A randomized cross-over study of the effects of macronutrient composition and meal frequency on GLP-1, ghrelin and energy expenditure in humans

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ABSTRACT

Objective: Little is known about human postprandial increase of energy expenditure and satiety-associated hormones in relation to both meal frequency and macronutrient composition.

Methods: Seven men and seven women (mean age 23 ± 1.5 years) were randomly assigned to the order of intake of a 750 kcal drink with the same protein content while having either 20 energy-percent (E%) or 55 E% from carbohydrates and the remaining energy from fat. Participants were also randomized to consume the drinks as one large beverage or as five 150 kcal portions every 30 min, starting in the fasting state in the morning. Energy expenditure (EE) was determined every 30 min by indirect calorimetry. Hormonal responses and suppression of hunger (by visual-analogue scales) were also studied. A p < 0.013 was considered statistically significant following Bonferroni-correction.

Results: The area under the curve (AUC) for EE was higher during the 2.5 h after the high-carbohydrate drinks (p = 0.005 by Wilcoxon) and also after ingesting one drink compared with five (p = 0.004). AUC for serum active GLP-1 was higher after single drinks compared with five beverages (p = 0.002). Although GLP-1 levels remained particularly high at the end of the test during the low-carbohydrate meals, the AUC did not differ compared with the high-carbohydrate occasions (low-carbohydrate: 58.9 ± 18 pg/ml/h, high-carbohydrate: 45.2 ± 16 pg/ml/h, p = 0.028). Hunger sensations were suppressed more after single beverages compared with five small drinks (p = 0.009).

Conclusions: We found higher EE during 2.5 h following one large drink compared with five smaller beverages. Since hunger was also suppressed more efficiently, and serum GLP-1 levels were higher after one compared with five smaller drinks, our findings do not support nibbling to avoid hunger or to keep up EE from morning to noon.

1. Introduction

Energy expenditure (EE) increases following a meal and many studies have tested effects of one as compared with several smaller meals on the metabolic rate. Most [1–4], but not all [2,5], found that more energy is spent after consuming large meals as compared with several smaller meals. Obesity has been linked to lowered diet-induced EE. However, it is often more convenient to fundamentally change proportions of the carbohydrate content in relation to amount of fat as sources of food that are very rich in protein are more scarce and sometimes also expensive. Kinabo et al. performed a study where they compared carbohydrate content and differences between one large or two smaller meals within the same trial [5]. They found no differences in EE when a single 1200 kcal or two 600 kcal meals were compared whether this was eaten as high-carbohydrate meals in 8 women, or as low-carbohydrate meals in another 10 women [5]. To the best of our knowledge, there are no reports from studies were several small meals have been compared with single isocaloric large meals in which varying carbohydrate/fat proportions were also tested.

The incretin glucagon-like peptide-1 (GLP-1) is released from L-cells in the small intestine following nutrient intake. GLP-1 induces satiety, inhibits gastric emptying and it can also induce release of insulin in
humans [10]. Indeed, GLP-1 analogues are already in use for treatment of type 2 diabetes, and these drugs reduce body weight and glucose levels [11]. The hormone ghrelin is also regulated by food intake in humans. However, the release of ghrelin from cells lining the stomach and small intestine is inhibited by the presence of nutrients. Indeed, many of the effects of ghrelin are opposite to those of GLP-1 as it acts to increase hunger and appetite [12].

We aimed to study potential interactions of the carbohydrate and fat content of the food with the meal distribution on postprandial EE. To potentially gain more insights of mechanisms we also measured the levels of serum ghrelin, serum GLP-1 and suppression of hunger. We recruited both men and women and randomly assigned the participants to a single 750 kcal soft drink with either 20% of the energy content from carbohydrates (E%) or 55 E% from carbohydrates but with the same protein content. We used a cross-over design to increase statistical power as compared to the study by Kinabo et al. [5]. To test the influence of meal distribution we also randomized the participants to consume the high- or low-carbohydrate beverages as one single drink in the morning or to drink it divided into five 150 kcal drinks every 30 min from morning until noon.

