Original Article
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Obesity

Increased Protein Intake and Meal Frequency Reduces Abdominal Fat During Energy Balance and Energy Deficit

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Objective: Unrefined, complex carbohydrates and lean protein diets are used to combat obesity, although it's unknown whether more frequent meals may improve this response. The effects of consuming traditional (~15%) versus higher (~35%) protein intakes as three or six meals/day on abdominal fat, postprandial thermogenesis (TEM), and cardiometabolic biomarkers in overweight individuals during 28 days of energy balance (BAL) and deficit (NEG), respectively were compared.

Design and Methods: Overweight individuals ($n = 30$) were randomized into three groups: two high-protein groups (35% of energy) consumed as three (HP3) or six (HP6) meals/day and one group consumed three meals/day of a traditional intake (TD3). Following a 5-day baseline control (CON), subjects consumed their respective diets throughout a 56-day intervention consisting of two, 28 day phases: a BAL followed by a NEG phase (75% of energy needs). Total body fat (BF) and abdominal BF (ABF), body weight (BW), TEM, and fasting biomarkers were assessed at the end of CON, BAL, and NEG phases.

Results: BW remained stable throughout CON and BAL in all groups, whereas BF ($P < 0.001$) and ABF ($P < 0.01$) decreased in HP groups and lean body mass (LBM) and leptin increased in HP6. Following NEG, BW decreased in all groups. BF, ABF, and leptin decreased in HP groups; LBM remained higher ($P < 0.05$), and TEM was highest in HP6 ($P < 0.05$).

Conclusions: Consuming increased protein (~35%) more frequently (6×) throughout the day decreases BF and ABF, increases LBM and TEM, and favorably affects adipokines more than current recommendations for macronutrients consumed over three meals/day in overweight individuals during both BAL and NEG.

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Introduction

Current US dietary guidelines recommend a carbohydrate (CHO) intake up to 65% of total kcals, moderate fat (20-35% of total kcals) and 10-35% of intake as protein (PRO) for proper weight control (1). However, recent data suggest that consuming protein at the higher acceptable range (~35%) lowers cardiometabolic disease risk (2,3,4,5) and may do so, independent of weight loss (6). Further clarification of the weight-loss-dependent versus -independent effects of diets with increased protein (>30%) or carbohydrate (>60%) intakes may have important implications for individuals attempting to improve health outcomes without undergoing caloric restriction and weight reduction.

Studies confirm a cardiometabolic protective effect of low-glycemic index (GI <50) CHO, increased PRO (~30%) diets compared to increased GI (>60) CHO diets (7,8). Recent data also show that the frequency and distribution of PRO are associated with reduced risk for metabolic syndrome (9), and the combined effects of increased dietary PRO and reduced GI diets enhance weight-loss maintenance in previously overweight adults (10). However, to date, few, if any, studies have emphasized CHO quality (low-GI) and PRO distribution (timing of meals) during energy balance (BAL). The current study was designed to systematically compare a higher PRO (~35% of kcals) diet, moderate in CHO (~40% of kcals) versus a lower PRO (~15% of kcals) diet, higher in CHO (~60% of kcals), both of which emphasized complex, low-GI (GI values of < 50) CHOs

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consumed throughout 28 days of BAL and deficit (NEG), respectively (56 days total).

Meal frequency (number of meals eaten) is another important factor underemphasized in weight-loss studies. Several studies have suggested that meal frequency is inversely related to body weight (11,12), whereas others have found no association (13) . Similarly, some (14-17), but not all (18), studies have reported an inverse relation between meal frequency and cardiometabolic risk. To our knowledge, no studies have directly compared a HP intake combined with increased meal frequency versus a traditional diet consumed as ''three meals'' per day on changes in abdominal fat and postprandial thermogenesis during BAL (weight maintenance) and NEG (weight loss). Our laboratory previously demonstrated that HP (25%, 40%) intakes more favorably affect body composition and cardiometabolic health compared with a traditional diet (PRO $<$ 20%) consumed over six meals per day (4).

The purpose of the current study was to examine the impact of macronutrient intake (PRO, 15% vs. 35%) and meal frequency (three vs. six meals/day) on body composition, postprandial thermogenesis, and plasma adipokines before and after 28 days each of BAL (28 days) and NEG (28 days) in overweight individuals. We hypothesize that higher protein intakes will elicit more favorable body composition, thermogenic, and cardiometabolic changes than higher carbohydrate intakes, and the magnitude of change will be greatest in those consuming the higher protein meals more frequently.

Materials and Methods

Participants

A total of 44 individuals were recruited from the Saratoga Springs, NY area, through newspaper advertisements and flyers and initially screened for participation, of which 36 were eligible for participation. Prior to randomization, six individuals withdrew from the study, resulting in 30 participants who started the study in the spring of 2004. Participants were nonsmoking, healthy men and women with no known cardiovascular or metabolic diseases as assessed by a medical history and a comprehensive medical examination by their physicians. All participants were inactive ($<$30 min, 2d/wk of structured physical activity), overweight or obese (BMI = 30.3 $\pm$ 5.9 kg/m^2; % body fat = 35.6 $\pm$ 6.6), middle aged (45.9 $\pm$ 9.4 years), and weight stable ($\pm$2 kg) for at least 6 months prior to beginning the study. Each participant provided informed written consent in adherence with the Skidmore College Human Subjects review board prior to participation, and the study was approved by the Human Subjects Institutional Review Board of Skidmore College. All experimental procedures were performed in accordance with the Federal Wide Assurance and related New York State regulations, which are consistent with the National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research and in agreement with the Helsinki Declaration as revised in 1983.

Experimental design

Study timeline. This study was a 62-day nutritional intervention (Figure 1) consisting of three distinct phases: a 5-day baseline control (CON); a 28-day BAL; and a 28-day NEG phase. All laboratory testing procedures (see below) were completed following the 5-day baseline CON (Day 1), BAL (Day 29), and NEG (Day 57) phases.

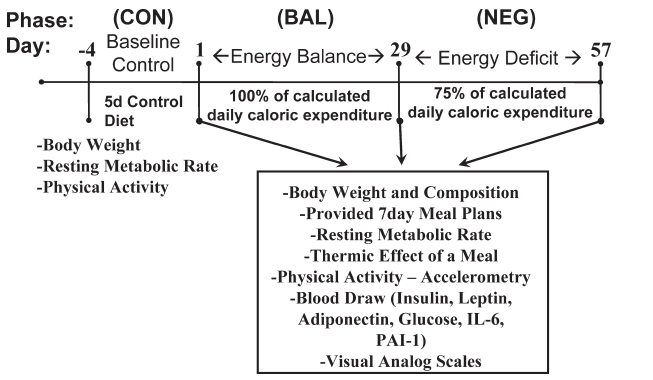

FIGURE 1 Study timeline. BAL, energy balance; NEG, energy deficit; IL-6, interleukin 6; PAI-1, plasminogen activator inhibitor-1; 5d, 5 day.

