Differential Acute Postprandial Effects of Processed Meat and Isocaloric Vegan Meals on the Gastrointestinal Hormone Response in Subjects Suffering from Type 2 Diabetes and Healthy Controls: A Randomized Crossover Study

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Abstract

Background: The intake of meat, particularly processed meat, is a dietary risk factor for diabetes. Meat intake impairs insulin sensitivity and leads to increased oxidative stress. However, its effect on postprandial gastrointestinal hormone (GIH) secretion is unclear. We aimed to investigate the acute effects of two standardized isocaloric meals: a processed hamburger meat meal rich in protein and saturated fat (M-meal) and a vegan meal rich in carbohydrates (V-meal). We hypothesized that the meat meal would lead to abnormal postprandial increases in plasma lipids and oxidative stress markers and impaired GIH responses.

Methods: In a randomized crossover study, 50 patients suffering from type 2 diabetes (T2D) and 50 healthy subjects underwent two 3-h meal tolerance tests. For statistical analyses, repeated-measures ANOVA was performed.

Results: The M-meal resulted in a higher postprandial increase in lipids in both groups (p < 0.001) and persistent postprandial hyperinsulinemia in patients with diabetes (p < 0.001). The plasma glucose levels were significantly higher after the V-meal only at the peak level. The plasma concentrations of glucose-dependent insulinotropic peptide (GIP), peptide tyrosine-tyrosine (PYY) and pancreatic polypeptide (PP) were higher (p < 0.05, p < 0.001, p < 0.001, respectively) and the ghrelin concentration was lower (p < 0.001) after the M-meal in healthy subjects. In contrast, the concentrations of GIP, PYY and PP were significantly lower after the M-meal in T2D patients (p < 0.001). Compared with the V-meal, the M-meal was associated with a larger increase in lipoperoxidation in T2D patients (p < 0.05).

Conclusion/Interpretation: Our results suggest that the diet composition and the energy content, rather than the carbohydrate count, should be important considerations for dietary management and demonstrate that processed meat consumption is accompanied by impaired GIH responses and increased oxidative stress marker levels in diabetic patients.

Trial Registration: ClinicalTrials.gov NCT01572402

Introduction

The current guidelines for the treatment of diabetic patients focus primarily on carbohydrate counts [1]. However, the postprandial metabolic response is likely also modified by the contents of other macronutrients in meals and by gastrointestinal hormone (GIH) release.

Dietary saturated fat is known to impair insulin sensitivity and to enhance hepatic glucose production [2]. Studies of patients suffering from diabetes have revealed that dietary fat delays gastric emptying, leading to a lag in glucose absorption [3,4]. On the other hand, studies suggest that in patients suffering from diabetes, higher fat meals acutely increase the glucose concentration and the requirement for insulin compared with meals containing similar...
carbohydrate but lower fat contents [5]. Additionally, addition of protein energy to a meal likely increases the postprandial glucose level [6].

Epidemiological studies suggest a positive association between high consumption of total and red meat and incident T2D [7,8]. Subjects who consumed any processed meats (salted fish and frankfurters) were 38% more likely to develop diabetes [9]. Previous studies support the concept that increased oxidative stress may play an important role in T2D manifestation [10]. Dietary fat quality has been proposed to be a critical factor. Several studies have suggested that a high intake of saturated fatty acids naturally present in meat contributes to the risk of glucose intolerance [11,12]. In an intervention study, humans suffering from metabolic syndrome who were consuming a high saturated fatty-acid diet displayed higher oxidative stress markers postprandially [13,14].

In contrast, some intervention trials demonstrated a greater improvement in insulin sensitivity, glycemic control and a reduction in oxidative stress markers in T2D patients consuming a vegetarian diet compared with those consuming a traditional diabetes diet [15,16]. The aim of our study was to determine the acute effects of meat and plant-based meals on postprandial GIH secretion. Thus, we designed this randomized crossover study to evaluate the acute (a time span of hours) postprandial response to two standardized meals containing the same caloric content but a different nutritional content: a processed meat (hamburger) meal rich in protein and saturated fat and a plant-based meal rich in carbohydrates. The objective of our study was to examine whether the acute intake of different types of isocaloric meals consumed in amounts typical of normal eating (food intake representative of real life) would be associated with different postprandial changes in glucose, lipid, immunoreactive insulin (IRI), GIH and oxidative stress marker levels in patients with T2D compared with healthy subjects. We investigated the hypothesis that a processed meat meal would lead to an abnormal postprandial increase in the levels of plasma lipids and oxidative stress markers and impaired GIH responses in T2D patients. Our results should contribute knowledge relevant to the dietary management of diabetic patients.

