significance at 240 min ($P = 0.012$) (Fig. 2). AUC data analysis for $PYY_{3\text{-}36}$ revealed significantly higher values following HPLF meal (2,396 ± 961) in comparison to MPMF (127 ± 564) meal ($P = 0.051$), and a tendency for a statistically significant difference between LPHF (2,151 ± 753) and MPMF (127 ± 564) meals ($P = 0.079$). Within meals, there was no significant effect of time on the postprandial $PYY_{3\text{-}36}$ responses, irrespective of meal composition ($P = 0.770$).

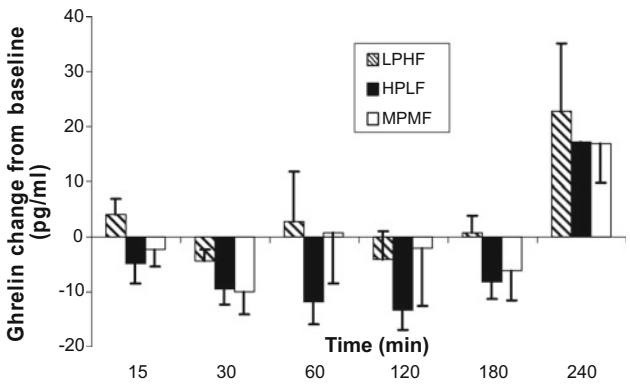

Fig. 1 Changes in plasma acylated ghrelin from baseline following HPLF, LPHF and MPMF meals in normal weight adult males

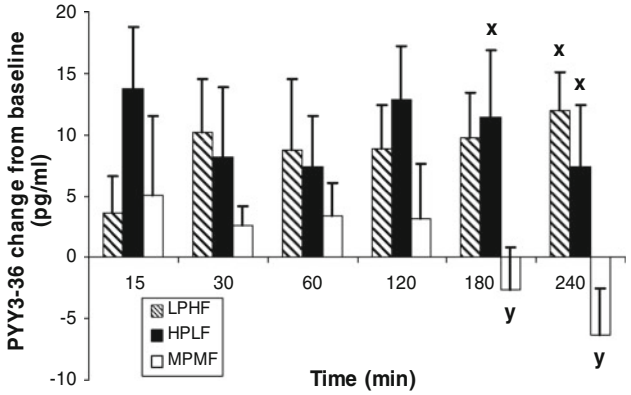

Fig. 2 Changes in plasma $PYY_{3\text{-}36}$ from baseline following HPLF, LPHF and MPMF meals in normal weight adult males. *x*, *y* Values, at the same time point, with different superscripts are significantly different at $P < 0.05$

Serum insulin

Postprandial insulin responses varied significantly as a function of time, following all three HPLF, LPHF and MPMF meals, reaching significant peaks at 30 min ($P < 0.01$ for all meals). Pooling data together, there was a trend for a significantly higher insulin increase above baseline following the HPLF (40.9 ± 5.07) meal in comparison to the MPMF (27.4 ± 4.61) meal ($P = 0.064$). When considering each time point separately, the HPLF meal was observed to induce a statistically significant greater insulin increase above baseline as compared with the LPHF meal at 15 min ($P = 0.006$), the MPMF meal at 240 min ($P = 0.045$) and both the LPHF and MPMF meals at 120 min ($P = 0.007$) with a tendency at 180 min ($P = 0.089$) (Fig. 3). Insulin AUC data analysis revealed significantly greater values following the HPLF (9,849 ± 1,456) meal in comparison to the MPMF (6,002 ± 1,282) meal ($P = 0.051$).

Serum glucose

There was a significant effect of time on the postprandial glucose responses, with significant peaks observed at 15 and 30 min, irrespective of meal composition ($P = 0.000$). When pooling all data together, there was no significant difference in glucose change from baseline between meals ($P = 0.280$). Considering each time point separately, a statistically significant difference was observed following

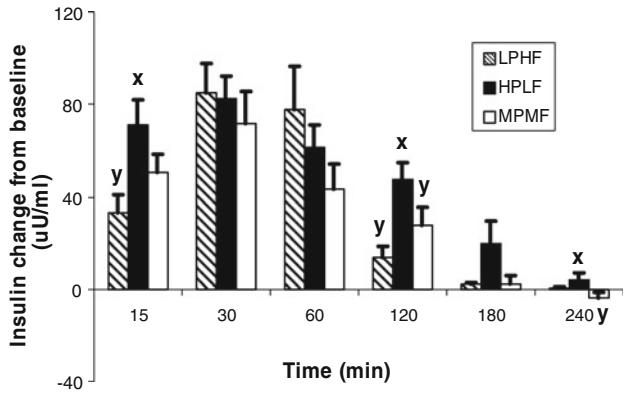

Fig. 3 Changes in serum insulin from baseline following HPLF, LPHF and MPMF meals in normal weight adult males. *x*, *y* Values, at the same time point, with different superscripts are significantly different at $P < 0.05$