On day -4, all participants were measured for height, body weight, total and regional body composition, resting metabolic rate (RMR), and level of physical activity. During the 5-day baseline CON phase (day -4 to day 0), each participant was required to follow a standardized meal plan consisting of 25% protein, 45% carbohydrate, and 30% fat (Table 1). This macronutrient distribution was chosen because it was between the TD3 and HP levels for PRO quantity and was meant to not inappropriately bias one group over the other at the start of the BAL phase. More importantly, this 5-day CON-phase diet allowed for the antecedent diet to be well-controlled and standardized for all study participants prior to the beginning of the BAL phase. Additionally, this meal plan was designed to maintain body weight and adjusted to predicted individual energy requirements using RMR (via indirect calorimetry) plus energy expenditure due to physical activity (kcals/day) estimated from a previously validated 3-month physical activity questionnaire (19). To further verify the accuracy of our physical activity energy expenditure estimates using the 3-month questionnaire, measured RMR was also multiplied by an activity factor ranging from 1.2 to 2.0 (average activity factor for all participants of 1.30 $\pm$ 0.5; mean $\pm$ SD). In addition, all participants wore an accelerometer (Caltrac) around their waist for 2 days during the 5-day CON phase (data not shown). In both cases, the activity factor and accelerometer, the derived estimates of physical activity energy expenditure were strongly correlated ($r >$ 0.80) with the physical activity factor estimates calculated from the 3-month questionnaires. As such, all participants remained weight stable throughout the 5-day CON phase (as well as during the entire 28-day BAL phase, see *Results* section, Figure 3A). Participants were asked to maintain their current level of physical activity throughout the study protocol. Following the 5-day CON phase, participants completed, in order, two, 28-day phases: a weight-maintenance, BAL phase (consumption of 100% of estimated energy needs; RMR $\times$ physical activity energy expenditure) followed by a weight loss, NEG phase (25% less than BAL). On the first day of BAL (day 1), the start of NEG (day 29) and end of NEG (day 57) subjects arrived at 0600, fasted, and were measured for body weight, body water (bioelectrical impedance spectroscopy), and total and regional body composition (DXA). Following 20-min supine in a quiet dimly lit room, RMR was measured for 30 min followed by a fasted blood draw for plasma adipokines, insulin, glucose and completion of visual analog scales (VAS) of hunger, desire to eat, and satiety (see *Laboratory Testing Procedures* below). Subjects then consumed

TABLE 1 Dietary intake during baseline control (CON), energy balance (BAL), and energy deficit (NEG) phases

	CON (Days 0 to −4)	BAL (Days 1 to 28)	NEG (Days 29 to 56)
Kcal/day			
TD3	1878 ± 269	1941 ± 316	1456 ± 237[a,b]
HP3	2075 ± 345	2157 ± 443	1559 ± 258[a,b]
HP6	2049 ± 350	2076 ± 369	1541 ± 263[a,b]
Protein (%)			
TD3	25 ± 1	16 ± 1[a]	17 ± 1[a]
HP3	25 ± 1	35 ± 1[a,x]	34 ± 1[a,x]
HP6	26 ± 1	33 ± 1[a,x]	33 ± 2[a,x]
Protein (g)			
TD3	118.0 ± 16.7	75.4 ± 10.9[a]	61.8 ± 10.4[a,b]
HP3	129.6 ± 21.6	181.5 ± 30.1[a,x]	132.2 ± 22.0[a,b,x]
HP6	132.6 ± 22.3	169.1 ± 28.9[a,x]	127.2 ± 21.3[a,b,x]
Fat (%)			
TD3	28 ± 1	24 ± 1[a]	24 ± 1[a]
HP3	29 ± 1	22 ± 1[a]	21 ± 1[a]
HP6	28 ± 1	22 ± 1[a]	21 ± 1[a]
Fat (g)			
TD3	58.0 ± 7.9	49.8 ± 6.7[a]	38.9 ± 6.3[a,b]
HP3	66.5 ± 12.1	50.4 ± 10.1[a]	36.4 ± 6.0[a,b]
HP6	63.5 ± 10.8	50.4 ± 8.3[a]	35.8 ± 6.1[a,b]
Carbohydrate (%)			
TD3	46 ± 1	60 ± 1[a]	59 ± 1[a]
HP3	45 ± 1	44 ± 1[x]	45 ± 1[x]
HP6	46 ± 1	45 ± 1[x]	46 ± 1[x]
Carbohydrate (g)			
TD3	215.6 ± 30.9	281.9 ± 40.4[a]	214.8 ± 35.3[b]
HP3	233.3 ± 38.9	228.2 ± 37.9	175.4 ± 29.3[a,b,x]
HP6	235.7 ± 40.3	230.5 ± 39.3	176.9 ± 30.2[a,b,x]
Glycemic index			
TD3	49 ± 4	40 ± 4	48 ± 4
HP3	49 ± 3	47 ± 3	49 ± 3
HP6	41 ± 4	41 ± 5	46 ± 3

Note. Values are means ± SD; CON, 5-day (days −4 to 0) baseline control; BAL, 28-day (days 1 to 28) energy balance; NEG, 28-day (days 29–56) energy deficit. Comparison between CON, BAL, and NEG for each diet group was obtained by repeated measures ANOVA. (a) Significantly different from CON, $P < 0.05$; (b) significantly different from BAL, $P < 0.05$. (x) Tukey's post hoc tests showed significantly different than TD3, $P < 0.05$.

a test meal specific to the test phase (CON, BAL, and NEG) and their intervention group (TD3, HP3, and HP6) (see "*Resting Metabolic Rate and Thermic Effect of a Meal*" below), followed by serial measures of indirect calorimetry to assess the acute thermic effect of a meal (TEM) (min 15-30, 45-60, 75-90) (Figure 2).