Methods and Materials

1. Study subjects and design

The protocol for this trial and supporting CONSORT checklist are available as supporting information; see Checklist S1 and Trial protocol S1 and S2. The study used a prospectively randomized crossover design and included a group of 50 patients suffering from T2D and 50 healthy controls. Their characteristics are presented in Table 1. The mean age was 55; about half of the subjects were men; and the mean duration of diabetes among the T2D patients was 9.8 years. Written informed consent was obtained from all participants prior to enrollment in the study; the study protocol, informed consent, and patient information were reviewed and approved by the Ethics Committee of the Thomayer Hospital and Institute for Clinical and Experimental Medicine in Prague, Czech Republic on November 9, 2011. On January 5, 2012 we started the telephone screening of potential participants. Subjects were screened in person and enrolled into the study between April 3, 2012 and April 20, 2012. The first patient entered the intervention phase on April 10, 2012 and the last

Figure 1. Enrollment of the participants and completion of the study.
doi:10.1371/journal.pone.0107561.g001
completed on May 5, 2012. There was no follow-up phase. The study flowchart is presented in Figure 1. Of the 178 patients with T2D and 276 healthy controls, who were screened, 50 participants of both groups were included and randomized, 2 participants with T2D and 1 healthy subject did not complete the trial. The reasons for study participant exclusion are given in Figure 1. Registration on ClinicalTrials.gov was initiated on April 3, 2012 (Identifier: http://clinicaltrials.gov/show/NCT01572402). The authors confirm that all ongoing and related trials for this intervention are registered.

2. Inclusion and exclusion criteria
The inclusion criteria were men and women aged between 30–70 years with a body mass index (BMI) between 27–50 kg/m². Patients diagnosed with T2D had been treated with diet and/or oral hypoglycemic agents (metformin and/or sulfonylureas) for at least one year. Healthy volunteers exhibiting normal glucose tolerance and without metabolic syndrome or diagnosed diabetes among first-line relatives were included. Subjects exhibiting renal, liver or thyroid disease, drug abuse, including alcoholism, and pregnant or breastfeeding women were excluded.

3. Study procedure
All measurements were performed on an outpatient basis after a 10- to 12-h overnight fast with only tap water allowed ad libitum. The diabetic patients did not take any of their diabetes medication the evening or the morning before the assessments. The postprandial state was measured after intake of a standard breakfast – one of two isocaloric test meals in a random order consisting of either a processed meat burger meal (a fastfood burger: cooked-pork seasoned meat in a wheat bun with sesame seeds, tomato, cheddar-type cheese, lettuce, spicy sauce, onion) or a plant-based burger meal (a couscous burger: boiled couscous, baked with onion, garlic, plant oil, spices, oat-flakes in a wheat bun with sesame seeds). For each intervention visit, the meals were delivered fresh from the manufacturer. The study nurses generated via enzymatic methods (Roche, Basel, Switzerland). Plasma lipid concentrations were determined using Insulin and C-peptide IRMA kits (Immunotech, Prague, Czech Republic), respectively. Plasma lipid concentrations were measured via enzymatic methods (Roche, Basel, Switzerland).

4. Analytic methods
4.1 Metabolic parameters. Plasma glucose was analyzed via the glucose-oxidase method using a Beckman Analyzer (Beckman Instruments Inc., Fullerton, CA, USA). Serum immunoreactive insulin and C-peptide concentrations were determined using Immunotech and C-peptide IRMA kits (Immunotech, Prague, Czech Republic), respectively. Plasma lipid concentrations were measured via enzymatic methods (Roche, Basel, Switzerland).

4.2 Gastrointestinal and appetite hormones. Protease and dipeptidyl peptidase-4 inhibitors were added to two samples at each time point. The concentrations of glucagon-like peptide –1 (GLP-1), gastric inhibitory peptide (GIP), pancreatic polypeptide (PP), peptide YY (PYY), leptin and ghrelin were determined via multiplex immunoanalyses based on xMAP technology using a MILLIPLEX MAP Human Gut Hormone Panel (Millipore, Billerica, MA, USA) and a Luminex 100 IS analyzer (Luminex Corporation, Austin, TX, USA) [17]. The assay sensitivities, expressed as the minimum detectable concentrations reported in the instructions for use by the manufacturer, are (in pg/mL): ghrelin 1.8; leptin 157.2; GIP 0.2; GLP-1 5.2; PP 2.4; and PYY 8.4. For our measurements, the sensitivity was set as the value of the lowest standard concentration: ghrelin 13.7; GIP 2.7; GLP-1 13.7; and PP 13.7. For other analytes, there were no values below the corresponding calibration range.

4.3 Oxidative stress markers. The levels of lipid peroxidation were determined via a home-made method using thiobarbituric acid reactive substances (TBARS) [18]. The activity of superoxide dismutase (SOD) was analyzed using a SOD assay kit (Sigma-Aldrich, St Louis, MO, USA). The serum level of ascorbic acid was measured via a home-made method [19]. The level of reduced glutathione in whole blood was determined using a glutathione HPLC diagnostic kit (Chromsystems, Munich, Germany).