2. Methods

2.1. Recruitment and interventions

The participants were consecutively recruited by local advertising with posters at Linköping University. Healthy non-obese subjects with normal thyroid function could participate. Exclusion criteria were significant medical conditions, regular medication (except birth control pills), and obesity. The mean age of participants was 23 ± 1.5 year (range from 20 to 26 years). Subjects were asked not to change their usual dietary or exercise habits during the course of the study. Thyroid hormone function was tested and judged as normal in all the participants ahead of participation.

Each subject performed four test occasions in a randomized order by drawing of ballots. There were two different test meals, one high-carbohydrate (HC) and one low-carbohydrate (LC) meal. The meals were ingested in two different settings, either as one single meal in the morning (denoted HC1 or LC1) or as five equally sized portions (denoted HC5 or LC5) every 30 min. The test meals consisted of standard milk (3% fat), cream (40% fat), frozen blueberries, raw eggs and surose in two different combinations to achieve 20% of the total energy from carbohydrates in the LC meal and a correspondingly 54.9% of total energy from carbohydrates in the HC meal. All the meals were homogenized using a blender. The final energy compositions of the meals are shown in Table 1.

During the test occasions in which five small portions were consumed, each serving was 95.4 g. All the test meals had a temperature of 18–22 °C when served. All four test meals for each subject were ingested within a six week period and the total trial-period was from September to November 2015.

The participants were studied at the Department of Clinical Physiology at Linköping University Hospital and each session started 08:00 AM after fasting from 10:00 PM the prior evening. Subjects were only allowed to drink water during this fasting period. Height, weight, blood pressure in the supine position and basic blood biochemistry was obtained at the start of the first test meal. Fourteen healthy participants were recruited, seven women and seven men. A venous cannula for drawing blood was inserted at the start of each test occasion. Each test occasion lasted from about 08:00 AM to 12:00 AM in total. This time period was chosen since it was assumed to represent a common interval between breakfast and the subsequent lunch. A common lunch meal would be expected to have a larger effect on the diet induced thermogenesis than remaining effects from the breakfast to noon period and thus measurements were carried out from morning to noon. Fig. 1 shows timelines of the investigations.

The EE was measured by indirect calorimetry (Quark RMR, Cosmed, Finland) and the equipment was calibrated daily. This calibration also includes analysis of a reference gas that ensures proper measurement of concentrations and hence validation of the function. All participants rested in the supine position 10 min before each measurement of EE. The result of the measurement was based on the mean value of the last five minutes of readings following individual stabilization for about 10 min. When consuming the five small meals the participants consumed each meal during five minutes. When consuming the LC1 or HC1 beverages the subjects were asked to finish the drinks within 10 min. EE rate was measured five times with 30-min intervals starting 30 min from start of the ingestion of the meal. This left a period of 20 min (single drink) or 25 min (small drinks) before each recording of EE after finishing the beverages. Venous blood samples were collected after each measurement of EE. We used data for the mean values at each time point from the high- and low-carbohydrate tests when we compared the effects of five drinks with one. Correspondingly, the mean values from one and five drinks, at each time point, were used when we calculated the differences between the low- and high-carbohydrate occasions.

Serum samples for hormones were collected at one and a half hours, two and a half hours and three hours after the basal fasting samples had been collected. The venous blood samples were taken at room temperature and centrifuged immediately before being frozen to −20°C within 30 min. Within a day the sera was transferred to a −80°C freezer until final analysis of hormones. Body composition was measured by air displacement plethysmography with BodPod equipment (Life Measurement Instruments, Concord, CA, USA). Hunger sensations were measured by using visual-analogue scales (VAS). Every 30 min, ahead of the measurement of EE, the participant draw a vertical line on a document between the statements “very hungry” and “not hungry at all” and the data were analyzed as percentage of the total length of the horizontal line. A high score thus corresponded to a strong suppression of hunger.