BAL began on day 1 with the administration of a TEM challenge using a macronutrient composition and quantity (size) that reflected a single meal eaten during the baseline CON diet (CON: 25% PRO; 45% CHO; 30% FAT). NEG (day 29) also started with a TEM testing day using a single meal that was identical in macronutrient composition and quantity based on their respective BAL diet from the previous 28 days. The final TEM test meal occurred immediately following the 4-week NEG phase (Day 57) and was also identical to the size and composition of a single meal each participant had been eating during the NEG phase (25% less than BAL).

For each participant, allocation to an intervention group (TD3, HP3, and HP6) was performed after completion of the 5-day baseline CON phase (Figure 1 and Table 1) and occurred in two phases so that the investigators were able to provide proper control and oversight of the nutritional interventions and administration of the laboratory testing. As such, this process was a quasi-random assignment in which groups were balanced for body mass index (BMI), body weight, and percent body fat (%BF). Of the 30 participants that started the study, 28 ($n = 4$ men; $n = 24$ women) completed (two drop-outs due to unwillingness to comply with meal plans) all testing procedures. The three groups were two high-protein groups (35% PRO, 45% CHO, and 20% FAT) consumed either as six meals (HP6, $n = 10$, age = 45 ± 9 years) or three meals (HP3, $n = 10$, age = 47 ± 9 years) per day and a third group consuming three meals per day in accordance with traditional dietary intakes (15% PRO, 60% CHO, and 25% FAT) (TD3, $n = 8$, 46 ± 11 years). There were no differences in caloric intake (total kcals/day), fat intake, or GI among the three groups during CON, BAL, and NEG (Table 1). This study design allowed us to compare the independent effects of macronutrient content (higher vs. traditional protein intake) and meal frequency (three vs. six meals/day) on all outcome variables.

As previously mentioned (see *Introduction*), it is important to note that we elected not to include a traditional diet of six meals/day (CR6) group based on the previous research from our laboratory showing that a similar overweight/obese, age-matched group of 19 subjects (9 female/10 male, age 43 ± 10 years), consuming six meals per day of traditional dietary intakes (similar to our TD3 group; <20% PRO, ~55% CHO, and ~25% FAT) for 3 months, decreased body weight, total and abdominal fat significantly less than a group of 27 age-matched subjects consuming higher protein (~40%) meals six times/day, similar to HP6 in the current study (4). Thus, we felt it unnecessary to duplicate our prior finding and did not include a TD6 group for that reason. Instead, our study was designed to systematically compare HP to HC diets, both containing nutrient-dense macronutrients, during both BAL and NEG on abdominal obesity, postprandial thermogenesis, and plasma adipokines and whether increased meal frequency with HP has an additive benefit in our group of obese/overweight individuals.

Laboratory testing procedures

All laboratory procedures were conducted between 0600 and 1000 am following a 12-h fast and a 48-h restriction of physical activity, caffeine, and alcohol intake. Upon arrival to the laboratory, height and body weight (Befour, model number FS0900) were measured with participants clothed in shorts and a T-shirt.

Total body and regional body composition. Total [fat mass and lean body mass (LBM)] and regional (abdominal fat mass) body composition was determined by dual energy x-ray absorptiometry (DXA; software version 4.1, model DPX-IQ; Lunar, Madison, WI) with subjects in the supine position as previously described (4). DXA scans were performed at CON (day 1), BAL (day 29), and NEG (day 57). Total body adiposity (FM) was expressed as %BF, and LBM was expressed as kilograms (kg). Regional abdominal adiposity was determined by creating a region of interest (ROI) for the abdomen using the ROI option (with ruler option) within the manual

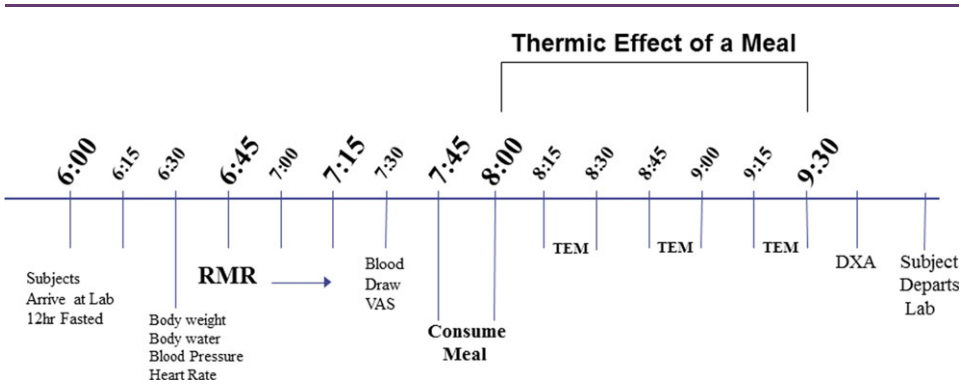

FIGURE 2 Laboratory procedures timeline. RMR, resting metabolic rate; TEM, thermic effect of meal; DXA, dual-energy x-ray absorptiometry; VAS, visual analog scales. All time is depicted during morning hours. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

analysis menu of the Lunar software. Abdominal adiposity is expressed as percent (%ab fat) and total (kg) abdominal fat as previously described (4). Test–retest intraclass correlation (r) and coefficient of variation (CV) for body composition analysis using DXA in our laboratory with $n = 12$ is LBM and FM $r = 0.99$, CV $= 0.64\%$, and $r = 0.98$, CV$= 2.2\%$, respectively, and for regional abdominal body composition analysis is %FAT $r = 0.99$, CV $= 2.4\%$.

Total body water. Total body water was estimated with participants lying supine on a nonconductive table in a thermal-neutral environment (22°C) with arms and legs slightly abducted. Participants were fasted (12 h) and refrained from alcohol, caffeine, and strenuous exercise for 24 h. All bio-impedance measurements followed standardized procedures and were obtained immediately following the 45-min RMR test using a multi-frequency bioelectrical impedance analyzer (Xitron 4000 Bioimpedance Analyzer TM, Xitron Technologies, San Diego, CA) during a logarithmic multifrequency sweep of frequencies ranging from 1 to 500 kHz. Total body water (TBW) was calculated using the equations of Deurenberg et al. (20).