Table 1. General characteristics of the Diabetic and Control Population.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients with T2D (n = 50)</th>
<th>Healthy Controls (n = 50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age – years</td>
<td>56±6</td>
<td>54±8</td>
</tr>
<tr>
<td>Male – No. (%)</td>
<td>23 (46)</td>
<td>23 (46)</td>
</tr>
<tr>
<td>Female – No.(%)</td>
<td>27 (54)</td>
<td>27 (54)</td>
</tr>
<tr>
<td>Smokers – No. (%)</td>
<td>11 (22)</td>
<td>7 (14)</td>
</tr>
<tr>
<td>Weight – kg</td>
<td>96.5±17</td>
<td>71±11</td>
</tr>
<tr>
<td>BMI – kg.m⁻²</td>
<td>33.3±5.6</td>
<td>24.4±2.5</td>
</tr>
<tr>
<td>Waist – cm</td>
<td>107±13.0</td>
<td>85±8.0</td>
</tr>
<tr>
<td>Hips – cm</td>
<td>115±12.0</td>
<td>98±5.0</td>
</tr>
<tr>
<td>HbA1c (IFCC) – %</td>
<td>7.0±3.2</td>
<td>5.6±2.4</td>
</tr>
<tr>
<td>HbA1c (IFCC) – mmol/mol</td>
<td>53.7±12.0</td>
<td>37.3±2.7</td>
</tr>
<tr>
<td>Fasting glucose level – mmol/l</td>
<td>8.0±3.1</td>
<td>5±0.44</td>
</tr>
<tr>
<td>Duration of diabetes – years</td>
<td>9.8±6.3</td>
<td></td>
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</table>

Data are means ± SD.
doi:10.1371/journal.pone.0107561.t001

PLOS ONE | www.plosone.org 3 September 2014 | Volume 9 | Issue 9 | e107561
5. Statistical analyses

For statistical analysis, repeated-measures ANOVA was performed. An estimate of the number of subjects that we recruited was carried out using a power analysis of repeated measurements via PASS 2005 statistical software (Number Cruncher Statistical Systems, Kaysville, UT, USA). The power analysis was completed for the most important variables and the number of subjects should provide the power >0.8 for all these variables. The factors of subject, group, meal and time were included in the analyses. The interactions between meal and time (meal×time) were calculated for each variable. ANOVA was followed by least significant difference (LSD) multiple comparisons post-hoc analysis. The original dependent variable was subjected to a power transformation to obtain constant variance and a symmetric distribution of the data and residuals [20]. The data are presented as the means with 95% CI. We have checked the carry-over effect using the model including the factor “sequence” and we have not found any significance of this factor for any dependent variables.

Results

1. Glucose, C-peptide, insulin and lipid responses

The plasma concentrations of glucose, IRI, C-peptide and lipids during the fasting state and their postprandial profiles are illustrated in Fig. 2. All of these measured parameters were significantly higher in the T2D patients than in healthy controls at nearly every time point. The fasting concentrations did not differ before the M- and V-meals in both groups. In both the diabetic and healthy subjects, the postprandial plasma levels of glucose were significantly higher after the V-meal than the M-meal at any one time point – at peak blood glucose level, after 30 min in healthy subjects and 60 min in patients with T2D. The two different meals induced relatively similar glucose responses in both groups when the time-course is considered. Although the M-meal resulted in a significantly lower IRI and C-peptide response than the V-meal in healthy subjects, these levels decreased more slowly, and after 180 min the IRI after the M-meal was significantly higher in both groups of subjects. In the T2D patients, the postprandial increases in IRI and C-peptide were not significantly different after both diets, and the peak of the IRI curve was considerably postponed. After the M-meal, hyperinsulinemia persisted longer in both groups. The plasma concentration of triglycerides inversely correlated to the plasma concentration of free fatty acids. The M-meal resulted in a significantly higher postprandial increase in triglycerides and a further decrease in free fatty acids in both groups, and these differences were more pronounced among the patients with T2D.

2. GIHs

The basal concentrations of nearly all of the GIHs were significantly increased in the T2D patients compared with the healthy controls. There was a considerable difference in the GIH response after ingestion of the M-meal compared with the V-meal in both healthy subjects and the T2D patients (Fig. 3). The V-meal resulted in a significantly higher GIH response than the M-meal but only among the T2D patients. In healthy subjects, the postprandial GIH levels were significantly lower after the V-meal than after the M-meal. The largest difference was detected in the postprandial concentrations of PP and PYY. In the T2D patients, the postprandial concentrations of GLP-1, GIP and PYY were significantly higher after the V-meal. The largest difference between the two meals was detected in the postprandial secretion of GLP-1, both with respect to quantity and dynamics.

3. Appetite hormones

The concentrations of ghrelin and leptin differed significantly between the T2D patients and the healthy controls throughout the entire meal assessment (Fig. 3). In the fasting state, the plasma concentration of ghrelin was 56% lower in the diabetic subjects (p<0.001), and the plasma concentration of leptin was 150% higher than the healthy controls (p<0.001). The plasma concentration of ghrelin was significantly lower and that of leptin was significantly higher in the T2D patients throughout the entire meal assessment, with no difference between the two isocaloric diets. The physiological postprandial suppression of ghrelin secretion was less pronounced in the diabetic subjects than in the healthy controls, and there was a rapid decrease in the ghrelin level beyond the first 60 min after consumption, and the postprandial decrease was significantly larger after the M-meal among healthy controls (p<0.001).

We have added a subanalysis by adding a “baseline ghrelin” factor to the model. The baseline ghrelin was the most important factor when included in the model with F = 187, p<0.0001. It means that the higher the baseline level of ghrelin is, the larger the drop. The difference between the two meals remained significant with F = 19.43, p<0.001.