Standard laboratory tests were analyzed according to routines at Department of Clinical Chemistry at the Linköping University Hospital. Active GLP-1 and active ghrelin were analyzed using Milliplex® MAP Human Metabolic Hormone Magnetic Bead Panels (Merck Millipore, Darmstadt, Germany) and a Luminex® 200 equipment (Luminex Corporation®, Austin, Texas, USA). A protease inhibitor cocktail containing aprotinin (P2714 Protease Inhibitor Cocktail, Sigma, Israel) and DPP-4 inhibitor (product number DPP4, Merck Millipore, Darmstadt, Germany) were added before centrifugation of the samples, according to the instructions from the manufacturer, to preserve active ghrelin and active GLP-1. Total coefficient of variation (intra + inter-assay) was 11.4% for serum GLP-1 and 5.0% for serum ghrelin.

2.2. Ethics

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Regional Ethics Committee of Linköping. Written informed consent was obtained from all subjects. The study was reported to https://clinicaltrials.gov with accession number...
1421 ± 101 kcal/24 h, men: 1988 ± 253 kcal/24 h, p = 0.001) than
66.0 ± 13 kg, p = 0.001) and higher fasting EE (women:

3.1. Baseline data of participants

Table 2

| Variable                  | Women          | Men            |
|---------------------------|----------------|----------------|
| Age (years)               | 23 ± 1.7       | 23 ± 1.3       |
| Weight (kg)               | 58.2 ± 9.3     | 81.2 ± 15      |
| BMI (kg/m²)               | 21.1 ± 3.5     | 24.3 ± 3.6     |
| Systolic BP (mmHg)        | 116.9 ± 8.3    | 127.0 ± 7.2    |
| Diastolic BP (mmHg)       | 69.7 ± 6.0     | 73.0 ± 5.3     |
| Hemoglobin (g/l)          | 132 ± 7.7      | 141 ± 8.4      |
| Plasma creatinine (µmol/l)| 66.3 ± 10      | 84.0 ± 11      |
| Plasma Apolipoprotein A1 (g/l) | 1.45 ± 0.41 | 1.17 ± 0.14 |
| Plasma Apolipoprotein B (g/l) | 0.780 ± 0.12 | 0.737 ± 0.096 |
| Serum T4 (µmol/l)         | 14.9 ± 1.5     | 17.4 ± 1.8     |
| Fasting EE (kcal/24 h)    | 1421 ± 101     | 1988 ± 253     |
| Serum ghrelin (pg/ml)     | 67.1 ± 36      | 56.9 ± 25      |
| Serum GLP-1 (pg/ml)       | 11.1 ± 22.8    | 9.5 ± 6.2      |
| Fat percentage (%)        | 29.2 ± 6.3     | 18.5 ± 7.6     |
| Fat-free mass (kg)        | 40.8 ± 3.6     | 66.0 ± 12.6    |

Abbreviations: BP blood pressure; EE energy expenditure by indirect calorimetry.

3.2. Effects of the interventions

Postprandial changes in EE, serum ghrelin, serum GLP-1 and hunger suppression by the drinks are shown in Figs. 2–5. The corresponding AUCs of the hormones during 2.5 h are presented in Tables 3 and 4. The EE increased more during the high-carbohydrate drinks and also when consuming the drinks as one large portion compared with five smaller portions (Fig. 1a and b, Table 3). There was no difference between genders regarding the specific postprandial increase in EE either for low- or high-carbohydrate macronutrient compositions or for one or five meals (p > 0.13 for all four comparisons).

In an exploratory analysis of potential influence of amount of body fat on EE by the beverages, as has earlier been demonstrated [6,7,16], we found a positive correlation between fat-mass and a trend also of fat-percentage to the increase of EE at the first postprandial measurement of EE (i.e. delta EE) when the low-carbohydrate drink was ingested as one large drink (fat mass: r = 0.72, p = 0.004, fat percentage: r = 0.59, p = 0.027). There were no corresponding such findings during the other three other test occasions (LCS, HCI and HCS). There was no major differences in the thermogenesis findings when comparing one beverage with five (p = 0.003), or the high- with low-carbohydrate drinks (p = 0.002), when data were re-calculated based on the women. The correlation between EE in the fasting state and fat-free mass was r = 0.98, p < 0.0001 (n = 14) which was suggestive of valid readings of both EE and body composition.