Resting metabolic rate and thermic effect of a meal. Assessed during CON (day 1), BAL (day 29), and NEG (day 57), RMR (kilocalories per minute) was measured between 0600 and 0700 am for 30 min after an overnight 12-h fast using the ventilated hood technique (21) with a computerized open-circuit indirect calorimeter (Parvomedics, Truemax 2400, Salt Lake City, UT). Participants were not allowed to sleep, and all measurements were obtained in the supine position following at least 20 min of quiet resting in a thermo-neutral (22-24°C), semi-dark room. Following the RMR, a TEM meal challenge was administered and acute postprandial thermogenesis was measured every other 15 min for 90 min (TEM 15-30; 45-60; 75-90 min). Steady state was achieved for all participants during the final 12 min of each 15-min measurement period; thus, only 12 min were used in the calculation of TEM (min 1-3 were discarded). The total 90-min TEM was calculated by taking an average of each 12-min TEM measurement and multiplying it by 30 min (0-30; 31-60; 61-90 min). Each of the three 30-min TEM periods was then summed for the 90-min TEM value. A 90-min TEM was chosen to capture the acute postprandial response only and has been shown to correlate highly with 3-h TEM measurements (22). The kcals for the TEM meal challenge during CON (Day 1) was based on one-third of each individual participant's estimated total daily energy expenditure calculated as their measured RMR multiplied by an activity factor ranging from 1.2 to 2.0 based on their physical activity questionnaire in conjunction with daily energy expenditure assessed by physical activity accelerometry for 2 days (Actical) and consisted of 25% PRO, 45% CHO, and 35% FAT. The kcals for the TEM meal challenge during CON were isocaloric among the three intervention groups. The kcals for the TEM challenge on the BAL test day (Day 29) was calculated similarly, with the exception of the high-protein six meal/day group (HP6), which was one-sixth (1/6) of the total daily energy requirements instead of one-third (1/3), and the macronutrient composition followed the nutritional guidelines (see *Meal Plans* below) for each group. This study design allowed for the direct comparison of differences in macronutrient distribution and quantity of meal frequency on the thermogenic response. The TEM challenge (kcals) for the NEG test day (Day 57) was 25% less than during the CON and BAL test days (TEM for HP6 remained at 1/6 of the NEG intake). Test–retest intraclass correlation (r) and CV in $n = 14$ is RMR (Kcal/min) $r = 0.92$, 4.2%, respectively.

Cardiometabolic variables

Plasma biomarkers. Venous blood samples (~20 ml) were obtained following the RMR. Blood was collected into EDTA-coated vacutainer tubes and centrifuged (Hettich Rotina 46R5) for 15 min at 2500 rpm at 4°C. Plasma was then separated and subsequently stored at −70°C in small aliquots until analyzed. Leptin (LEP), adiponectin (ADI), tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), plasminogen activator inhibitor-1 (PAI-1), and insulin were determined using commercially available ELISA kits (Millipore and DSL). Plasma glucose concentrations were determined using a glucose analyzer using the glucose oxidase technique (GM7 Analyser, Analox Instruments, Lunenberg, MA). Additionally, insulin sensitivity was estimated with the ISI-HOMA (23), which has a strong correlation ($r = 0.69$; $P < 0.05$) with the hyperinsulinemic–euglycemic clamp technique:

$$ISI - HOMA = \frac{405}{glucose_{\text{fasting}} \times insulin_{\text{fasting}}}$$

Feelings of hunger, satiation, and desire to eat

Visual analogue scales (VAS) were used to evaluate hunger, satiation, and desire to eat scores. Each VAS was 100 mm in length and anchored at each end. Participants were instructed to place a

mark on the 100 mm line to indicate their levels of hunger, satiety, and desire to eat. For hunger, a mark at 0 mm indicated no hunger, whereas a mark at 100 mm indicated extreme hunger. For satiety, a mark at 0 mm indicated no feeling of fullness, whereas a mark at 100 mm indicated an extreme feeling of fullness. For desire to eat, a mark at 0 mm indicated no desire to eat, and a mark at 100 mm indicated extreme desire to eat. For each of the three measures (hunger, satiety, and desire to eat), the degree in which each sensation was felt was quantified by measuring how far the mark was from the 0 mm mark. For this measurement, a standard millimeter ruler was used and all scores were computed by the same investigator. VAS scales were completed on CON (day 1), BAL (day 29), and NEG (day 57) just prior to the TEM meal challenge.

Nutritional interventions and 3-day food diaries

Nutritional interventions. Each participant's diet was strictly controlled and monitored by a research team member and/or a registered dietician on a daily basis. Participants were provided with an individual meal plan specific to their nutrient needs that was based on their measured RMR and physical activity level (see *Experimental Design* above). Participants consumed their daily meals throughout each phase (CON, 5 days; BAL, 28 days; and NEG, 28 days) in specific accordance with their individual meal plans. In addition, each participant was provided a monthly allowance to purchase foods to comply with their meal plans during CON, BAL, and NEG phases. All nutrition plans for each group (TD3, HP3, and HP6) during BAL and NEG were similar in fat (20-25% total energy) and low in glycemic index (<50). It is important to highlight that the composition of all diets emphasized unprocessed, unrefined, high nutrient-density whole foods and were thus high in fruits, vegetables, unsaturated plant oils, and lean sources of protein. A 7-day menu plan was provided to all participants for the entire 62-day intervention. These meal plans included seven choices for each breakfast, lunch, and dinner (and mini-meals for HP6) that were modified for each person's specific caloric needs and designed by a registered dietician (RD) using the Food Processor SQL Edition (version 10.7.0, ESHA Research, Salem, OR, 2010) using common foods (see Supplementary information Table S1). It is important to highlight that each individual meal consumed during the 28-day BAL and NEG phases was designed to deliver the same macronutrient distribution pattern as the overall nutritional plan for each group (Table 1).

HP3 and TD3 meal plans. Participants in HP3 were provided with pre-packaged ''protein foods'' to supplement their three meals in the form of either powder shakes they mixed with water, ready-to-drink shakes or bars (Myoplex Carb Control; 150 kcals, 3.5 g fat, 5 g carbohydrate, 25 g protein; EAS, Abbott Laboratories). During BAL, HP3 subjects consumed an average of 180 g of protein/day (~60 g per meal) during the duration of the study. This amount was reduced by 25% (or 135 g of protein/day) during NEG. Participants in TD3 were provided ''healthy snacks'' in the form of ''no sugar added applesauce'' and other American Heart Association approved cereals and granola bars to supplement their meals according to traditional dietary intakes.

HP6 meal plan. The HP6 group was also provided with ''protein foods'' to supplement their three healthy snacks in the form of either powder shakes they mixed with water, ready-to-drink shakes or bars (Myoplex Carb Control; 150 kcals, 3.5 g fat, 5 g carbohydrate, 25 g protein; EAS, Abbott Laboratories). During BAL, HP6 subjects consumed an average of 172 g of protein/day that included 25 g of protein at each of three snacks and 32 g of protein at each of three meals during the duration of the study. This amount was reduced by 25% (or 129 g of protein/day) during NEG.