4. Oxidative stress parameters

The fasting concentrations of all of the oxidative stress markers differed between the groups (Fig. 4). In the T2D patients,
Figure 2. Postprandial changes in plasma concentrations of glucose, immunoreactive insulin, C-peptide, triglycerides and free fatty acids in patients with diabetes (T2D, full line) (n = 48) and healthy controls (HC, dashed line) (n = 49) after ingestion of the V-meal (circles) and the M-meal (squares). Data are expressed as mean with 95%CI. Repeated-measures ANOVA was performed. A: Plasma glucose: Healthy controls: Factors diet: p = 0.0001; time: p < 0.0001; interaction diet × time: p = 0.0001; T2D: diet: p = 0.3767; time: p < 0.0001; interaction diet × time: p = 0.708. B: Immunoreactive insulin: Healthy controls: Factors diet: p = 0.9156; time: p < 0.0001; interaction diet × time: p < 0.0001; T2D: diet: p = 0.1084; time: p < 0.0001; interaction diet × time: p = 0.098. C: C-peptide: Healthy controls: Factors diet: p < 0.0001; time: p < 0.0001; interaction diet × time: p < 0.0001; T2D: diet: p = 0.0713; time: p < 0.0001; diet × time: p = 0.6615. D: Triglycerides: Healthy controls: Factors diet: p = 0.0001; time: p < 0.0001; interaction diet × time: p < 0.0001; T2D: diet: p < 0.0001; time: p < 0.0001; interaction diet × time: p = 0.0192. E: Free fatty acids: Healthy controls: Factors diet: p < 0.0001; time: p < 0.0001; interaction diet × time: p = 0.0003; T2D: diet: p < 0.0001; time: p < 0.0001; interaction diet × time: p = 0.058.

doi:10.1371/journal.pone.0107561.g002
Figure 3. Postprandial changes in plasma concentrations of gastrointestinal and appetite hormones in patients with diabetes (T2D, full line) (n = 48) and healthy controls (HC, dashed line) (n = 49) after ingestion of the V-meal (circles) and the M-meal (squares). Data are expressed as mean with 95%CI. Repeated-measures ANOVA was performed. A: GLP-1: Healthy controls: Factors diet: p = 0.1072; time: p < 0.0001; interaction diet × time: p = 0.2731; T2D: diet: p < 0.0001; time: p < 0.0001; interaction diet × time: p < 0.0001. B: GIP: Healthy controls: Factors diet: p = 0.0128; time: p < 0.0001; interaction diet × time: p = 0.0001; T2D: diet: p = 0.0005; time: p < 0.0001; interaction diet × time: p < 0.0001. C: PP: Healthy controls: Factors diet: p < 0.0001; time: p < 0.0001; interaction diet × time: p = 0.0016; T2D: diet: p = 0.5702; time: p < 0.0001; interaction diet × time: p = 0.0124. D: PYY: Healthy controls: Factors diet: p < 0.0001; time: p < 0.0001; interaction diet × time: p = 0.0768; T2D: diet: p < 0.0001; time: p < 0.0001; interaction diet × time: p = 0.004. E: Ghrelin: Healthy controls: Factors diet: p < 0.0001; time: p < 0.0001; interaction diet × time: p = 0.9983; T2D: diet: p < 0.0001; time: p < 0.0001; interaction diet × time: p = 0.4988.

doi:10.1371/journal.pone.0107561.g003
compared with the control subjects, the levels of TBARS, a measure of lipoperoxidation, were increased by 67% (p < 0.001), while the levels of ascorbic acid, reduced glutathione and SOD activity were decreased by 5%, 13% and 48%, respectively (p < 0.001).

During the postprandial phase, consumption of the M-meal was associated with a significantly greater increase in the TBARS levels in diabetic patients than that of the V-meal. In the healthy controls, no postprandial dynamics of the TBARS levels were detected. The SOD activity in the healthy controls was significantly increased after the V-meal compared with the M-meal. There was no significant change in the plasma concentration of SOD during the postprandial phase in diabetic subjects after either meal. The plasma concentrations of reduced glutathione and ascorbic acid did not change significantly in either patients with diabetes or healthy subjects after either meal.

Discussion

1. Postprandial changes in the plasma concentrations of glucose, lipids and insulin

Our results suggest that after two isocaloric meals containing different carbohydrate contents (M- and V-meals), the postprandial plasma glucose levels during the first 180 min after meal ingestion differed significantly only with respect to the peak glucose levels. Some human studies suggest that other macronutrients can modify the postprandial insulin demand and the glyceremic response and therefore exert an additional impact on the postprandial increase in the glucose levels, as shown in healthy subjects [21] as well as patients suffering from T1D [6] or T2D [22].

Concerning the lipid levels, the significantly higher postprandial increase in the triglyceride levels and the smaller decrease in the free fatty acid levels detected in both groups after the M-meal is not surprising. Free fatty acids are known to impair insulin sensitivity and to enhance hepatic glucose production [2].
Decreases in the levels of these components leads to improvements in insulin sensitivity and glucose tolerance based on intervention studies [23].

We initially detected significantly lower IRI and C-peptide responses after the M-meal than the V-meal in healthy subjects. However, these levels decreasing more slowly, and after 180 min, the level of IRI was significantly higher in both groups after the M-meal. This result indicates that after the M-meal, hyperinsulinemia persisted longer in both groups.

