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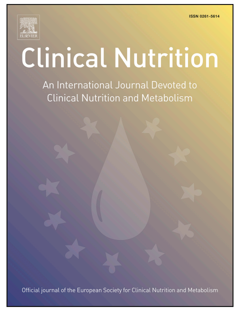

Energy-restricted, high-protein diets more effectively impact cardiometabolic profile in overweight and obese women than lower-protein diets

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Title: Energy-restricted, high-protein diets more effectively impact cardiometabolic profile in overweight and obese women than lower-protein diets

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Abbreviations

BMI ; Body mass index

HP ; High-protein

Clinical trial registration: The clinical trial has been registered in ClinicalTrials.gov (Identifier: NCT02160496).

ABSTRACT

Background & Aims: High-protein energy-restricted diets have demonstrated efficacy in promoting weight loss in overweight and obesity.However, the protein percentage that achieves optimal efficacy and acceptability remains unknown. We sought to assess the effects of three energy-reduced diets with different percentages of calories from protein (20%, 27%, and 35%) on weight loss and lipids. Secondary outcomes included diet acceptability and compliance.

Methods: Six-month, randomized study included women aged18-80 years with BMI of 27.5-45 kg/m^2 and who were not taking lipid-lowering drugs. We randomly assigned 91 women to one of three calorie-reduced diets with: protein, 20%, 27%,or 35%(80% from animal protein); carbohydrates, 50%, 43%,or 35%;fat, 30%. Dietary intervention involved individual visits with a nutritionist every 2 weeks during the first 3months. We performed a follow-up visit at 6 months.

Results: Eighty women aged 44.0±9.08 years with BMI of 37.7±3.39 kg/m^2 completed the study. At 3 months, weight loss was -8.16±4.18 kg, -9.66±5.28 kg, and -10.7±4.28 kg in the 20%, 27%, and 35%-protein groups, respectively (P=0.16). These figures slightly and homogeneously increased at 6 months. Around 65% of women following 35%-protein diet lost ≥ 10% of body weight vs. ~33% in 20%-protein group (P=0.023). Significant decreases occurred in fat mass, lipids and insulin resistance, especially in the 35%-protein group (P<0.05 vs. 20%protein). This improvement was not fully explained by weight loss. Triglyceride change was negatively correlated with animal-protein intake. All groups provided similar responses to an acceptance, palatability, and satisfaction questionnaire.

Conclusions: An energy-restricted diet with 35% protein, mostly of animal origin, more effectively impacts cardiometabolic profile than an energy-restricted diet with lower protein content although no clear benefit between diets in terms of overall weight loss was observed. The high-protein diet displayed an excellent safety profile and acceptability. This trial was registered in ClinicalTrials.gov as NCT02160496.

Keywords: diets; energy restriction; protein; lipids; weight loss.

INTRODUCTION

Lifestyle intervention, including a calorie-reduced diet and adequate physical activity, is the first line of treatment for overweight and obesity (1). However, the optimal macronutrient composition of energy-restricted diets has caused intense debate in recent years and remains an unsolved issue. In numerous clinical trials high-protein (HP) diets resulted in greater weight loss over 3-6 months (or even longer) than more conventional high-carbohydrate,low-fat diets (2-4). A recent meta-analysis of 24 high-quality intervention trials including a total of 1063 participants compared energy-restricted, isocaloric, HP (12-18% of energy), low-fat diets with standard-protein (25-35% of energy) and low-fat diets with regard to weight loss (5); HP isocaloric diets provided some additional weight-loss benefits compared to low-protein isocaloric diets. Similar results were obtained in previous meta-regression and meta-analysis investigations of this topic (2,6). Further, energy-restricted HP diets increase resting energy expenditure due to the preservation of fat-free mass, increase satiety, reduce total cholesterol and triglyceride levels, improve insulin resistance (5,7-10), and lead to better long-term maintenance of weight (7,11-13).

Recently published guidelines for the management of overweight and obesity reinforce the importance of reducing dietary-energy intake (1), but the role of diet composition was completely disregarded (1,14). Both the WHO and the Food and Nutrition Board of the Institute of Medicine (National Academy of Sciences, United States), which issues the RDA, established that the dietary reference intake of protein is 0.8 g/kg per day in adults (15,16).Guidelines from the American Association of Clinical Endocrinologists, the American College of Endocrinology, and the Obesity Society recommend a HP diet for healthy eating. Nevertheless, the protein consumption advises for weight-loss intervention ranges from 15% to 35% of the total daily calorie intake, which is quite ambiguous and it has not been accurately stated (17).

The protein content (as a percentage of total calories and as a total amount of protein per kg) required for optimal weight loss and long-term weight control with good acceptability and compliance has not yet been established. Although randomized clinical trials have tested standard-protein diets versus HP diets (12-45% of calories from protein) (12,18), to our knowledge, no clinical trial has randomized different HP diets to identify the most-effective protein content.

There is currently not much evidence regarding the effects of energy-restricted diets with high absolute amounts of protein on weight loss and maintenance. Thus, the primary efficacy endpoint was to assess the effects of three energy-restricted diets with different moderate-to-high percentages of calories from protein (20%, 27% and 35%-protein diets, mainly 20%- vs. 35%-protein diets), in combination with exercise promotion, on weight loss and lipid metabolism after 3-months intervention. We also aimed to explore the proportion of patients achieving targets of percentage change in body weight ≥ 10% at 3-month visit. Secondary endpoints included the percent change in weight from baseline to follow-up week 24, the percent change in glucose, HOMA, total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol and apo B from from baseline to month 3. Acceptability and compliance associated with each diet were also explored.

MATERIALS AND METHODS

Subjects

Women were recruited by public advertisement on a local television station and in local newspapers. Respondents were screened with a questionnaire to establish compliance with inclusion criteria. The questionnaire was administered during information sessions at the hospital where all research was conducted (Hospital Universitario Miguel Servet, Zaragoza, Spain). Of the 824 individuals who attended the information session, 603 completed the questionnaire. Body weight, height, medical history, current medications, and geographic distance to our hospital were assessed. The inclusion criteria included age 18-80 years, body mass index (BMI) 27.5-45 kg/m^2, and steady weight (±3 kg) in the previous 3 months. The exclusion criteria included uncontrolled hypothyroidism, type-2 diabetes (glycated hemoglobin > 8%), any other disease that could interfere with the ability to comply with the study protocol, and current lipid-lowering or anti-diabetic drugs. Respondents taking supplements of phytosterols, omega-3-fatty acids, or any obesity drug were also excluded.

Of the participants that met the study criteria, 91 were randomly selected for randomization to one of three diets (Figure 1). All subjects provided written informed consent to participate in the study. The study protocol was approved by the ethical committee of our institution (Comité de Ética e Investigación Clínica de Aragón);all procedures were in accordance with the ethical standards of that committee. This clinical trial was registered in ClinicalTrials.gov under identifier NCT02160496.

Ninety-one women were randomized to one of three reduced-calorie diets in April 2014. The groups did not differ in terms of clinical or biochemical characteristics ($P \geq 0.05$ for all variables among diets groups). Participants were mostly middle-aged women (43.7±9.74, 45.1±8.52 and 43.2±9.17 years in 20%, 27% and 35%-protein groups respectively) with a mean BMI of 33 kg/m^2 (33.2±3.31, 33.0±3.51 and 32.4±2.96 kg/m^2 in 20%, 27% and 35%-protein groups respectively) who were metabolically healthy, as expected given our inclusion and exclusion criteria. Baseline characteristics are included in Table 2. Eleven participants (12% of all participants; six, two, and three women from the 20%-, 27%-, and 35%-protein groups, respectively) withdrew from the study during the first 3 months (the dietary intervention phase; Figure 1) due to personal issues (N=5), change in place of residence (N=2), long distance from the hospital (N=1), and unknown reasons (N=3). Subjects who withdrew from the study did not differ from the remaining participants in terms of any clinical characteristics according to sensitivity analysis. Fifteen participants were lost to follow-up at the 6-month visit (nine, four, and two women from the 20%-, 27%-, and 35%-protein groups, respectively). The study flow chart is shown in Figure 1.