Meal timing. The timing of meals was an important component of the study, particularly the evening meal, which needed to be consumed by 20:00 h for the HP3 and TD3 and within 2 h of going to bed at night (21:00-22:00 h) for the HP6, as well as the morning meal that needed to be consumed within the first hour of waking in the morning for all three groups. HP6 participants were instructed to eat approximately every $2^1/_2$ −3 h during the day, for example, breakfast between 06:00 h and 08:00 h, morning mini-meal 09:30 h and 11:00 h, lunch 12:00-13:00 h, afternoon mini-meal 15:00-16:00 h, and dinner 17:00 h and 19:00 h and bedtime mini-meal 21:00 h and 22:00 h.

Subjects in the HP3 and TD3 were required to eat breakfast between 06:00 h and 09:00 h, lunch 11:00 h and 14:00 h, and dinner 17:00 h and 20:00 h. In addition, participants recorded food and drink logs every day. All three diets were low in the glycemic index (GI values <50). Verification of meal timing and dietary adherence was documented via the 3-day food diaries, described below, and through personal contact with either the PI and/or a registered dietician on a daily basis with each subject and revealed a very high compliance rate (>98%).

Dietary compliance. All participants were provided verbal and written instructions regarding the meal frequency, appropriate portion sizes, and specific foods that met their respective dietary guidelines and preferences. The PI and/or a registered dietician met weekly with study participants on an individual basis to answer questions, clarify dietary guidelines, and verify compliance with the diets. Thus, dietary compliance was reinforced and monitored through daily subject–researcher contact that involved an inspection of food logs by an investigator and distribution of specific foods as described above (see *Meal Plans* above), weekly inspection of nutrition journals, weekly return of empty supplement packets, monthly group meetings, and 3-day food diary analysis.

Three-day food diary. To further verify compliance with the nutrition plans, each subject's food intake, including meal timing, was assessed for 3 days at three different time points (CON; days −3 to −1: BAL; days 25-27: NEG; days 58-60) using the Food Processor SQL Edition (version 10.7.0, ESHA Research, Salem, OR, 2010), as previously described (24). All dietary analyses were performed by the same laboratory technician and reported in Table 1.

Statistical Analysis

Statistical analyses were performed using the SPSS software (Ver. 19; IBM-SPSS). Significance was set at $P < 0.05$. All values are reported as means ± SEM unless noted otherwise. Prior to the start of the study, subject number was determined from a power analysis based

TABLE 2 Descriptive characteristics of study participants

	Traditional diet three meals/day TD3 ($n = 8$)	High-protein three meals/day HP3 ($n = 10$)	High-protein six meals/day HP6 ($n = 10$)
Sex (M/F)	1/7	3/7	0/10
Age (years)	46 ± 11	47 ± 9	45 ± 9
Height (cm)	162.4 ± 4.7	167.4 ± 9.0	162.7 ± 6.1
Weight (kg)	83.9 ± 25.8	80.4 ± 13.4	78.7 ± 13.5
Body fat (%)	37.5 ± 5.4	34.4 ± 7.2	37.2 ± 6.6
Body mass index	31.6 ± 8.3	28.5 ± 3.1	29.9 ± 5.9
Resting heart rate (bpm)	65 ± 8	67 ± 11	66 ± 9
Diastolic blood pressure (mmHg)	78 ± 6	84 ± 6	81 ± 7
Systolic blood pressure (mmHg)	123 ± 9	122 ± 9	122 ± 8
Plasma insulin (μU/ml)	16.4 ± 5.8	9.5 ± 5.3	10.1 ± 8.7
Plasma glucose (mmol/l)	5.5 ± 0.6	5.2 ± 0.5	4.8 ± 0.5
Plasma leptin (ng/ml)	19.8 ± 3.5	14.5 ± 3.3	13.6 ± 2.4
Plasma TNFα (pg/ml)	4.4 ± 0.5	5.1 ± 1.7	5.1 ± 0.8
IL-6 (pg/ml)	29.9 ± 12.1	54.3 ± 24.6	50.0 ± 20.0
PAI-1 (ng/ml)	4.4 ± 1.9	4.7 ± 1.5	3.5 ± 0.9
Adiponectin (μg/ml)	32.3 ± 5.0	24.0 ± 3.4	28.7 ± 3.7

Note. All values are means ± SD. No significant differences existed among groups at baseline.

on our major outcome variables (postprandial thermogenesis and total and regional body composition) as reported by our previous study (5) with an alpha level set to 0.05 and a power of 0.8. This analysis determined we would require $n = 30$ participants (10 per treatment group) to detect significant differences. Absolute changes in body weight, percent total body fat, and abdominal body fat (ABF) (kg) were calculated as the baseline value (CON) subtracted from the BAL and NEG follow-up values (Figures 3A-C, respectively). A 3 × 3 factor repeated measures ANOVA (diet: TD3, HP3, HP6 and time: CON, BAL, NEG) was run to determine differences among groups and time points. Where significant main effects were identified, post hoc comparisons (Tukey's test) were performed to locate differences.

Results

Baseline characteristics and attrition

Two participants (one male and one female) in the TD3 group withdrew within 1 week of beginning the study due to unwillingness to comply with the meal plans; thus, 28 participants completed the study. Participant characteristics and selected outcome variables at baseline did not differ among groups (Table 2). All groups met the classification criteria for overweight and obesity (BMI ≥ 28.5, %bodyfat ≥ 34) (25). Men ($n = 4$) and women ($n = 24$) did not differ in their responses to any of the variables measured.

Assessment of energy intake

Dietary intakes are shown in Table 1. We observed a main effect of time for total caloric intake and percent fat intake; however, there were no differences among the groups at any time point. By design, total caloric intake declined by 25% during NEG and fat intake decreased during both BAL (% and grams) and NEG (grams) in all groups ($P < 0.05$). Macronutrient composition during BAL and NEG phases was significantly different ($P < 0.05$) among TD3 and both HP groups. Specifically, protein intake decreased in TD3 and increased in HP groups and carbohydrate (% and grams) increased significantly in TD3 compared to HP groups ($P < 0.05$).