This finding is in accordance with a glucose clamp study of non-diabetic human subjects indicating that increases in the FFA levels lead to insulin resistance within several hours [24]. Several studies confirmed that meals containing higher protein and/or fat contents exert an additional impact on the postprandial insulin demand and the increase in the glucose levels. In patients suffering from type 1 diabetes, high-fat meals containing identical carbohydrate and protein contents required a larger dose of insulin than low-fat meals [5]. Furthermore, addition of protein increases the insulin demand [6].

2. Postprandial changes in the plasma concentrations of GIHs and appetite hormones

Carbohydrate, fat and protein in the lumen of the gut have been demonstrated to stimulate the secretion of a broad range of GIHs [25].

The incretin effect, the postprandial augmentation of insulin secretion by gut hormones, has been primarily associated with the secretion and insulinitropic effect of two GIHs – GIP and GLP-1. The incretin effect is thought to mediate approximately 50–70% of the overall insulin response after a mixed meal or glucose ingestion in healthy subjects [26–27].

It is well understood that in patients with diabetes, the incretin effect is diminished secondarily [28,29], and this result appears to be due to impaired beta cell sensitivity [30].

The mechanisms underlying the loss of incretin activity remain incompletely understood, but several hypotheses have been proposed, including hyperglycemia- and hyperlipidemia-associated receptor desensitization [31,32].

Studies that have examined the GIP and GLP-1 responses after meals differing with respect to caloric and macronutrient content report inconsistent results regarding the contribution of meal composition to the stimulation of these hormones. One study reported a higher GIP response after a mixed meal than after an oral glucose load [33]. Another study demonstrated that fat preferentially stimulated GIP secretion, whereas carbohydrates stimulated GLP-1 secretion, both regardless of the diabetes status [34]. Dietary protein exerted a variable effect on incretin release [35,36].

Our results suggest that the intake of each type of isocaloric meal consumed in amounts typical of normal eating was associated with different postprandial changes in the GIP and GLP-1 plasma levels. In the T2D patients, the V-meal resulted in a larger release of GLP-1 and GIP than the M-meal. This result is in accordance with a study suggesting that the GLP-1 response after fat intake is impaired in T2D patients [37]. In contrast, in healthy individuals, the GLP-1 and GIP responses after ingestion of the V-meal were smaller than after the M-meal. Our data suggest that carbohydrates appear to represent the strongest activator of GLP-1 and GIP secretion among T2D patients, whereas healthy subjects display higher GLP-1, GIP, PYY and PP responses to the mixed meal containing higher fat and protein contents.

In our study, we demonstrated increased basal and postprandial levels of GIP and GLP-1 in the T2D patients compared with the healthy patients. This finding is in accordance with a study that evaluated the GLP-1 secretion levels and indicated relatively unchanged or even elevated secretion in response to oral nutrition intake in subjects suffering from diabetes [38].

Additionally, a recent meta-analysis revealed that T2D patients do not exhibit reduced GLP-1 secretion in response to an OGTT or a meal test [39]. With respect to GIP secretion, most studies quantifying GIP secretion reported that the GIP levels are normal or even higher in T2D subjects compared with healthy controls [40]. However, reductions in the GLP-1 levels were found in patients suffering from a long disease duration and poor compensation [41]. A recent meta-analysis revealed a negative influence of HbA1c levels on plasma GLP-1 responses [39].

Other gastrointestinal peptides play a role in the regulation of energy intake, appetite and overall energy homeostasis in humans. They are secreted by different cells in the intestine and from the pancreas, and their release is modulated by food ingestion. PYY is co-secreted predominantly from the endocrine L-cells in the ileum together with GLP-1. PYY plays an important regulatory role in GIT function [42].

PP is secreted from entodermic cells in the pancreas. Both PYY and PP, when infused intravenously, reduce appetite and energy intake in healthy humans [43]. They are secreted in response to all three macronutrients, with fat being the most potent stimulus. In healthy humans, it was shown that the presence of fat in the small intestine induced stimulation of PYY and PP in a manner dependent on fat digestion and the presence of free fatty acids [44]. This finding is in accordance with our results: in healthy subjects, we detected significantly greater secretion of PYY and PP after ingestion of the M-meal compared with the V-meal.

The course of PYY secretion consisted of two phases. The initial rise in the PYY level is likely due to a link to the proximal small intestine, and the continuous rise reflects the direct contact of lipids with the distal small intestine [45]. This result was not detected in our diabetic group of patients; their postprandial response with respect to both peptides was significantly higher after the V-meal. Similarly, a previous study suggested that both insulin resistance and abnormal glucose metabolism impaired the PYY response to fat intake [37]. To the best of our knowledge, this is the first report of the different secretory responses of PP after both isocaloric meals in T2D patients compared with healthy controls.

Ghrelin stimulates appetite and food intake and is secreted in the stomach. In healthy individuals, the ghrelin plasma concentrations increase during fasting and are suppressed by meal intake [46], likely via postprandial hyperinsulinemia [47,48].