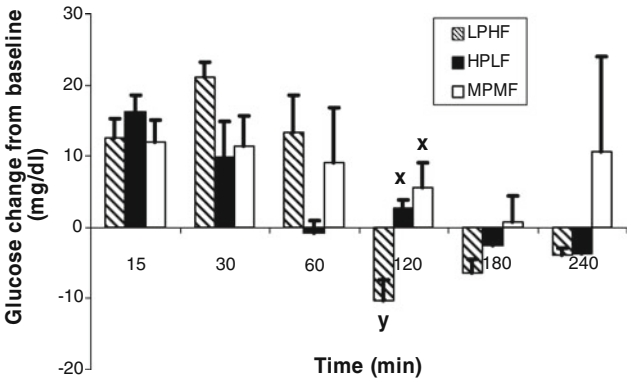

Fig. 4 Changes in serum glucose from baseline following HPLF, LPHF and MPMF meals in normal weight adult males. *x*, *y* Values, at the same time point, with different superscripts are significantly different at $P < 0.05$

HPLF and MPMF meals in comparison to the LPHF meal only at 120 min ($P = 0.001$) (Fig. 4) and a tendency for a statistically significant difference in glucose AUCs was noted between LPHF (146 ± 262) and MPMF (1,548 ± 859) meals ($P = 0.079$).

Serum triglycerides

There was no significant difference in triglyceride increase above baseline between the three meals, whether when pooling data together or considering each time point separately or comparing triglyceride AUCs ($P > 0.1$).

Correlation among variables

Following each meal separately, pooling the data at all time points, differences in the correlation analyses were noted. The HPLF meal showed significant negative correlations between acylated ghrelin and insulin ($r = -0.264$, $P = 0.049$), acylated ghrelin and triglycerides ($r = -0.360$, $P = 0.007$) and glucose and triglycerides ($r = -0.364$, $P = 0.006$). Following the MPMF meal, significant negative correlations were observed between $PYY_{3\text{-}36}$ and acylated ghrelin ($r = -0.462$, $P = 0.000$) and acylated ghrelin and triglycerides ($r = -0.415$, $P = 0.001$), while significant positive correlations were noted between $PYY_{3\text{-}36}$ and triglycerides ($r = 0.336$, $P = 0.011$) and insulin and triglycerides ($r = 0.284$, $P = 0.034$).

Discussion

The present study investigated the meal composition capable of affecting more favorably the appetite hormones ghrelin and PYY, when subjects are consuming LC meals. This is, to our knowledge, the first study to show that on short-term exposure to LC meals the circulating concentrations of postprandial ghrelin and PYY respond more favorably to a high protein to fat ratio than a high fat to protein one. We have previously reported acute changes in plasma ghrelin and PYY levels following the ingestion of carbohydrates-rich meals in humans [20, 21, 24]. However, in this study, carbohydrates were kept at a minimum concentration of 35% in order to minimize their effects on circulating levels of insulin and ghrelin.

Results of the present study showed that the meal with the highest protein content led to a statistically significant reduction in postprandial ghrelin levels below baseline, which was maintained until 120 min. Additionally, comparing the three meals, the reduction of acylated ghrelin below baseline was significantly more pronounced following the HPLF meal as compared to the LPHF meal. The effects of HP diets on weight loss have been previously reported with varied concentrations of proteins given in a single meal [25] or in a mixed diet [26, 27], both in healthy and obese subjects. However, this effect was not studied under low carbohydrates conditions. El Khoury et al. [20] and Alawar et al. [21] reported a significant and long-lasting decline in postprandial acylated ghrelin levels following the ingestion of a HP meal in healthy males and females, respectively. Similarly, Blom et al. [28] observed a maximum decrease in acylated ghrelin levels, at 120 min, following the ingestion of a HP breakfast in healthy males.