3.3. Statistics and power calculation

Statistical estimates were calculated using IBM SPSS Statistics 23 software (IBM Corporation, Somers, New York, USA). Comparisons of single variables within and between groups were done with Wilcoxon signed ranks test to avoid effects of potentially skewed distributions. Statistical significance for paired comparisons was considered to be present if p < 0.013 after Bonferroni correction, i.e. p = 1-(1-α)^1/k where α is the traditional p-value of 0.05 and k = 4 (variables tested: EE, ghrelin, GLP-1 and hunger suppression). Linear correlations were done by using the Pearson product-moment method. The areas under the curves (AUC) were calculated based on the trapezoidal rule. When done by using the Pearson product-moment method. The areas under the curves (AUC) were calculated based on the trapezoidal rule. When calculating the AUC for hunger suppression by the drinks, we used data for the change from the fasting VAS value (postprandial level divided by the fasting value on that day) to avoid effects of the variation of the hunger sensations in the fasting state between occasions. Since participants ate lunch and dinner at their own choice after the tests, we expected no carry-over effects from the beverages tested in the study. We used one-way ANOVA to test if all four individual test-occasions differed with respect to EE, ghrelin, GLP-1 and suppression of hunger. In this single analysis a p < 0.05 was considered statistically significant.

Based on our earlier studies of EE in humans, [13,14] the study had > 80% power to detect a 5% postprandial increase of EE following a single meal compared with five small meals with a study size of 12 participants. We had no previous data for calculating the power to detect changes in serum GLP-1 or ghrelin. Given the results from other studies on postprandial changes in levels of serum GLP-1 and ghrelin [10,15], we assumed that power to detect differences in levels of these hormones would be at least as high as for detecting differences in EE.
on EE per kg of FFM for each individual.

Serum ghrelin levels were suppressed after the drinks (Fig. 3a and b, p = 0.005 comparing 1 with five drinks, p = 0.002 comparing carbohydrate contents, i.e. differences from baseline with the first postprandial measurement at 1.5 h). Ghrelin displayed no differences in AUC for carbohydrate contents or drink distributions (Tables 3 and 4). Serum ghrelin at 2.5 h tended to be higher after high-carbohydrate beverages compared with low-carbohydrate drinks (low-carbohydrate drinks: 28.3 ± 18 pg/ml, high-carbohydrate drinks: 40.1 ± 22 pg/ml, p = 0.033).

AUC for serum GLP-1 was higher after single drinks compared with five beverages (p = 0.002, Table 3, Fig. 4a) and tended to be higher during ingestion of low- compared with high-carbohydrate beverages (p = 0.028, Table 4, Fig. 4b).

Hunger sensations were suppressed stronger from morning until noon after ingestion of the single beverages compared with five small drinks (p = 0.009, see Table 3 and Fig. 5a). There were no differences in AUCs for suppression of hunger when comparing low- with high-carbohydrate drinks (Table 4). However, the hunger sensations were suppressed more during the low- compared with the high-carbohydrate drinks at the very last estimation point (p = 0.009, * in Fig. 5b).

One-way ANOVA was also used in an analysis of all four separate test occasions. We found that GLP-1 AUC was numerically highest during the single meal low-carbohydrate occasion (p = 0.034 for comparison of all four groups) but there were no statistically significant differences regarding EE (p = 0.7), ghrelin (p = 0.8) or suppression of appetite (p = 0.14).