Body weight, composition, fat distribution, and body water

Mean changes in body weight, total BF and ABF, LBM, as well as total body water during BAL and NEG interventions are shown in Table 3 and Figure 3. We observed main effects of time for body weight, %BF, total BF and ABF, and time X group effects for body weight and a trend for LBM ($P = 0.07$). Interestingly, post hoc analysis revealed that body weight declined to a greater extent in HP3 compared with TD3, ABF declined more in HP6 compared to TD3, and LBM increased in HP6 compared to the other two groups. Within all groups, body weight and body water were maintained during BAL as was intended by the design of the study. Following NEG, body weight decreased on a relative basis in all groups ($P < 0.05$) (Figure 3A). Relative changes in total BF (% and kg) and ABF (kg) remained lower in HP3 and continued to decrease in HP6 ($P < 0.05$) but remained unchanged from CON in TD3 (Figure 3B and C, respectively).

Resting metabolic rate and thermic effect of a meal

Resting metabolic rate remained unchanged in all three groups throughout the entire 62-day study (see Supplementary information Table S2). By experimental design, there was a main effect of time ($P < 0.01$) with kcals consumed during the TEM test meal (Table 4). Additionally, we observed a time × group interaction ($P < 0.01$), whereby HP6 consumed 50% less kcals during the TEM test meal

TABLE 3 Body weight and composition at baseline control (CON), energy balance (BAL), and energy deficit (NEG) test days

	CON (Day 0)	BAL (Day 29)	NEG (Day 57)
Body weight (kg)			
TD3	83.9 ± 9.1	83.8 ± 9.3	82.4 ± 9.1[a,b]
HP3	80.4 ± 4.2	79.7 ± 4.1	77.2 ± 4.1[a,b,x]
HP6	78.7 ± 4.3	78.6 ± 4.3	76.5 ± 4.5[a,b]
Body fat (%)			
TD3	37.5 ± 2.1	36.5 ± 2.2	36.4 ± 2.2
HP3	34.4 ± 2.3	32.5 ± 2.4[a]	32.4 ± 2.3[a]
HP6	37.2 ± 2.1	35.0 ± 2.4[a]	34.4 ± 2.3[a,b]
Abdominal fat (kg)			
TD3	3.5 ± 0.6	3.4 ± 0.6	3.3 ± 0.6
HP3	3.7 ± 0.3	3.4 ± 0.3[a]	3.3 ± 0.3[a]
HP6	3.7 ± 0.5	3.3 ± 0.5[a]	3.1 ± 0.5[a,b,x]
Lean body mass (kg)			
TD3	44.0 ± 2.1	43.8 ± 2.1	43.4 ± 1.9
HP3	49.8 ± 3.6	49.9 ± 3.5	48.9 ± 3.3[a]
HP6	45.7 ± 1.9	46.6 ± 1.8[a]	46.3 ± 2.0[a,x,y]
Fat mass (kg)			
TD3	28.6 ± 3.3	27.3 ± 3.2	27.0 ± 3.1
HP3	27.3 ± 2.1	25.4 ± 2.2[a]	24.8 ± 2.1[a]
HP6	29.4 ± 3.0	27.5 ± 3.2[a]	26.7 ± 3.2[a,b]
Total body water (kg)			
TD3	38.2 ± 2.4	37.3 ± 2.3	37.7 ± 2.4
HP3	38.6 ± 2.6	39.2 ± 2.9	39.1 ± 2.6
HP6	36.1 ± 1.7	36.3 ± 1.6	36.9 ± 1.9[a]

Note. Values are means ± SEM. Comparison between CON, BAL, and NEG for each diet group was obtained by repeated measures ANOVA. (a) Significantly different from CON, $P < 0.05$; (b) significantly different from BAL, $P < 0.05$. (x) Tukey's post hoc tests showed significantly different than TD3, $P < 0.05$; (y) Tukey's post hoc test showed significantly different than HP3, $P < 0.05$.

compared to TD3 and HP3 at both BAL and NEG test days. However, despite consuming ~50% less kcals during the TEM test meal, total postprandial thermogenesis (total kcals expended during 90-min TEM above the RMR) was similar for HP6 compared to TD3 and HP3 across all three test days (CON, BAL, and NEG) (Table 4 and Supplementary Figure S1). There were no main effects of time and time × group interactions for total postprandial thermogenesis (Table 4). Within-group analysis showed that HP3 significantly increased ($P < 0.05$) postprandial thermogenesis on BAL compared with CON and NEG. In contrast, postprandial thermogenesis was significantly blunted ($P < 0.05$) on NEG compared with CON and BAL test days in TD3. Expressing the data as kcals expended (postprandial thermogenesis) to kcals consumed, the magnitude of increased postprandial thermogenesis (TEM) was significantly greater (group × time interaction, $P < 0.05$) in HP6 at both BAL and NEG test days compared with TD3 and HP3 (Figure 4). This finding suggests a significantly heightened ($P < 0.01$) postprandial thermogenic response in HP6 during BAL and NEG despite consuming one-half the amount of food compared to TD3 and HP3 groups. It is important to note that TEM remained higher at the end of our 90-min measurement period, suggesting an underestimation of the total TEM response (data not shown).

Cardiometabolic biomarkers

Plasma glucose and insulin and ISI-HOMA responses showed no main effects of time and time × group interactions (data not shown). Plasma leptin and ADI showed no time or time × group interactions. Within-group analysis showed leptin increased at BAL in HP6 and decreased significantly in both HP groups following NEG compared with BAL but remained unchanged at BAL and NEG test days in TD3 (see Supplementary information Table S3). Plasma ADI increased from BAL to NEG in both HP3 and HP6 ($P < 0.05$) but remained unchanged at all test days in TD3 (see Supplementary information Table S3). Plasma TNFα, IL-6, and PAI-1 remained unchanged within and among groups for all test days (CON, BAL, and NEG) (data not shown).