The decrease in plasma ghrelin following the HPLF meal could be attributed to the significantly higher postprandial insulin response following that meal. Previous studies have shown that proteins containing certain amino acids are insulin secretagogues [29]. The proteins used in the present study came primarily from soy and whey, rich in the branched-chain amino acids valine and isoleucine, both reported to induce a large insulin response. In support of this explanation, the present study showed a significant

negative correlation between acylated ghrelin and insulin only following the HPLF meal. Other studies have shown insulin to have a suppressive effect on plasma ghrelin levels [30–32]. This inhibitory effect was reported to take place irrespective of either plasma free fatty acid levels [30] or plasma glucose concentrations [31]. In fact, insulin and ghrelin were described to exert opposite effects on the hypothalamic neuropeptides involved in food intake and energy expenditure regulation. Peripherally produced acylated ghrelin promotes neuropeptide Y (NPY) and orexin gene expressions and inhibits Proopiomelanocortin (POMC) and alpha-melanocyte stimulating hormone (α-MSH) expressions via activation of the GHS-R [33]. On other hand, insulin increases POMC and inhibits NPY gene expressions [34]. The mechanism of the inhibitory effect of insulin on ghrelin secretion is still unclear. It was suggested that insulin could act directly on ghrelin-secreting cells and subsequently suppress ghrelin release [31].

In this study, the LPHF meal did not have a significant effect on postprandial ghrelin responses. This is in agreement with previous studies showing that HF meals induce a "muted" ghrelin response as compared with HC meals [18, 35]. Mohlig et al. [30] and Poppitt et al. [36] also found no decrease in ghrelin levels following a HF meal in healthy male subjects. Thus, in LC meals, 40% of energy as protein was able to elicit a decrease in postprandial ghrelin levels, whereas 10 and 25% of energy as protein failed to elicit such a response.

In the present study, $PYY_{3\text{-}36}$ increased significantly and to a similar extent on both HPLF and LPHF meals. These findings suggest a threshold level for dietary proteins and fats at which PYY concentrations are affected. This is substantiated by reports in the literature, which revealed that pure fat intake induces the greatest and most sustainable increase in PYY circulating levels as compared with pure proteins and glucose whether taken orally or infused intra-duodenally [12, 37]. In addition, a HF meal, providing 74% of its total calories from fat, induced a greater total PYY response in obese men and women than a HC meal [22]. Studying proteins, Batterham et al. [23] showed that HP meals (65% of calories from proteins) resulted in the greatest increase in plasma total PYY and $PYY_{3\text{-}36}$ levels as compared with HC and HF meals, both in obese and normal-weight male subjects. Similar results were reported by Helou et al. [24] on obese hyperinsulinemic female volunteers, where the HF meal (50% of caloric value from fat) promoted a greater, while the HP meal (50% of caloric value from protein) a longer-term, increment in postprandial $PYY_{3\text{-}36}$ levels. However, in a recent study on healthy volunteers, PYY acute responses to high protein meals (25% protein, 45% carbohydrates and 30% fat) were found to be similar to those following adequate protein meals (10% protein, 60% carbohydrates and 30% fat) [38]. Thus, adequate concentrations of both dietary proteins and fats are needed in order to induce a significant PYY response. The mechanisms by which protein and fat increased $PYY_{3\text{-}36}$ release were not investigated in the current study. However, cholecystokinin (CCK) could be suggested as a potential mediator, even if not measured in the current study. Previous studies have shown that dietary proteins [39] and fats [40] increase CCK release, which is a stimulant of PYY [41]. Thus, in a meal density of 1 kcal/mL, 40% of energy as protein or 55% of energy as fat was able to elicit the greatest PYY postprandial release.

In the present study, a significant inverse relationship was observed between $PYY_{3\text{-}36}$ and acylated ghrelin following the MPMF meal. This inverse association was also reported in the study of Monteleone et al. [42], who found that postprandial changes in circulating ghrelin were significantly and negatively correlated to postprandial changes in circulating PYY. It was suggested that PYY may counter regulate ghrelin release via its growth hormone secretagogue receptor (GHS-R), expressed in the vagus nerves and in the arcuate nucleus of the hypothalamus [43]. It is well-known that PYY and ghrelin negative interaction occurs at the level of vagal afferents, nodose ganglion and the hypothalamus. PYY penetrates to the hypothalamus via semi-permeable capillaries [43], and acts on vagal nerves mainly via Y2 receptors [44].

This study has the following limitations: it did not assess hunger feelings using a visual analogue scale, in parallel with the decrease in ghrelin and the increase in PYY, and did not report on the actual food intake at the next meal. Moreover, the % energy contribution of carbohydrates could be lowered further, to reach a ketotic level used by many consumers of LC diets. Another limitation is the texture of meals, being liquid rather than solid, as liquid meals are more readily emptied and absorbed than solid meals [45], which might affect the hormonal postprandial responses different from solid meals. However, the use of liquid texture enabled a strict control of the macronutrient content of the meals.

In conclusion, in weight-reducing LC regimens, HP diets, low in fat content, may have more favorable effects on appetite regulation possibly due to their suppressive effect on ghrelin levels, concomitant with their positive long-lasting PYY-releasing properties.

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Conflict of interest statement The authors declare that they have no conflict of interest.

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