Study design

This study consisted of a 3-month weight-loss intervention phase followed by a 3-month follow-up period. A screening visit was performed to assess inclusion/exclusion criteria, and a randomization visit was scheduled for 7 days later for subjects who met the inclusion criteria. Clinical and anthropometric parameters were assessed at baseline and after 3 and 6 months. Biochemical assessments were performed at baseline and after 3 months of dietary intervention, as described below. The study had a three-arm design with subjects randomly assigned to one of three calorie-reduced diets: 20% protein, 27% protein, or 35% protein. Once all screening visits were concluded, all subject data were recorded in a data file. The first woman to be included in the study was allocated to the 20%-protein diet, the second to the 27%-protein diet, the third to the 35%-protein diet, and so on. Participants were blinded to their assigned macronutrient composition. The dietician who formulated the diets and carried out the individual consultations was aware of each participant’s group assignment, but the rest of the staff was blinded to that information.

Each participant’s caloric prescription represented a deficit of 600 kcal/day as calculated from energy intakes estimated by multiplying the activity factor (energy expenditure for various activities established by the WHO) by the resting energy expenditure calculated with the Harris-Benedict equation. In general, the prescribed energy intake was 1200-1500 kcal/day. The three diets had the following distribution of calories: protein, 20%, 27%,or 35%; carbohydrates, 50%, 43%,or 35%;fat,30% in all diets. Thus, higher protein content was achieved by reducing carbohydrate content. Approximately 80% of protein came from animal sources, mainly lean lamb meat, which was partially provided to participants to promote compliance. Diets included a wide variety of foods typical of the Mediterranean diet and participants were provided with daily menus (Supplemental Table 1). The dietician provided participants with recipes and shopping counseling to improve intervention compliance and to achieve weight-loss goals. A single dietician performed individual consultations every 2 weeks to reinforce the intervention and to motivate weight loss. After the 3-month dietary intervention, a 3-month follow-up phase was implemented during which participants were advised to follow the same regimen as during the short-term study. No individual consultations with the dietician were performed during this phase.

All participants were provided with physical-activity advice that was in accordance with their physical status. Patients were counseled to increase exercise in each monitoring visit based on the training reported in each visit to promote weight loss. Physical activity advice was quite heterogeneous due to different women fit condition (i.e.: walk one hour a day or running 30 minutes three times a week).

Dietary assessments were performed at baseline and at 3 months and 6 months after randomization. Participants were asked to complete a 3-day weighed food record before each visit to focus their dietary intervention, to monitor dietary changes, and to check compliance with the diet during the study. Total energy and nutrient intakes were calculated with EasyDiet® (Biocentury, S.L.U, Barcelona, Spain), which is based on Spanish food-composition tables (19). A brief validated exercise questionnaire was also administered at baseline and after 3 and 6 months to monitor activity changes (20). Participants completed a satisfaction questionnaire at baseline and after the 3-month intervention phase to address issues regarding hunger, satisfaction, and health.

Body weight and composition

Anthropometric measurements (body weight and waist circumference) were evaluated at three time points: at baseline (randomization visit), at 3 months (after the weight-loss phase), and at 6 months (after the follow-up phase). Body weight was measured in subjects without shoes to the nearest 0.1 kg with a calibrated scale. Height was assessed to the nearest millimeter with a wall-mounted stadiometer. BMI was calculated as weight in kilograms divided by the square of height in meters. Waist circumference was measured with anthropometric tape midway between the lowest rib and the iliac crest.

Body composition was assessed via bioelectrical impedance through the bipolar foot-to-foot technique (Tanita TBF 410 GS, Omron Corporation®, Tokyo, Japan) (21). Abdominal fat deposits were also measured via bioelectrical impedance (Tanita ViScan AB-140, Omron Corporation®) by evaluating visceral fat (22). Measurements were performed in the abdominal area with the patient in the supine position with her hands on her chest. Abdominal-fat composition was always determined at the navel, with an area 10 cm around it clear. As established by the manufacturer, abdominal visceral fat was expressed on a scale of 0 to 35. All measurements were taken in accordance with the recommended guidelines: no food or drink 3 h prior to measurements, no exhausting exercise 12 h prior to measurements, and no alcohol or caffeine consumption 24 h prior to measurements.

Clinical and laboratory parameters

Clinical parameters (medical history and physical examination) were evaluated at the screening visit, after the weight-loss phase (at 3 months), and after the follow-up period (at 6 months). Blood pressure was measured in triplicate with a validated semiautomatic oscillometer (Omron M3, Omron Corp., Hoofddorp, the Netherlands). Blood samples were drawn by venipuncture after 12 h fasting at the randomization visit and at the 3-month visit. The levels of total cholesterol, triglycerides, and HDL cholesterol were measured with standard enzymatic methods. LDL cholesterol levels were estimated with the Friedewald formula when serum triglycerides were < 400 mg/dL. The levels of non-HDL cholesterol were calculated as the levels of total cholesterol minus the levels of HDL cholesterol. We used HOMA-IR as a marker for insulin resistance (23). Blood glucose levels were measured with the glucose-oxidase method. Insulin levels were measured via radioimmunoassay. HOMA-IR was estimated as fasting serum glucose (mg/dL) × plasma insulin (μU/mL)/405. Glycated hemoglobin levels were determined via high-performance liquid chromatography.

Statistical analyses

A total sample size of 30 subjects per group was calculated to have 80% power to detect a between treatment-group difference in mean percent change in weight of 20% with a 5% 2-sided significance level and assuming a common standard deviation of 10%, and a 5% non-evaluable primary endpoint. The primary efficacy analysis was conducted in all randomized patients with an evaluable primary endpoint at the 3 months visit. All subjects who completed the study were included in the data analysis, independent of reported dietary compliance, as indicated by food records, or weight loss according to intention-to-treat analysis. Continuous variables are expressed as mean±SD when normally distributed or as median [25thpercentile-75thpercentile] otherwise. Categorical variables are reported as percentages. ANOVA and Kruskal-Wallis tests were performed for the comparison of multiple independent variables. Weight loss variation after 3 months by comparing 20% and 35%-protein diets was performed thought *t*-test.

When applicable, *post hoc* adjusted comparisons were performed with the Bonferroni correction. Categorical variables were compared using the chi-squared test by including inter-group comparison. Pearson's and Spearman's tests of correlation were applied as appropriate. Differences in paired clinical and biochemical variables were calculated with the dependent t-test for paired samples or with the Wilcoxon test. Approximately 15% of patients had missing weight values at the 6 months visit. Multiple imputation with 5 imputations was used, achieving 95% to 99% relative efficiency and ensuring in-range values. Repeated measures analysis of ANOVA or Friedman were used to assess the differences in dietary parameters among baseline, 3 months after weight loss intervention and 6 months follow-up visits by also including inter-groups comparison. To identify variables associated with changes in lipid and glucose metabolism after dietary intervention, we applied multiple linear regression with weight loss, dietary parameters, and physical activity as independent variables. We explored those variables associated to ≥ 10% of weight loss after 3-months of dietary intervention thought binary logistic regression by including baseline weight, age, physical activity and type of diet. All statistical analyses were performed with SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) and significance was set at $P < 0.05$.