Hunger, satiation (feelings of fullness), and desire to eat

Feelings of fullness and desire to eat did not differ among or within groups at any time point (data not shown). However, there was a

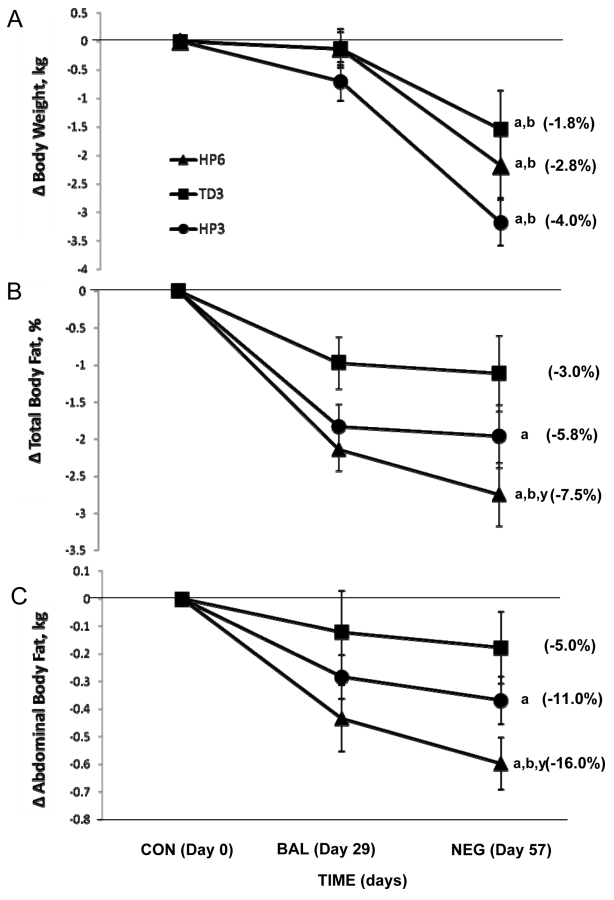

FIGURE 3 Percent change in body weight (**A**), total percent body fat (**B**), and abdominal body fat (**C**) between traditional diet eaten three times per day (TD3; filled square), high-protein diet eaten three times per day (HP3; filled circle), and high-protein diet eaten six times per day (HP6; filled triangle). CON, control diet; BAL, energy balance; NEG, energy deficit. Values are expressed as means ± SEM.(a) Significantly different from CON, $P < 0.05$; (b) significantly different from BAL, $P < 0.05$; (y) total and abdominal body fat (Figure 3**B** and **C**) were significantly lower at BAL and NEG in HP6 compared to TD3, $P < 0.05$. Values in parentheses ( ) indicate the mean delta change for each group.

TABLE 4 Kcals consumed and postprandial response during thermic effect of a meal test

	Total Kcals consumed for TEM test			Total postprandial response during TEM test (Kcals/90 min; Kcals/day)		
	CON (Day 0)	BAL (Day 29)	NEG (Day 57)	CON (Day 0)	BAL (Day 29)	NEG (Day 57)
TD3	539.0 ± 54.9	512.0 ± 47.1	387.7 ± 31.5[a,b]	18.0 ± 3.5	24.6 ± 3.2	16.4 ± 3.5[a,b]
				288.0 ± 56	393.6 ± 51.2	262.4 ± 56[a,b]
HP3	545.8 ± 32.1	537.6 ± 21.4	395.2 ± 12.2[a,b]	13.7 ± 2.7	22.1 ± 1.4[a,c]	16.7 ± 2.3
				219.2 ± 43.2	353.6 ± 22.4[a,c]	267.2 ± 36.8
HP6	535.8 ± 26.8	258.3 ± 11.5[a,y]	201.3 ± 11.2[a,b,y]	16.7 ± 4.4	21.3 ± 2.6	16.4 ± 1.8
				267.2 ± 70.4	340.8 ± 41.6	262.4 ± 28.8

Note. Values are means ± SEM. (a) Significantly different from CON, $P < 0.05$; (b) significantly different from BAL, $P < 0.05$. (c) Significantly different from NEG, $P < 0.05$; (y) significantly different from TD3 and HP3, $P < 0.05$.

time × group interaction for hunger ratings. Specifically, during NEG, hunger ratings were significantly greater ($P < 0.05$) for TD3 compared with HP groups (see Supplementary information Table S4). These findings imply that a diet higher in protein consumed as three or six meals/day during energy restriction is associated with less feelings of hunger compared with a diet higher in carbohydrate.

Discussion

Herein, we report the following novel findings—following BAL: (1) body weight remained stable in all groups; however, total and ABF decreased in HP groups (HP3 and HP6) only and LBM increased in HP6, whereas TD3 remained unchanged. Following NEG: (2) total and ABF remained lower in HP3 and HP6, and LBM remained elevated in HP6, and (3) postprandial thermogenesis (TEM) during BAL and NEG was similar between TD3 and HP3, but significantly greater (67-100%, $P<0.01$), on a relative basis, in HP6 at BAL and NEG test days compared with TD3 and HP3. The increased TEM response in HP6 may partly explain the enhanced total and ABF loss in this group. Moreover, the TEM response at BAL and NEG was still significantly elevated above RMR in all groups at the termination of the 90-min test period (Table 4 and Supplementary Figure S1). Given that previous work from our laboratory showing men and women significantly reduce ABF similarly in response to nutritional interventions (4,5), we included both genders in the current study. Interestingly, the greatest ABF loss and thermogenic response occurred in HP6, the group comprised entirely of women, which we feel strengthens the efficacy of our study design because women have been shown to be less responsive to ABF loss following weight-loss interventions compared to men (26).

By study design, body weight remained stable throughout BAL and decreased during NEG in all groups (~2 kg total or .5 kg/week). During NEG, the modest weekly weight loss was due to a 25% reduction in kcal intake (−3500 kcal deficit each week), which is considered ideal for enhancing long-term maintenance of weight loss (27), reinforces healthy nutritional intake habits (28), and verifies participant compliance. Although total and ABF were not different between HP6 and TD3 from beginning of BAL (Day 1) to end of NEG (see Table 3), both were significantly reduced on a relative basis in HP6 compared to TD3 (see Figure 3) and occurred, in the absence of significant differences in total energy intake or expenditure suggesting a "metabolic advantage" from consuming HP6 versus TD3 diets. Reductions in total and ABF and increased LBM in HP6 may be due to improved nutrient partitioning, as well as increased plasma levels of leucine (29). Layman et al. (29) propose that enhanced quality and quantity of protein (PRO) during weight loss increases plasma leucine, which in turn stimulates muscle PRO synthesis and may increase fat oxidation, both of which support our findings of reduced ABF and increased LBM in HP6 versus TD3. However, our study design does not rule out the possibility of eating frequency or an interaction of the two as playing a role in mediating these responses.