### 4. Discussion

#### 4.1. Energy expenditure

We confirmed that a single large beverage in the morning caused a larger increase of EE than five smaller isoenergetic drinks during 2.5 h. Our data are in line with a study on this topic by Tai et al. in 1991 [3] who studied the effects on EE of eating 750 kcal (54.5 E% carbohydrates) either as one meal or divided into six equal portions every 30 min for three hours in seven healthy women. Our study also included men and women and we tested both high- and a low-carbohydrate drinks. We suggest that the contrasting findings in our study to the trial by Kinabo et al. [5] in which no difference between large and small meals was found, related to using real-time measurement of EE by Quark RMR equipment rather than the Douglas bag.
technique and/or increased statistical power by the cross-over setting in which participants served as their own controls. Also we tested five smaller meals as compared with two in the study by Kinabo et al. [5]. We found no differences in postprandial increase of EE when data from men and women were compared. We want to acknowledge, however, that to find small differences between genders in this respect, larger studies than the one presented here would likely be necessary. However, our study did find differences in body composition between genders also in this trial of non-obese subjects. Earlier studies have suggested that the diet-induced thermogenesis is lowered in obese subjects [6,7,16]. However, in a specific analysis of this clinically relevant topic we found a positive correlation between fat-mass to the increase of EE during the single large low-carbohydrate meal. It should be noted that this analysis was not part of the main aims of our trial and hence it would need to be explored further in more dedicated studies of the potential importance of macronutrient compositions in relation to fat-mass for achieving a high postprandial EE. Our contrasting findings to other trials could relate to the notion that we studied relatively young participants who were non-obese and healthy rather than the more profoundly obese subjects as in other trials [7,16].

Table 3
Areas under the curve for metabolic rate and for hormones during the test-occasions comparing one large or five small drinks.

| Variable                        | AUC for one large meal | SD | AUC for five small meals | SD | P between occasions |
|---------------------------------|------------------------|----|--------------------------|----|---------------------|
| EE (kGal)                       | 556                    | 101| 535                      | 96 | 0.004               |
| Serum ghrelin (pg/mL/h)         | 128                    | 66 | 129                      | 70 | 0.92                |
| Serum GLP-1 (pg/mL/h)           | 64.1                   | 20 | 40.0                     | 16 | 0.002               |
| Suppression of hunger (AU)      | 4.04                   | 1.3| 3.09                     | 1.01 | 0.011              |

Abbreviations: AU, arbitrary units; AUC, area under the curve; EE, energy expenditure by indirect calorimetry; P, p-value; SD, standard deviation.

Data for ghrelin was missing in one subject and data for GLP-1 was missing in two subjects, for technical reasons.

A limitation with regard to generalizability of this finding, however, was the fact that we only related fat mass to increase in EE in the morning. Different findings could apply during other periods of
Table 4
Areas under the curves for energy expenditure, suppression of hunger and for hormones when comparing carbohydrate and fat contents.

| Variable                        | AUC for high-carbohydrate drinks | SD | AUC for low-carbohydrate drinks | SD | P between occasions |
|---------------------------------|----------------------------------|----|---------------------------------|----|--------------------|
| EE (kCal)                       | 557                              | 97 | 534                             | 100| 0.005              |
| Ghrelin (pg/ml/h)               | 136                              | 70 | 121                             | 68 | 0.20               |
| GLP−1 (pg/ml/h)                 | 45.2                             | 16 | 58.9                            | 18 | 0.028              |
| Suppression of hunger (AU)      | 3.47                             | 1.1| 3.79                            | 1.0| 0.30               |

Abbreviations: AU, arbitrary units; AUC, area under the curve; EE, energy expenditure by indirect calorimetry; P, p-value; SD, standard deviation.