RESULTS

Dietary intake

Dietary assessments at baseline and at 3 months are presented in Table 1. There were no significant between-group differences at baseline. Energy restriction at the 3-month visit was approximately 650 kcal, as calculated from energy expenditure, and was homogeneous across the groups (P=0.68); participants achieved a mean energy intake of approximately1200 kcal/day. Protein consumption reported at the 3-month visit was

23.3±3.21%, 27.4±3.04%, and 31±4.94% of total energy intake for participants assigned to the 20%-, 27%-, and 35%-protein diets, respectively (*P*<0.001). Consumption was thus very close to the goal for each group. Absolute protein consumption at the beginning of the study did not differ among groups, but significant differences occurred at the end of the study (68.9±10.1 g/day, 83.0±11.6 g/day, and 95.8±16.9 g/day in the 20%-, 27%-, and 35%-protein groups, respectively; *P*<0.0001). These differences were exclusively due to significant increases in the amounts of animal-source protein consumed by the 27%- and 35%-protein groups; there were no differences in the amount of protein of vegetable origin consumed (Table 1).

Fat consumption (monounsaturated, polyunsaturated, and saturated fat) decreased homogeneously across the three groups. Carbohydrate intake increased in the 20%- and 27%-protein groups (7.46±10.2% vs. baseline, *P*=0.002 and 4.82±8.30% vs. baseline, *P*=0.006, respectively). However, carbohydrate consumption slightly decreased with respect to baseline in the 35%-protein group to 33.6±4.43% of total energy. As expected, changes were heterogeneous among groups mainly due to different protein consumption that was achieved by reducing carbohydrates (*P* = 0.001 among three diets). Alcohol consumption was very low (median nearly 0 g per day) at the beginning of the study in all groups (*P*=0.77); this consumption slightly decreased even further in all groups at 3 months (*P*=0.75).

Dietary assessment at 6-months follow-up visit is exposed in Supplemental Table 2. Diet was quite similar to that reported after 3 months of weight loss intervention in the three groups.

Weight and body composition

Three months of dietary intervention led to weight reductions of-8.16±4.18%, -9.66±5.28%, and -10.7±4.28% in the 20%-, 27%-, and 35%-protein diet groups, respectively (Table 2). Although weight loss tended to increase with higher protein consumption, no statistically significant differences in inter-groups analysis were detected among groups. Nevertheless, participants in the 35%-protein group achieved the greatest weight loss. The goal of 10% weight loss was met by 33.3%, 41.5%, and 65.4% of participants in the 20%-, 27%-, and 35%-protein groups, respectively (P= 0.023 by comparing 20%-protein vs. 35%-protein diets) (Figure 2). *Post hoc* analysis showed statistically significances between 20 and 35%-protein diet weight loss variation after 3 months (P = 0.041). Type of diet showed significantly influence on ≥ 10% weight loss target achievement adjusting by baseline weight and physical activity by determining a 27.9% of variance (Table 3). Participants homogeneously lost approximately 1% more weight at the 6-month follow-up visit than at the 3-month visit, without significant differences between the two study phases or among groups (P = 0.374 comparing three time-points among groups) (Supplemental Table 3 and Figure 3).

The most total fat mass and visceral fat was lost by the 35%-protein group; total fat-mass loss in this group significantly differed from loss in the other two groups (P<0.0001 among groups) (Table 2). Participants in all diet groups also experienced a slight loss of fat-free mass (P≥0.05 among groups). Fat mass and visceral fat mass change strongly and positively correlated with weight loss (R=0.68, P=0.009 and R=0.56, P<0.0001, respectively) in all diet groups.

The levels of physical activity level did not significantly differ at baseline among groups (P=0.91). These levels increased by 108±240%, 148±170%, and 146±141% in the 20%-, 27%-, and 35%-protein groups, respectively, at 3 months. Participants reported decreased physical activity at the 6-month visit versus the 3-month assessment:-91.5% [-100%-(-58.0%)], -67.4% [-100%-(-42.4%)], and -60.6% [-77.6%-(27.4%)] in the 20%-, 27%-, and 35%-protein groups, respectively (P=0.034 among the three groups and *P*=0.011 for 20% protein vs. 35% protein). Weight variation was negatively correlated with physical-activity change (R=-0.39, P=0.0001) in all groups by involving higher weight loss with higher physical activity with respect to baseline.

Blood pressure

Systolic blood pressure diminished at 3 months visit versus baseline in the 27%- and 35%-protein groups (*P*=0.008 and *P*=0.021, respectively); no significant differences occurred in the 20%-protein group (*P*=0.30) (Table 2). However, systolic blood-pressure changes at 3 months and at 6 months did not significantly differ among groups ($P \geq 0.05$ among groups) (Tables 2 and 3). Diastolic blood-pressure changes were homogeneous among groups, with no significant differences at 3 months or at 6 months.

Lipids

Changes in lipid profile between baseline and 3 months markedly differed among groups. The 20%-protein group experienced no significant changes relative to baseline ($P \geq 0.05$). The 27%-protein diet was associated with a mild reduction in the levels of total cholesterol, triglycerides, LDL cholesterol, and non-HDL cholesterol at 3 months ($P \geq 0.05$ for all lipid parameters with respect to baseline), while the 35%-protein group displayed significant reductions in the levels of cholesterol, triglycerides, LDL cholesterol, and non-HDL cholesterol at 3 months ($P < 0.0001$ for all lipid parameters except LDL cholesterol with respect to baseline; Table 2). The levels of total cholesterol, triglycerides, and non-HDL cholesterol were significantly lower in the 35%-protein group than in the 20%-protein group (*P* = 0.013, *P* = 0.016 and *P* = 0.044 respectively). Change in triglyceride levels was negatively correlated with consumption of animal protein (R=-0.24, *P*=0.036) by involving a greater triglycerides concentration reduction with a higher animal protein consumption. Linear regression indicated that animal-protein intake was associated with change in triglyceride levels across all groups regardless of weight loss and changes in physical activity (B=-0.67; 95% CI: -1.25, -0.10; *P*=0.020), determining 5.6% of variance. An inverse association between vegetal-protein intake and decrease in triglyceride levels was also detected(R=0.23, *P*=0. 047).

Glucose metabolism and other biochemical parameters

Glucose metabolism changed over the course of the study, especially in the 35%-protein group. Blood glucose levels were significantly lower at 3 months than at baseline only in participants following the 35%-protein diet (*P*=0.035; Table 2). Although a clear trend in blood glucose levels was observed, these levels did not significantly differ among diets (Table 2). HOMA-IR index decreased significantly with respect to baseline only in the 27%- and 35%-protein groups (*P*=0.010 and *P*=0.001, respectively). We uncovered a weak and non-significant correlation between glucose levels and weight loss when all women were included in the analysis (R=0.21, *P*=0.059). Linear regression demonstrated that changes in HOMA-IR were associated with allocated diet independent of weight loss and changes in physical activity at 3months (B=-33.7; 95% CI: -62.6, -4.72; *P*=0.023), determining 4.7% of variance.

Satisfaction questionnaire and adverse events during the study

All groups provided similar responses to all questions on the questionnaire administered at the 3-month visit (Supplemental Table 4). Very high scores revealed general satisfaction with diet, health status, and willingness to comply with study directions. Intention to withdraw from the study scored nearly zero for the 80 patients in all diets that completed the study. There were no differences in the incidence of adverse events among groups (Supplemental Table 5).