We detected postprandial suppression of ghrelin secretion during the first 60 min after meal ingestion. The postprandial decrease depended significantly on the baseline level of ghrelin, which means that the higher the baseline level of ghrelin is, the larger the drop. Furthermore, it was significantly greater after the M-meal in healthy subjects. This finding is in accordance with a study that demonstrates that fat digestion is required for the suppression of ghrelin [44]. The related changes in ghrelin could be deemed positive with respect to the meat meal. We detected lower fasting and postprandial plasma concentrations of ghrelin and a diminished postprandial suppression of ghrelin secretion in the T2D patients, with no difference between the two meals in time.

3. Postprandial changes in the plasma concentrations of oxidative stress markers

The postprandial state, characterized by hyperglycemia and hyperlipidemia, is associated with increased oxidative stress in...
patients suffering from diabetes [49]. In our group of T2D patients, we detected significantly higher fasting concentrations of all oxidative stress markers and significantly lower concentrations of blood antioxidants. Previous studies support the concept that increased oxidative stress may play an important role in T2D manifestation [10]. Dietary fat quality has been proposed to be a critical factor. Several studies have suggested that a high intake of saturated fatty acids naturally present in meat contributes to the risk of glucose intolerance [11,12]. Based on an intervention study, humans suffering from metabolic syndrome who were consuming a high saturated fatty acid diet displayed higher levels of oxidative stress markers postprandially [13,14]. We found that in diabetic patients during the postprandial phase, the processed meat meal was associated with a significantly greater increase in the products of oxidative damage, namely TBARS, compared with the plant based meal. This finding could also be due to the method of cooking at a high temperature. It has been reported that most of the lipid-peroxidation products ingested from popular foods are derived from meat products and high-fat processed foods such as red meats cooked at high temperatures [50]. Another study has shown that adding antioxidants to hamburger meat could significantly reduce the formation of lipid-peroxidation products during cooking [51].

4. Strengths and limitations of the study

The strength of our study was that we took a physiological approach to measure the postprandial state. We selected two simple isocaloric meals at amounts corresponding to those ingested during a typical meal. The M-meat was rich in both saturated fat and protein; thus, we cannot separate the effect of each macronutrient. This study also had several limitations. First, the T2D patients exhibited a significantly higher body weight and BMI compared with the control subjects, and these differences may have affected some of the responses reported. The object of our study was to compare the effect of the two different meals within each group (patients with T2D and healthy subjects). In healthy subjects, no effect of adiposity on the postprandial GIP and GLP-1 levels has been reported [52]. The effect of obesity on other GIHs is unclear.

In this study, we primarily aimed to compare the two meals rather than the two groups of subjects.

Finally, the 3-h study duration might be insufficient for complete absorption of the nutrients, and this limitation may have led to an underestimation of the measured parameters.

Conclusions

In conclusion, according to our hypothesis, our results suggest that a processed meat meal is accompanied by an impaired GIH response and increased oxidative stress marker levels in diabetic patients. Our results revealed comparable glycemic responses to isocaloric meals containing different carbohydrate contents, despite the distinct peak glucose concentrations. This finding illustrates that the diet composition and the energy content, rather than the carbohydrate count or the glycemic load, should be important considerations for the dietary management of diabetes.

Supporting Information

Checklist S1 CONSORT Checklist. (DOC)

Protocol S1 Trial Protocol in English. (DOC)

Protocol S2 Trial Protocol in Czech. (DOC)

Acknowledgments

We thank Thomas O’Hearn for great help with text corrections and we thank our nurses for their technical assistance and care provided to the study participants.

Author Contributions

Conceived and designed the experiments: LB HK HM TP. Performed the experiments: HM OO OT JV. Analyzed the data: LB HK HM TP. Contributed reagents/materials/analysis tools: LB HK HM LK OO. Performed the experiments: HM OO OT JV LK. Analyzed the data: LB HK HM MH.

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PLOS ONE | www.plosone.org 9 September 2014 | Volume 9 | Issue 9 | e107561

PLOS ONE | www.plosone.org 9 September 2014 | Volume 9 | Issue 9 | e107561


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Postprandial Oxidative Stress and Gastrointestinal Hormones: Is There a Link?

Abstract

**Background:** Abnormal postprandial elevation of plasma glucose and lipids plays an important role in the pathogenesis of diabetes and strongly predicts cardiovascular mortality. In patients suffering from type 2 diabetes (T2D) postprandial state is associated with oxidative stress, cardiovascular risk and, probably, with impairment of both secretion and the effect of gastrointestinal peptides. Evaluating postprandial changes of gastrointestinal hormones together with changes in oxidative stress markers may help to understand the mechanisms behind the postprandial state in diabetes as well as suggest new preventive and therapeutical strategies.

**Methods:** A standard meal test has been used for monitoring the postprandial concentrations of gastrointestinal hormones and oxidative stress markers in patients with T2D (n = 50) compared to healthy controls (n = 50). Blood samples were drawn 0, 30, 60, 120 and 180 minutes after the standard meal.