Given daily energy intake and expenditure were tightly controlled throughout the 62-day intervention and were similar among groups, differences in body composition may be due to changes in macronutrient distribution (%) of the diets and not to changes in total caloric intake or energy expenditure, as others have documented previously (10, 30). Close attention was given to ensure that all participants

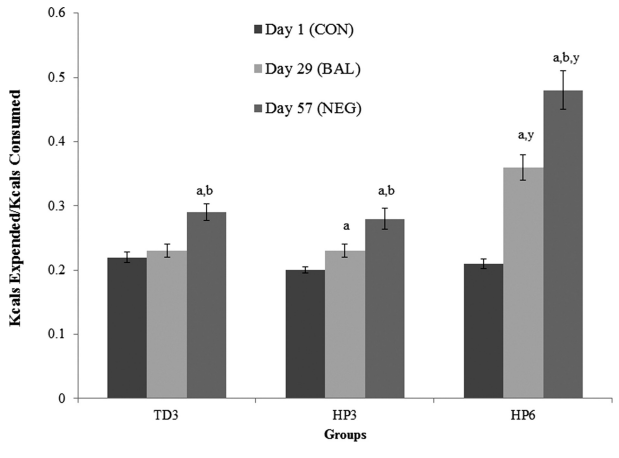

FIGURE 4 Total Kcals expended divided by Kcals consumed during TEM after the control period (CON; far left bar), energy balance (BAL; middle bar), and energy deficit (NEG; far right bar) by group. Traditional diet eaten three times per day (TD3), high-protein diet eaten 3 times per day (HP3), and high-protein diet eaten 6 times per day (HP6). Values are expressed as means ± SEM. (a) Significantly different from CON (Day 1) ($P < 0.05$); (b) significantly different from BAL (Day 29) ($P < 0.05$). (y) Kcals expended to Kcals consumed was significantly greater at BAL and NEG in HP6 compared to TD3 and HP3, $P < 0.05$.

consumed similar high-quality foods (see *Methods*) and only the ratio of macronutrients and frequency of meals consumed among the three groups differed. This novel finding highlights the benefits of HP6 in the absence of change in total macronutrient (kcal) intake on ABF loss and may have profound influence on public health policy regarding nutrient intake recommendations for adults.

The most likely explanation for enhanced body composition in HP6 was an increased TEM response during BAL and NEG compared to TD3. In fact, postprandial thermogenesis significantly decreased (5%) in TD3 during NEG. When expressed as a ratio of total kcals expended to kcals consumed, the increase in thermogenesis was more pronounced in HP6 (128%) compared to HP3 and TD3 (Figure 4). Extrapolating the 90-min TEM (TD3 and HP3@three meals/day; HP6@six meals/day in total kcals/day) to all meals eaten during the BAL and NEG period (56 days total) would result in an expenditure of 3444.0, 3259.2, and 6333.6 total kcals for TD3, HP3, and HP6, respectively, and clearly highlights the profound impact of increased dietary protein and meal frequency on thermogenesis and body composition.

Our finding corroborates others showing that an increased ratio of protein intake (7) increases energy expenditure and extends these findings by showing that, for the first time, increased protein intake and meal frequency elicit an additive thermogenic response. Interestingly, an earlier study showed the TEM increases in a nonlinear fashion and may be significantly increased by meal patterning to maximize thermogenesis and minimize energy storage (22). Thus, both responses may be contributory to the novel finding of enhanced body composition during BAL and NEG in HP6 subjects in a relatively short period that included no exercise training. Our findings highlight the importance of focusing nutritional intake recommendations on macronutrient distribution and eating frequency rather than absolute quantity of macronutrients consumed. It is important to note that RMR in HP6 was trending upward, although not significantly, which may be related to the increased LBM (0.9 kg) or explained by other factors (i.e., VO_{2max}, fat mass) known to independently contribute to changes in RMR (31).

It is interesting to speculate whether lack of favorable changes in body composition in TD3 may be related to changes in plasma adipokines. In the present study, plasma leptin decreased and adiponectin increased during NEG in HP groups but was unchanged in TD3. Our findings provide support for HP to enhance adipokines, which are associated with enhanced weight loss (32) and decreased total and abdominal body fat (33) .

Interestingly, sensation of hunger in HP groups was significantly less than TD3 at the end of NEG, suggesting that overweight/obese individuals consuming HP diets seem more responsive to hunger cues than those consuming higher CHO diets. Our finding is somewhat contradictory to a recent study (13) showing that HP consumed more frequently throughout the day has no added benefit on hunger ratings in obese men. The plausible reasons for these differences are that Leidy et al. (13) used only men and performed their laboratory measures following a 7-week energy restricted diet, which was more severe than the current study (−750 kcals/day vs. only −500 kcals/day in our study). We believe that a modest energy deficit (500 kcals/day) is more achievable and less likely to cause weight regain than a more severe restriction (750 or > kcals/day). Leidy et al.'s recommendation to consume a HP diet (25% or greater) in only three meals/day would require an excessive amount of PRO to be consumed at each meal and does not support research showing that 20-30 g of PRO at any one meal provides a maximally stimulating protein dose to elicit protein synthesis (34). Instead, we feel it is prudent to recommend consuming moderate amounts (20-30 g) of PRO more frequently during the day to optimally stimulate PRO synthesis and maximize nutrient absorption.

Major strengths of the present study included: (a) randomization of subjects to the three experimental conditions; (b) carefully monitoring the diet of all subjects during both BAL (28 days) and NEG (28 days); (c) close supervision of limiting physical activity and exercise; and (d) standardization of measurement procedures that included habituation of all subjects to the indirect calorimetry ventilated hood technique. The main limitations of our study included: (a) our relatively small sample size (especially among men, $n = 4$) and thus, larger groups are needed to confirm our findings; (b) menopausal status was not accounted for in our group of women; (c) our measure of abdominal obesity using DXA does not distinguish between subcutaneous and visceral adipose tissue stores and thus future studies are needed to determine the precise effects of macronutrient distribution and meal frequency on visceral adiposity; (d) only measuring the acute (90 min) thermic effect of feeding, which may have underestimated the total TEM response; (e) not directly including a group that consumed a traditional diet in six meals per day; (f) a reduction of dietary fat content consumed across the phases (CON, BAL, and NEG); and (g) not performing a cross-over design of BAL and NEG, realizing that it was not feasible given that our measures of metabolism and body composition would require an extended recovery and washout period.

In summary, consuming increased amounts of dietary protein (35% vs. 15%), more often (six meals vs. three meals/day) decreased abdominal fat and increased postprandial thermogenesis and LBM compared to traditional dietary intakes of protein consumed as three meals/day. These results were achieved even though total kcals consumed was identical between groups for the entire 62-day study. Our data indicate, for the first time, that macronutrient composition (increased dietary protein), nutrient quality (low glycemic index and unprocessed carbohydrates), and frequency of eating (six times per day) is more important than total energy intake for overweight/obese men and women to reduce abdominal obesity and enhance postprandial thermogenesis. O

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