DISCUSSION

The main findings of this randomized, single-blind study are that a HP diet with >30% of calories from protein (mainly of animal origin): 1) produced no clear benefit between diets in terms of overall weight loss, although ~ 65% of participants achieving ≥ 10% weight reduction after 3 months of intervention (P = 0.023); 2) had excellent acceptability and compliance by participants; 3) induced marked improvements in the levels of atherogenic lipoproteins and insulin resistance during the study; and 4) led to higher fat mass loss and associated improvements in lipid and glucose metabolism that were higher than those obtained with a 20%-protein diet; 5) obtained clinical benefits by associating physical activity promotion which was homogeneous across diet groups. This could have an essential role in the cardiometabolic profile improvement observed. Weight loss slightly improved during the 3 months of follow-up without further assistance from a nutritionist; this effect was apparent across the three diets groups. Dietary changes achieved after 3 months of weight loss intervention were maintained at 6-months follow up visit. Other studies reported larger weight reductions with diets up to 30-35% in protein content by comparing with results from standard amounts of protein (5,6). However, the current investigation is the first comparison of the effects of three moderate-HP diets, enabling us to identify the most effective and well-tolerated diet and to demonstrate that a calorie-restricted diet with 30-35% of total calories from protein may be preferable to other moderate -HP diets with lower protein content.

While 35% protein consumption was prescribed to the members of one group, they reported a consumption of 31.0±4.94% (95.8±16.9 g/day), which implies an intake of approximately 1.25 g of protein/kg/day. This mismatch is common in dietary-intervention studies, and most pertinent studies have noted this issue (5); a diet with >31-32% protein may therefore be quite difficult to achieve. Thus, a diet with >30-32%

protein content would be the most effective recommendation for energy-restricted diets, even strongly restricted diets such as those including 1200 kcal/day.

The key mechanisms underlying protein-induced weight loss are not fully understood. Decreases in caloric intake due to increased satiety despite energy-intake restriction have been described with HP diets; these decreases were attributed to reduced secretion of gastric ghrelin and increased release of intestinal satiety hormones such as GLP-1 and peptide tyrosine tyrosine (2,23). Here, we did not detect any differences in satiety or caloric intake among diet groups. Participants reported very low amounts of hunger on the questionnaire despite a reported mean consumption of ~1200 kcal/day, which probably indicates a threshold effect that can be obtained even with moderate-protein diets.

Physical activity is known to enhance the effects of energy-restricted diets. Layman et al. demonstrated that subjects following a diet of 1.6 g/kg/day of protein plus exercise training lost more weight that those consuming 0.8 g/kg/day plus exercise training (-8 kg vs. -6 kg, respectively) (24). In our study, women only received a general exercise increase counseling so physical activity change was quite heterogeneous. We observed a good correlation between physical activity and weight loss; higher levels of physical activity were achieved by members of the 27%- and 35%-protein groups versus members of the 20%-protein although there were not significant differences among diets. Despite this enhanced weight loss, HP consumption in an energy-restricted diet seems to be an independent determinant of weight-loss success.

The effects of various sources of protein in HP energy-restricted diets on weight loss have not been exhaustively explored. Observational studies usually report that higher intakes of animal protein, especially red and processed meat, are associated with weight gain, mainly in women, as well as elevated risks of coronary heart disease and diabetes (25,26). However, higher intakes of animal protein from other origins, such as milk or fish, are associated with significantly lower risks of coronary heart disease and obesity, suggesting that saturated fat and dietary patterns associated with red and processed meat, rather than animal protein itself, may underlie such deleterious effects (24,27).

Protein consumption has been said to favor the excretion of ketone bodies and satiety, an effect that would be strongly influenced by the amino-acid composition of protein (18,28). Leucine and lysine are the only two amino acids that are ketogenic, and lean meat contains large amounts of them. Further, protein quality is essential to diet-induced thermogenesis. Animal protein induces more protein oxidation than vegetable protein by increasing energy expenditure (18,29). Protein from lean lamb is of higher quality than protein from pork meat, and therefore an increase in energy expenditure at the expense of diet-induced thermogenesis may be an important mechanism of weight loss-enhance ability of high protein diets with a high amount of animal sources as observed in our study. This issue requires further investigation.

Our results confirm substantial improvements in fasting triglyceride levels, blood glucose levels, and insulin resistance with increasing amounts of protein in the diet that were not fully explained by weight loss and physical activity. These findings are supported by a recent meta-analysis of randomized controlled trials in overweight and obese adults (30). The pooled meta-analysis of 24 studies (1623 participants) identified a statistically significant decrease in triglyceride levels (standardized mean difference-0.51, P=0.002) and a non-significant reduction in LDL cholesterol levels (30). Analysis of 10 studies that included 718 participants uncovered a significant reduction in fasting insulin concentration (standardized mean difference -0.20, P=0.020) and a non-significant reduction in blood glucose levels with HP diets compared with standard protein diets (30). The benefits of HP diets have been explained by reductions in dietary carbohydrate intake and by the greater preservation of fat-free mass (31,32).

Our study shows some limitations that involve the lack of using a dual-energy X-ray absorptiometry scan or computed tomography to assess body composition. The relatively small sample size could have limited the significance of weight loss-enhance ability of the highest protein diet. Diet compliance assessment would be more precise by determining the urine microalbumin and nitrogen concentration. Futhermore, this study involved counseling to increase physical activity which could have an additional role in the cardiometabolic profile improvement observed.

In conclusion, this randomized study with a follow-up visit at 6 months indicates that an energy-restricted diet with 35%protein, mostly of animal origin, leads to better cardiometabolic profiles than HP energy-restricted diets with lower protein content. Although there was no clear benefit between diets in terms of overall weight loss, a higher fat mass loss was observed in those women following the highest protein diet. The 35% diet implemented here was associated with excellent safety and acceptability. Lipid profiles and insulin resistance particularly improved in members of the 35%-proteingroupand did not directly correlate with weight loss. A high percentage of animal protein, especially protein from lean lamb meat, could increase diet-induced thermogenesis or the maintenance of satiety. Further research will be required to confirm these effects.

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STATEMENT OF AUTHORSHIP

RMG conducted research, analyzed data and performed statistical analyses, wrote the paper,and has primary responsibility for its final content. VMB conducted research and analyzed data. SPC conducted research. AMB, LBR, IZM, and ICO conducted research and provided essential reagents and materials. AC conducted research, provided essential reagents and materials, and wrote the paper. FC designed and conducted research, analyzed data, wrote the paper,and has primary responsibility for its final content. All authors have read and approved the final manuscript.

CONFLICT OF INTEREST STATEMENT

No authors have conflicts of interest to declare.

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Figure 1. Schematic representation of randomization and study course[1].

[1]BMI denotes body mass index.

Figure 2. Weight loss achievement after 3-months of dietary intervention according to randomized diet[1].

[1]*P* refers to differences between 20% and 35%-protein diets calculated by chi-squared test.

Figure 3. Body weight evolution across study to type of diet[1].

[1]*P* refers to inter-groups differences among 3 time-points calculated by repeated measures of ANOVA

Table 1. Dietary characteristics of participants according to randomized diet at baseline and after 3 months of dietary intervention[1].