1 Data for ghrelin was missing in one subject and data for GLP-1 was missing in two subjects, for technical reasons.

4.2. Effects on hormones and suppression of hunger

Carbohydrates are often claimed to induce a particularly high GLP-1 secretion [10]. However, we found at least as high GLP-1 levels after the low-as after the high-carbohydrate drinks. Since protein intake was the same during these tests, our data suggest that a drink with a high-fat content and little carbohydrates do not give a weaker stimulus for the release of GLP-1 than high-carbohydrate beverages in healthy non-obese participants. Indeed, in the ANOVA analysis it was a single low-carbohydrate drink that showed highest GLP-1 levels compared to the other three test occasions. Since GLP-1 reduces appetite the high levels of this hormone were in line with stronger suppression of hunger during the low-compared with the high-carbohydrate drinks at the very last measurement point. Serum levels of GLP-1 were highest after the single-drink occasions and this was reflected by the corresponding VAS results displaying strong suppression of hunger. Among many biological effects, GLP-1 also inhibits gastric emptying [10] which corroborates this. It is possible that the high AUC of low-carbohydrate drinks was achieved by prolonged stimulation of the release from L-cells due to slower rate of gastric motility. This would indeed comply well with the shape of the curve during the last part of the test as seen in Fig. 4b.

Serum ghrelin levels were suppressed 1.5 h after ingestion of the first beverages but the AUCs did not differ between the tests. An analysis of the last time-point during the tests comparing carbohydrate contents, where the graph showed error bars that were not overlapping, yielded a p-value of 0.009. This post-hoc finding suggests that there was stronger suppression of hunger sensations by low-carbohydrate food when compared with isoinenergetic intake of high-carbohydrate drinks 2.5 h after the start of food consumption. This could be an effect of delayed gastric emptying by fat, an effect often ascribed to release of cholecystokinin which also induces a sense of satiety [18]. The data are in line with an earlier study showing very weak suppression of hunger by high-carbohydrate snacks consumed in the non-hungry state [19]. However they are in contrast to another trial that showed weaker inhibition of hunger after a high-fat breakfast compared with isoeenergetic low fat meal at noon in nine healthy males [20]. Although satiety was measured by an almost identical VAS that was used in our study, the contrasting findings could relate to more modest differentiation of fat- and carbohydrate-contents. It could also have been affected by the fact that the meals were not adjusted with respect to both caloric content and mass [20].

4.3. Limitations

We acknowledge potential shortcomings of our trial. As already pointed out, we only studied effects of the meals from morning to lunch. Earlier studies have shown that the timing of the meals could affect postprandial metabolic rate [21], and hence also hormone responses might also be affected. The reason for this choice of a three hour time-period was that it is most common to consume another main meal of the day at noon. Hence postprandial EE during the remainder of the day will mainly be affected by such a lunch meal, and other potential later meals, rather than the food intakes during the morning hours.

Several studies have confirmed higher EE following the consumption of food in a concentrated fashion as compared with nibbling [3,4,22,23], but this has not always been the case [5,24–26]. However, we know of no study that specifically compared such effects of isoinenergetic low- and high-carbohydrate beverages with the same volume and mass, as presented herein. We only included healthy non-obese adults who were less than 30 years of age. We call for more studies on the postprandial effects on EE and satiety related hormones in larger cohorts with varying ages and body compositions, and also in patients, to gain data of higher relevance for dysmetabolic patients.

4.4. Conclusions

We found that mean EE was larger after single drinks that were based on both low- and high-carbohydrate contents, when compared with the same energy intakes from five smaller beverages. We also showed that AUC for serum GLP-1 did not differ following low-carbohydrate drinks compared with isocaloric high-carbohydrate beverage in these non-obese men and women. In a comparison of all four test-occasions separately, we found that it was the single large low-carbohydrate meal that increased AUC for serum GLP-1 strongest. Ghrelin levels in serum tended to be more suppressed after 2.5 h by low-carbohydrate drinks that also had a more sustained appetite suppression than the high-carbohydrate beverages in the latest part of the tests. Since also the suppression of appetite was stronger by one as compared with five smaller meals, our results do not support nibbling as an effective means to either keep up EE, or to suppress hunger sensations, from morning to noon.

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Conflicts of interests

There are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported herein.

Author contributions

FHN obtained resources for the study and designed it with HG. SI and NV ran the trial and collected the data with help of ES and analyses with the statistician MF. All authors participated in analyzing the data and drafting of the article and revised it critically for important intellectual content. All authors contributed with their specific technical skills in the conduct of the study and approved the final article.

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