**Results:** Both basal and postprandial plasma concentrations of glucose and insulin proved to be significantly higher in patients with T2D, whereas plasma concentrations of ghrelin showed significantly lower values during the whole meal test. In comparison with healthy controls, both basal and postprandial concentrations of almost all other gastrointestinal hormones and lipoperoxidation were significantly increased while ascorbic acid, reduced glutathione and superoxide dismutase activity were decreased in patients with T2D. A positive relationship was found between changes in GIP and those of glucose and immunoreactive insulin in diabetic patients (p < 0.001 and p < 0.001, respectively) and between changes in PYY and those of glucose (p < 0.01). There was a positive correlation between changes in GIP and PYY and changes in ascorbic acid in patients with T2D (p < 0.05 and p < 0.001, respectively).

**Conclusion/Interpretation:** Apart from a positive relationship of postprandial changes in GIP and PYY with changes in ascorbic acid, there was no direct link observed between gastrointestinal hormones and oxidative stress markers in diabetic patients.

**Trial Registration:** ClinicalTrials.gov NCT01572402

Introduction

The postprandial dysmetabolism plays an important role in the pathogenesis of type 2 diabetes (T2D) and its complications. Abnormal postprandial elevation of plasma glucose and lipids is closely tied to insulin resistance and may occur in the absence of overt T2D. Postmeal hyperglycemia and hyperlipidemia increases the risk of cardiovascular diseases in diabetic patients and may predict cardiovascular risk more strongly than fasting values or even long-term parameters such as glycated hemoglobin [1].

In patients with T2D, acute hyperglycemia and hypertriglyceridemia lead to endothelial dysfunction, induce oxidative stress, increase the inflammatory milieu, affect coagulation, and, probably, impair secretion and diminish effect of gastrointestinal peptides [2].

Incretin hormones, which are released from the gastrointestinal tract in response to nutrient ingestion to enhance glucose-dependent insulin secretion, aid the overall maintenance of glucose homeostasis through slowing of gastric emptying, inhibition of glucagon secretion and control of body weight [3]. Two incretins - glucagon-like peptide-1 (GLP-1) (which has received the most pharmacological attention), and gastric inhibitory peptide (GIP) - were found to exert major glucoregulatory actions [4]. The impaired incretin effect may contribute to delayed and attenuated


Editor: Zane Andrews, Monash University, Australia

Received October 23, 2013; Accepted July 2, 2014; Published August 20, 2014

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Funding: This work was supported by Ministry of Health, Czech Republic, conceptual development of research organisation ("Institute for Clinical and Experimental Medicine – IČM, IN 00023001"), by the Internal Grant Agency of the Ministry of Health of the Czech Republic (NT/11236-4) and by the Grant Agency of Charles University – GAUK No 702312. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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insulin response during a meal in T2D [5,6,7]. The mechanism which would make clear the diminished effect of gastrointestinal hormones in patients with T2D is not completely understood. It is not clear whether the loss of incretin secretion is a cause or rather a consequence of hyperglycemia.

Appetite hormones, ghrelin and leptin, are also known to play a prominent role in glucose homeostasis and the regulation of energy. Changes in plasma concentrations of ghrelin and leptin in diabetic patients are strongly associated with hyperinsulinemia and are probably of great importance for the pathogenesis of diabetes [8].

According to recent studies, oxidative stress is supposed to be the link between acute postprandial hyperglycemia and cardiovascular risk in patients with T2D [9]. In some studies, several markers of oxidative damage such as TBARS [10], isoprostanes [11] and protein carbonyls [12] have been found to increase 2–3 hours after an oral glucose load (OGTT). However, there is still lack of information about the relationship of oxidative stress, gastrointestinal and appetite hormones, particularly during the postmeal phase.

Evaluating the effect of gastrointestinal hormones together with changes in oxidative stress markers may contribute to better understanding of the mechanisms underlying the postprandial state in patients suffering from T2D and thus suggest new preventive and therapeutical strategies. A standard meal test was used for monitoring the postprandial concentrations of gastrointestinal hormones and oxidative stress markers in patients with T2D compared to healthy controls. To the best knowledge of the authors, they are the first ones to try to find a link between postprandial oxidative stress and gastrointestinal hormones in a clinical and physiological setting.

Materials and Methods

Study subjects and design

The study group consisted of 50 patients with T2D and 50 healthy controls. Their characteristics are featured in Table 1. The mean age was 55 years, approximately 50% of the subjects were men, the mean duration of diabetes in diabetic subjects was 9.8 years. The study protocol was approved by the Ethics Committee of the Thomayer Hospital and Institute for Clinical and Experimental Medicine in Prague, Czech Republic. All participants have signed a written informed consent. Clinical Trial.gov number, NCT01572402. The protocol for this trial and supporting CONSORT checklist are available as supporting information; see checklist S1 and Protocol S1.

Eligibility criteria for participants were set as following: age 30 to 70 years, both genders. Inclusion criteria – diabetes duration at least 1 year, BMI 27–50 kg/m2, exclusion criteria – insulin therapy.

Procedures

All measurements were taken on an outpatient basis, after 10-h to 12-h overnight fasting with only tap water allowed ad libitum. In this single-center study the samples were collected at the Laboratory of Clinical Pathophysiology in Institute for Clinical and Experimental Medicine.

Standard meal tests.