	20% Protein diet				27% Protein diet				35% Protein diet				*P* among 3 diets[3]
	Baseline N = 30	3 months N = 24	Δ%	*P*[2]	Baseline N = 31	3 months N = 29	Δ%	*P*[2]	Baseline N = 30	3 months N = 27	Δ%	*P*[2]	
Energy, kcal	1896 [1649-2121]	1143 [1096-1259]	-34.4±14.6	<0.0001	1800 [1479-2091]	1169 [1132-1236]	-28.5±20.2	<0.0001	1856 [1521-2223]	1202 [1139-1294]	-31.4±32.1	<0.0001	0.675
Protein, %	21.1±5.04	23.3±3.21	2.22±5.24	0.054	22.1±5.54	27.4±3.04	5.36±6.40	<0.0001	20.1±5.76	31.0±4.94	10.9±7.15	<0.0001	< 0.0001
Animal protein, %	16.3±5.45	16.9±3.58	0.54±5.78	0.658	17.4±6.05	21.9±2.88	4.48±6.58	0.002	15.2±6.54	25.8±5.08	10.6±7.87	<0.0001	< 0.0001
Vegetal protein, %	4.74±1.53	6.42±1.13	1.68±1.70	<0.0001	4.58±1.63	5.50±1.27	0.93±2.18	0.035	4.84±1.38	5.17±0.97	0.33±1.65	0.333	0.054
Total Fat, %	44.9±6.73	35.2±5.37	-9.66±8.25	<0.0001	45.1±5.91	35.3±6.19	-9.78±8.94	<0.0001	43.5±5.07	35.4±4.92	-8.13±7.60	<0.0001	0.742
Monounsaturated fat, %	21.0±3.30	17.7±3.73	-3.38±5.00	0.004	20.2±3.61	18.1±3.42	-2.16±4.60	0.022	19.6±3.30	17.4±2.29	-2.25±4.08	0.013	0.591
Polyunsaturated fat, %	6.76±2.02	4.90±0.86	-1.86±1.90	<0.0001	6.93±3.63	4.71±0.87	-2.21±3.47	0.003	6.66±2.16	5.00±1.36	-1.67±2.81	0.008	0.785
Saturated fat,%	13.3±3.65	9.37±1.78	-3.94±3.59	<0.0001	14.0±2.65	9.13±2.76	-4.90±3.50	<0.0001	13.8±2.56	9.81±2.07	-3.95±3.68	<0.0001	0.546
Carbohydrates, %	33.7±7.61	41.2±7.55	7.46±10.2	0.002	32.4±6.75	37.2±5.77	4.82±8.30	0.006	35.9±6.72	33.6±4.43	-2.28±6.97	0.123	0.001
Sugar, g	70.3±21.9	71.7±20.8	6.05 [-29.6-31.9]	0.805	67.6±20.0	63.7±13.1	-10.2 [-21.1-13.4]	0.298	78.1±27.0	64.0±17.1	-22.4 [-46.1-17.0]	0.068	0.268
Fiber, g	18.5±8.97	24.3±5.00	50.7 [6.99-78.5]	0.006	16.3±5.51	19.3±6.26	42.1 [-21.9-61.6]	0.070	18.6±6.11	20.4±5.25	17.7 [-23.7-36.1]	0.305	0.087

[1]Values are mean ± standard deviation or median [percentile 25-percentile 75] as applicable. [2]*P* refers to differences between baseline and after 3-months dietary intervention in each diet. It is calculated by paired two-sample t-tests or Wilcoxon, as appropriate. [3]*P* refers to differences in 3-months variation among diets. It is calculated by ANOVA or Kruskal-Wallis tests, as appropriate.

Table 2. Changes in clinical and biochemical characteristics according to diet group after 3 months of dietary intervention[1].

	20% Protein diet				27% Protein diet				35% Protein diet				*P*[3]
	Baseline N = 30	3 months N = 24	Δ% 3- months from baseline	*P*[2]	Baseline N = 31	3 months N = 29	Δ% 3- months from baseline	*P*[2]	Baseline N = 30	3 months N = 27	Δ% 3- months from baseline	*P*[2]	
Weight, kg	86.4±8.35	78.8±8.94	-8.16±4.18	< 0.0001	87.9±9.33	79.4±9.41	-9.66±5.28	< 0.0001	85.1±8.39	76.6±8.44	-10.7±4.28	< 0.0001	0.164
Waist circumference, cm	99.6±7.06	89.5±7.48	-9.30±5.83	< 0.0001	99.4±9.41	90.4±8.37	-9.26±5.21	< 0.0001	101±11.6	88.7±9.97	-11.6±7.83	< 0.0001	0.306
Systolic blood pressure, mmHg	122±12.1	117±23.6	-3.64±17.2	0.303	124±19.7	115±16.7	-7.54±13.5	0.008	119±12.6	113±14.4	-4.54±10.9	0.021	0.582
Diastolic blood pressure, mmHg	77.3±9.03	75.0±8.70	-1.10±12.4	0.443	82.1±11.5	80.7±11.9	-2.62±12.7	0.184	79.3±9.58	78.9±8.97	0.44±12.9	0.733	0.745
Total cholesterol, mg/dL	210±40.7	202±41.2	0.16±10.7	0.954	225±28.1	215±31.8	-3.49±10.0	0.061	217±37.8	195±37.0	-8.10±9.26	< 0.0001	0.016[a]
HDL cholesterol, mg/dL	56.3±13.7	52.6±12.6	-4.77±13.2	0.075	56.4±9.56	53.4±9.74	-4.26±13.1	0.086	59.9±14.3	50.3±9.73	-12.4±10.7[b]	< 0.0001	0.031
Triglycerides, mg/dL	120±41.6	117±74.1	13.2±55.8	0.861	135±76.7	121±60.7	-4.17±30.4	0.073	124±49.8	94.1±26.2	-17.7±24.6	0.0001	0.020[a]
LDL cholesterol, mg/dL	129±41.6	127±32.4	1.18±14.6	0.905	142±23.6	132±28.4	-2.82±13.6	0.198	132±29.9	126±31.0	-3.52±12.5	0.144	0.436
Non-HDL cholesterol, mg/dL	153±34.8	150±35.9	2.44±14.0	0.532	166±24.3	158±24.9	-3.39±11.8	0.098	157±30.0	145±31.9	-6.29±11.6	0.006	0.046[a]
Glucose, mg/dL	91.7±12.5	87.5±9.99	-1.95±10.3	0.231	88.8±16.0	85.6±9.45	-2.37±17.3	0.134	85.9±8.26	81.2±9.17	-4.87±12.4	0.035	0.713
HOMA-IR	2.56 [1.50-3.58]	1.85 [1.49-3.18]	-17.8 [-45.4-55.5]	0.316	2.16 [1.64-4.39]	1.99 [1.40-2.68]	-19.4 [-41.7-4.57]	0.010	2.27 [1.47-2.92]	1.33 [0.89-2.09]	-39.4 [-54.9- (-10.2)]	0.001	0.121
HbA1c, %	5.50±0.29	5.43±0.23	0.64±2.88	1.000	5.43±0.39	5.41±0.36	-0.33±4.03	0.573	5.42±0.28	5.42±0.28	-0.02±2.73	0.901	0.900
GGT, U/L	18.5 [13.8-27.2]	16.5 [12.3-23.0]	-3.85 [-20.8-7.92]	0.267	21.0 [16.0-34.0]	18.0 [13.0-35.0]	-8.33 [26.8-6.51]	0.065	21.0 [14.5-30.5]	15.0 [12.0-24.0]	-14.3 [-36.4- (-4.55)]	0.001	0.186
ALT, U/L	16.5 [12.8-22.3]	15.0 [11.3-21.8]	-7.74 [-29.7-17.5]	0.321	16.0 [12.0-26.3]	14.0 [12.0-22.5]	-7.69 [-25.7-13.3]	0.115	15.0 [13.5-23.0]	14.0 [12.0-23.0]	-8.33 [-29.4-12.1]	0.143	0.965
Uric acid, mg/dL	5.10±1.38	5.10±1.17	0.93±18.5	0.711	5.36±1.25	5.39±1.14	0.66±15.3	0.795	4.98±1.02	5.09±0.96	2.84±14.4	0.627	0.862
Fat mass, kg	34.8±6.33	30.8±6.31	-10.5±12.0	< 0.0001	36.0±6.89	30.7±7.06	-15.2±9.13	< 0.0001	35.6±7.01	28.8±6.04	-18.3±12.2	< 0.0001	0.047[a]
Fat free mass, kg	46.9±5.12	45.4±2.97	-3.90 [-6.65-(-2.68)]	0.074	47.6±4.60	45.0±3.20	-4.71 [-7.88-(-2.78)]	< 0.0001	46.4±2.94	43.8±3.23	-5.13 [-7.13-(-2.68)]	< 0.0001	0.420
Visceral fat, level	9.20±1.94	7.92±2.13	-12.0±12.5	< 0.0001	10.2±3.04	8.24±2.08	-15.8±17.3	0.0001	9.21±2.26	7.41±2.12	-19.2±14.8	< 0.0001	0.246

Physical activity level, METs/min	693 [384-1386]	1340 [433-1868]	108±240	0.022	693 [462-1386]	1575 [594-3804]	148±170	< 0.0001	693 [429-1386]	1422 [1172-2517]	146±141	< 0.0001	0.073

[1] Values are mean±standard deviation (SD) or median [percentile 25-percentile 75] as applicable. [2]*P* refers to differences calculated by dependent t-test for paired samples or Wilcoxon test, as appropriate. [3]*P* refers to differences calculated by ANOVA or Kruskal-Wallis tests, as appropriate; adjusted by Bonferroni correction for multiple testing if applicable. “a” denotes $P < 0.05$ by comparing 20% vs. 35%-protein diets. BMI denotes body mass index; HOMA-IR, homeostasis model assessment-estimated insulin resistance; HbA1c, glycated hemoglobin; GGT, gamma glutamil transferase; ALT, alanine aminotransferase.

Table 3. Binary logistic regression analysis for ≥ 10% weight loss achievement after 3 months of dietary intervention*.

≥ 10% Weight loss	β Standarized coefficient	*P*	Odds ratio	Confidence interval (95%)		Corrected R^2
Physical activity at 3 months	0.001	0.004	1.001	1.000	1.001	27.9
Type of diet	0.632	0.046	1.882	1.012	3.500	
20%-protein diet**	-	-	-	-	-	
27%-protein diet	-0.082	0.899	0.921	0.260	3.260	
35%-protein diet	1.214	0.054	3.368	0.980	11.57	

* Linear regression model adjusted by baseline weight. ** 20%-protein was considered as reference category.

Supplemental Table 1. Examples of 1200 kcal-menus of three prescribed diets.*

	20%-Protein diet	27%-Protein diet	35%-Protein diet
Breakfast	Skimmed milk (200 ml) Coffee or tea Whole bread (45g) Butter (5g)	Skimmed milk (250 ml) Coffee or tea Whole bread (30g) Olive oil (5g)	Coffee or tea Whole cereal (40g) Walnut (5g) 2 Skimmed yoghurts
Mid-morning snack	Banana (160g)	Strawberries (175g) Light cheese (35g)	Orange juice (125 ml) Whole cereal (15 g) Tuna fish in brine (40g)
Lunch	Salad: tomato, lettuce, onion, carrot (100g) Green bean (150g) with potato (100g) Chicken (white meat, boneless skinless) (100g) Whole bread (30g) Olive oil (15g) Skimmed yoghurt	Salad: tomato, lettuce, onion, carrot (100g) Pasta (115g) with vegetables (50 g) natural tomato sauce (50g) Grilled turkey cooked (130g) with red / green peppers (50g) Olive oil (10g) Skimmed yoghurt	Salad: tomato, lettuce, onion, carrot (100g) Boiled lamb (135g) with red / green peppers (100g) and potatoes (50g) Olive oil (10g) Pear (160g)
Afternoon snack	Apple (130g)	Pineapple (120g)	Skimmed yoghurt Strawberries (175g)
Dinner	Salad: tomato, lettuce, onion, carrot (100g) Broccoli (150g) with potato (100g) Cooked ham (40g) Whole bread (30g) Olive oil (10g) Skimmed yoghurt	Vegetables (pumpkin, onion and carrot) purée (200 g) with potatoes (50g) Baked sardines (130g) Whole bread (30g) Olive oil (10g) Skimmed yoghurt	Vegetables (pumpkin, onion and carrot) purée (200 g) with potatoes (50g) Baked salmon (200g) Olive oil (10g) Skimmed yoghurt

*Food amount refers to raw weight.

Supplemental Table 2. Dietary characteristics of participants according to randomized diet at 6 months-follow-up visit[1].

	20% Protein diet			27% Protein diet			35% Protein diet		
	6 months N = 16	Δ% 6-months from baseline	*P*[2]	6 months N = 25	Δ% 6-months from baseline	*P*[2]	6 months N = 25	Δ% 6-months from baseline	*P*[2]
Energy, *kcal*	1179 [1151-1241]	-34.8±20.9	0.005	1240 [1145-1328]	-31.1±15.4	<0.0001	1172 [1107-1226]	-31.0±21.8	0.030
Protein, *%*	23.4±1.76	4.83±3.57	0.007	27.0±3.15	5.24±6.91	0.027	30.4±6.58	10.0±7.02	0.001
Animal protein, *%*	15.8±2.39	3.09±1.86	0.017	20.9±3.96	3.65±8.11	0.18	24.9±7.28	9.92±6.96	0.002
Vegetal protein, *%*	7.58±0.82	1.75±2.49	0.13	6.13±1.85	0.58±2.83	0.13	5.35±0.96	0.16±1.87	0.21
Total Fat, *%*	31.7±5.46	-10.7±8.19	0.22	31.3±3.71	-12.6±5.19	0.002	31.8±5.19	-12.3±9.07	0.37
Monounsaturated fat, *%*	15.1±2.46	-5.36±3.44	0.42	15.1±1.79	-5.32±2.98	0.08	14.6±1.83	-6.46±2.92	0.36
Polyunsaturated fat, *%*	5.05±0.74	-1.41±2.45	0.06	4.74±0.86	-1.13±1.85	0.017	4.70±0.82	-2.04±2.89	0.58
Saturated fat,*%*	8.59±3.63	-3.13±3.85	020	8.37±2.40	-5.58±2.71	< 0.0001	9.28±3.61	-3.60±5.24	0.11
Carbohydrates, *%*	44.6±5.37	6.21±5.92	< 0.0001	41.6±4.96	7.71±7.04	< 0.0001	37.9±5.38	2.69±5.73	< 0.0001
Sugar, *g*	70.8±15.0	-17.2 [-32.8-(-14.1)]	0.21	70.4±12.0	-0.91 [-19.2-29.0]	0.77	67.2±16.6	-23.3 [-37.2-25.8]	0.77
Fiber, *g*	27.7±3.76	-11.6 [-18.0-40.5]	0.81	20.6±6.71	16.4 [-10.5-56.9]	0.32	22.5±7.22	5.69 [-33.9-32.9]	0.96

[1]Values are mean ± standard deviation or median [percentile 25-percentile 75] as applicable. [2]*P* refers to differences between baseline, 3 and 6 months visits calculated by repeated measures analysis of ANOVA or Friedman, as appropriate.

Supplemental Table 3. Percentage changes in anthropometric characteristics according to diet group by comparing 6-months to baseline and 3-months assessments[1].

Δ %	20% Protein diet N = 16		27% Protein diet N = 25		35% Protein diet N = 25		P^2	
	Δ% 6-months from baseline	Δ% 3 to 6 months	Δ% 6- months from baseline	Δ% 3 to 6 months	Δ% 6- months from baseline	Δ% 3 to 6 months	Δ% 6-months from baseline	Δ% 3 to 6 months
Weight	-9.44±4.98	-0.62±2.28	-11.2±7.42	-0.52±4.43	-11.4±7.98	-1.23±5.39	0.653	0.974
Waist circumference	-10.0±5.53	0.80±3.37	-9.21±7.17	0.52±4.79	-10.3±9.27	2.12±7.25	0.872	0.589
Systolic blood pressure	-4.10±9.50	1.93±16.4	-6.18±13.7	3.08±10.0	-1.63±10.2	2.85±7.02	0.413	0.953
Diastolic blood pressure	6.99±11.6	6.08±11.8	-2.22±12.3	4.29±8.86	0.10±13.0	0.11±10.6	0.097	0.204
Fat mass	-9.64±14.8	-0.98±4.93	-16.4±13.4	0.55±11.6	-20.0±18.6	-3.37±16.6	0.149	0.571
Fat free mass	-5.32 [-8.40-(-2.61)]	-0.33 [-3.55-4.23]	-5.98 [-8.52-(-3.40)]	0.24 [-1.55-2.50]	-4.64 [-8.41-(-2.02)]	-0.34 [-2.86-1.86]	0.537	0.630
Visceral fat	-12.7 [-21.2- (-10.0)]	0 [-6.67-0.00]	-11.8 [-30.0-(-9.32)]	0 [-11.9 -7.50]	-22.6 [-33.3-(-12.5)]	0 [-13.8-0.00]	0.154	0.778

[1]Values are mean±standard deviation (SD) or median [percentile 25-percentile 75] as applicable. [2]*P* refers to differences calculated by ANOVA or Kruskal-Wallis tests, as appropriate.

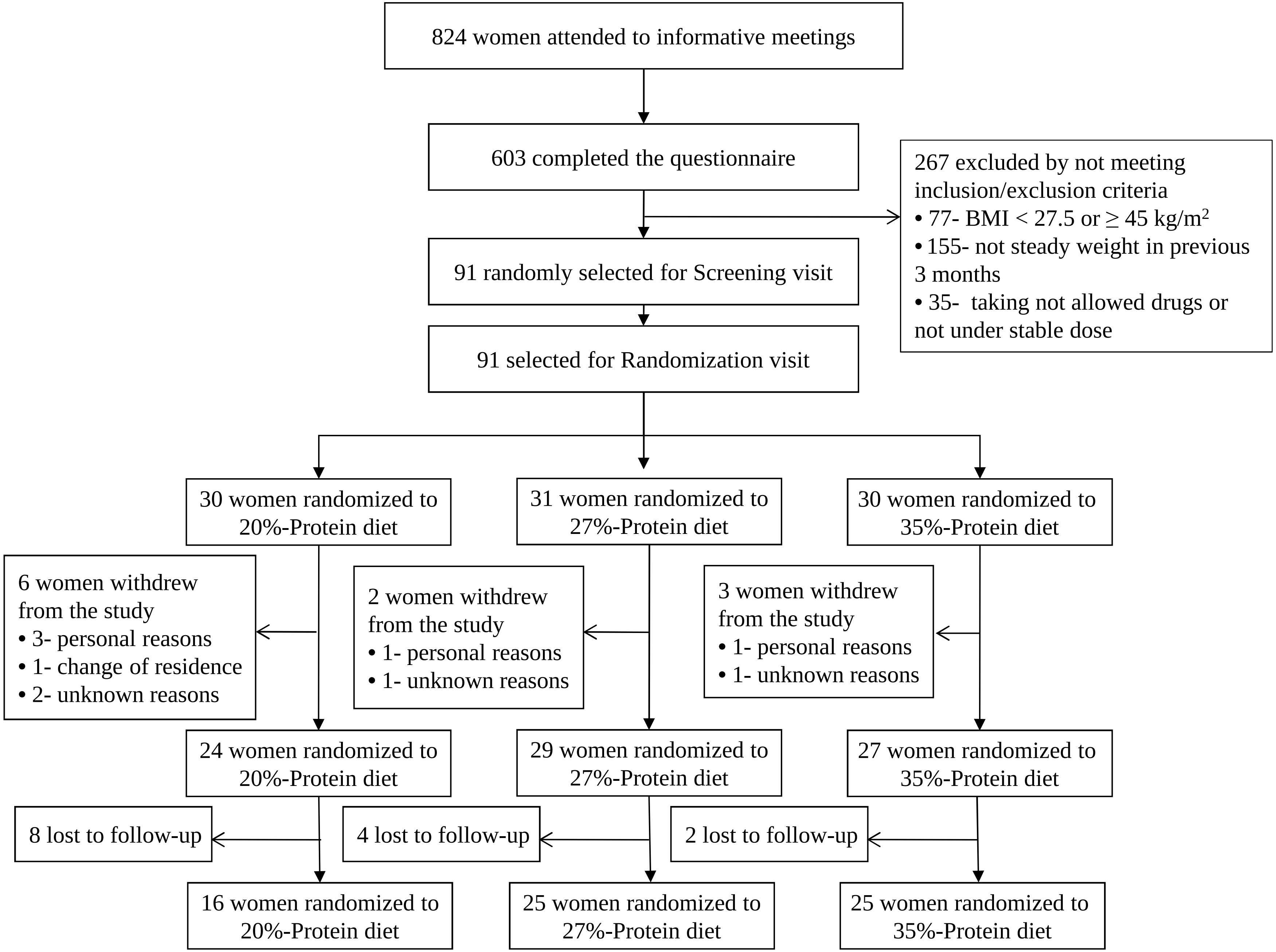
Supplemental Table 4. Satisfaction questionnaire performed to participants after 3-months of dietary intervention[1].

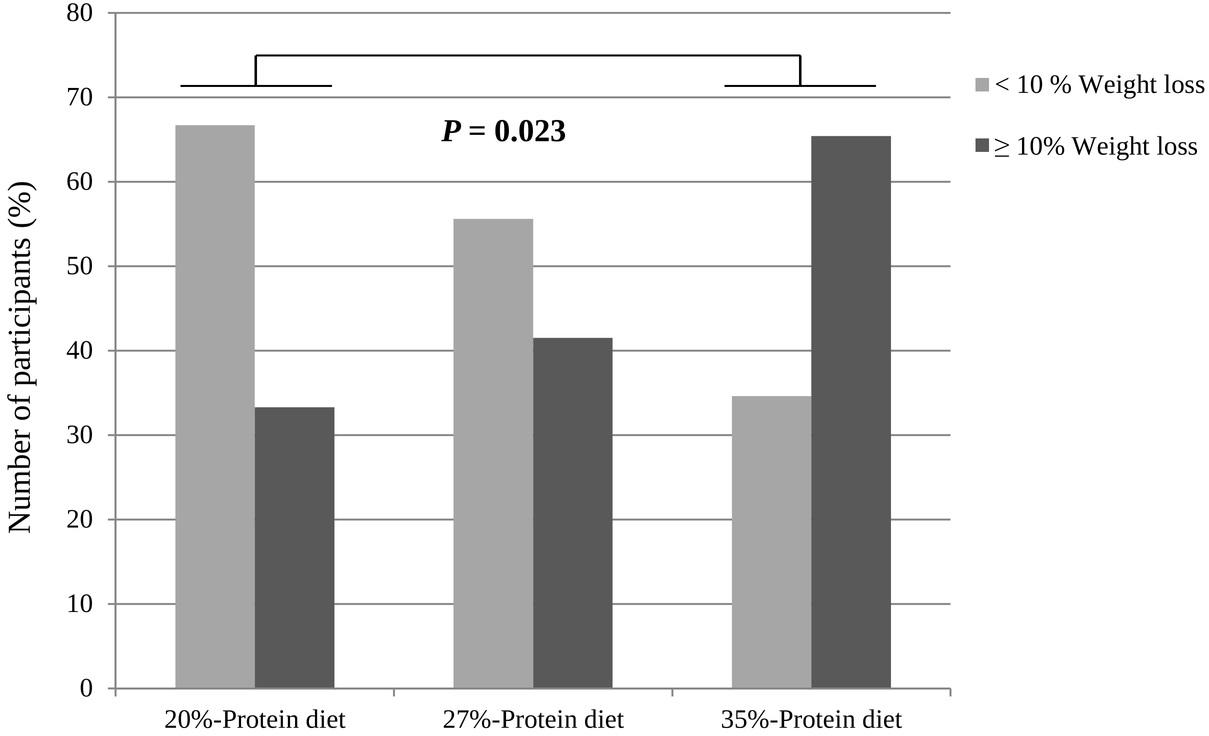
Scale, 0-10	20% Protein diet N = 24	27% Protein diet N = 29	35% Protein diet N = 27	*P*[2]
Health status	7.79±1.96	7.63±1.78	8.31±1.03	0.40
Hunger during study	2.14±2.09	2.54±2.14	2.39±1.69	0.84
General satisfaction with diet	9.78 [8.38-10.0]	9.25 [8.63-10.0]	9.25 [8.38-9.63]	0.61
Intention to withdraw from the study	0 [0-0]	0 [0-0.5]	0 [0-1.63]	0.31
Willingness to unlimited follow-up the diet	9.50 [7.63-10.0]	9.25 [8.00-10.0]	9.00 [8.00-9.63]	0.62
Compliance acceptability	9.00±0.88	8.44±1.17	8.28±1.10	0.16

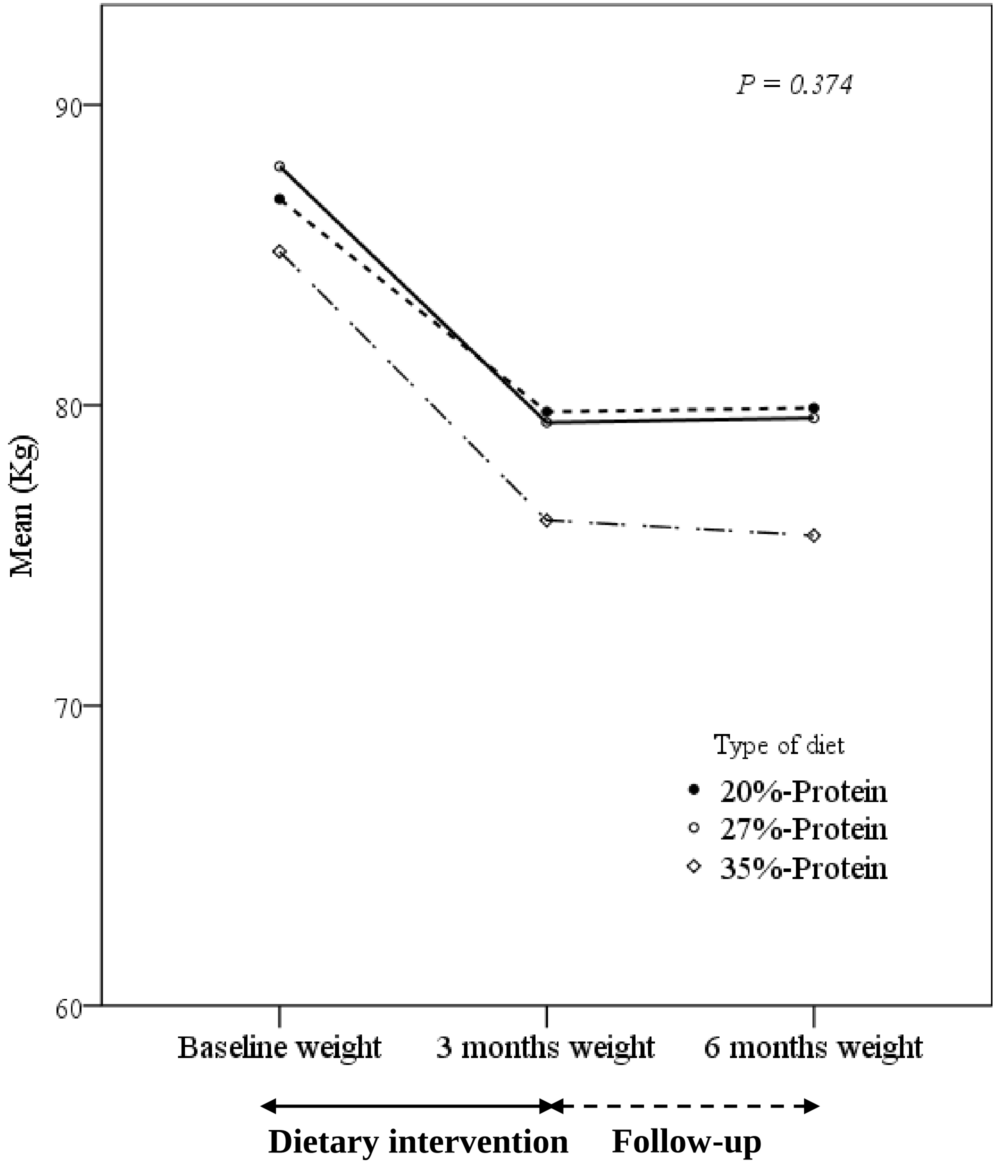
[1] Values are mean±standard deviation (SD) standard deviation or median [percentile 25-percentile 75] as applicable. [2]*P* refers to differences calculated by ANOVA or Kruskal-Wallis tests, as appropriate.

Supplemental Table 5. Adverse events reported by participants across the study according to randomized diet group.

Severity	Adverse event description	Number of participants who reported the adverse event	Randomized diet		
			20% Protein diet N = 30	27% Protein diet N = 31	35% Protein diet N = 30
Serious adverse events	Motorcycle accident	1	1	0	0
	Appendicitis	1	0	1	0
Adverse events	Constipation/ Constipation worsening	7	2	3	2
	Renal colic	2	1	1	0
	Anxiety	3	1	1	1
	Gastroenteritis	1	1	0	0
	Hypotension	2	0	1	1
	Sprained ankle	2	0	1	1
	Lower back pain	2	0	0	2
	Otitis	1	1	0	0
	Flu	2	0	1	1


824 women attended to informative meetings
603 completed the questionnaire
267 excluded by not meeting inclusion/exclusion criteria
• 77- BMI < 27.5 or ≥ 45 kg/m2
• 155- not steady weight in previous 3 months
• 35- taking not allowed drugs or not under stable dose
91 randomly selected for Screening visit
91 selected for Randomization visit
30 women randomized to 20%-Protein diet
31 women randomized to 27%-Protein diet
30 women randomized to 35%-Protein diet
6 women withdrew from the study
• 3- personal reasons
• 1- change of residence
• 2- unknown reasons
2 women withdrew from the study
• 1- personal reasons
• 1- unknown reasons
3 women withdrew from the study
• 1- personal reasons
• 1- unknown reasons
24 women randomized to 20%-Protein diet
29 women randomized to 27%-Protein diet
27 women randomized to 35%-Protein diet
8 lost to follow-up
4 lost to follow-up
2 lost to follow-up
16 women randomized to 20%-Protein diet
25 women randomized to 27%-Protein diet
25 women randomized to 35%-Protein diet


80
70
60
50
40
30
20
10
0
Number of participants (%)
P = 0.023
< 10 % Weight loss
≥ 10% Weight loss
20%-Protein diet
27%-Protein diet
35%-Protein diet


P = 0.374
90
80
70
60
Mean (Kg)
Type of diet
20%-Protein
27%-Protein
35%-Protein
Baseline weight
3 months weight
6 months weight
Dietary intervention
Follow-up