Postprandial state was tested after stimulation with a standard breakfast (The Baguette Cheese Gourmet produced by Crocodile, 453 kcal, 45% carbohydrates, 17% proteins, 38% lipids). This is the part of the randomised clinical study, where we observed the postprandial effect after three different sandwiches in the random order in patients with T2D and healthy controls. Presented data relate to the cheese sandwich only. The nurses engaged in the study generated the random sequence of the meals and assigned participants to interventions. Neither the study staff nor the participants could be blinded to the content of the meals. The participants ate the sandwiches in the laboratory under the observation by nurses.

Plasma glucose, immunoactive insulin, C-peptide, triglycerides, free fatty acids, oxidative stress markers and gastrointestinal hormones were all measured after 0, 30, 60, 120, and 180 minutes.

Analytic methods.

Blood samples were drawn in the fasting state and then 30, 60, 120 and 180 minutes after the standard meal. Protease and Dipeptidyl peptidase-4 inhibitors were added into two samples at each time point. Plasma glucose was analysed using the Beckman Analyzer glucose-oxidase method (Beckman Instruments Inc., Fullerton, CA, USA). Serum immunoreactive insulin and C-peptide concentrations were determined using Insulin and C-peptide IRMA kits (Immunotech, Prague, Czech Republic). Plasma lipids were measured using enzymatic methods (Roche, Basel, Switzerland).

Gastrointestinal and appetite hormones: Concentrations of GLP-1, GIP, amylin, pancreatic polypeptide (PP), peptide YY (PYY), leptin and ghrelin were determined by multiplex immunoanlyses based on the xMAP technology using MILLIPLEX MAP Human Gut Hormone Panel (Millipore, Billerica, MA, USA) and Luminex 100 IS instrument (Luminex Corporation, Austin, USA).

Oxidative stress markers: The amount of lipid peroxidation was determined as thiobarbituric acid reactive substances (TBARS) using a modified method according to Yokode [13]. The activity of superoxide dismutase (SOD) was analyzed by superoxide dismutase assay kit (Cayman Chemical, MI, USA). The serum level of ascorbic acid was measured by the spectrophotometric method as previously described [14]. The whole blood level of reduced glutathione was determined with the Glutathione HPLC diagnostic kit (Chromsystems, Munich, Germany).

Statistical analyses

For statistical analysis, repeated-measures ANOVA was used. The factors of group, subject and time were included in the model. Interactions between group and time (group × time) were calculated for each variable. Within each group, paired comparison t-tests were calculated to test whether the changes from baseline to 30’, from 30’ to 60’, from 60’ to 120’ and from 120’ to 180’ were statistically significant. Pearson correlations were calculated for the relationship between changes in oxidative stress markers and changes in gastrointestinal hormones. Data are presented as mean with 95% CI.

Results

The number of participants included and dates defining the periods of recruitment and follow-up are shown in Figure 1. The authors have not observed harms or unintended effects of consumed meals of any kind in participants.

The plasma concentrations of glucose, lipids, IRI and C-peptide in fasting and postprandial state after the standard meal test are illustrated in Figure 2. All these measured parameters were significantly higher in diabetic subjects than in healthy controls at virtually every time point after the standard meal. Plasma concentrations of triglycerides were inversely related to plasma concentrations of free fatty acids in both diabetic and healthy subjects.
Gastrointestinal hormones

Both basal and postprandial concentrations of almost all gastrointestinal hormones were significantly higher in patients with T2D compared to healthy controls (see Figure 3). The most notable differences between diabetics and healthy controls were observed in postprandial secretion of amylin, GLP and PP, in both

Table 1. General characteristics of the Diabetic and Control Population.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Diabetics (n = 50)</th>
<th>Controls (n = 50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age – years</td>
<td>56±6</td>
<td>54±8</td>
</tr>
<tr>
<td>Male - No. (%)</td>
<td>23 (46)</td>
<td>23 (46)</td>
</tr>
<tr>
<td>Female - No. (%)</td>
<td>27 (54)</td>
<td>27 (54)</td>
</tr>
<tr>
<td>Smokers – No. (%)</td>
<td>11 (22)</td>
<td>7 (14)</td>
</tr>
<tr>
<td>Weight – kg</td>
<td>97±17</td>
<td>71±11</td>
</tr>
<tr>
<td>BMI – kg.m⁻²</td>
<td>33.3±5.6</td>
<td>24.4±2.5</td>
</tr>
<tr>
<td>Waist – cm</td>
<td>107±13</td>
<td>85±8</td>
</tr>
<tr>
<td>Hips – cm</td>
<td>115±12</td>
<td>98±5</td>
</tr>
<tr>
<td>HbA1c (DCCT) – %</td>
<td>7.0±3.2</td>
<td>5.6±2.4</td>
</tr>
<tr>
<td>HbA1c (IFCC) – mmol/mol</td>
<td>53.7±12.0</td>
<td>37.3±2.7</td>
</tr>
<tr>
<td>Fasting glucose level – mmol/l</td>
<td>8.0±3.1</td>
<td>5.0±0.4</td>
</tr>
<tr>
<td>Duration of diabetes – years</td>
<td>9.8±6.3</td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SD.

doi:10.1371/journal.pone.0103565.t001

Figure 1. Enrollment of the participants and completion of the study.
doi:10.1371/journal.pone.0103565.g001
quantity as well as dynamics (Figure 3). However, there were differences in dynamics between individual gastrointestinal peptides. The increase in postprandial secretion of GLP and PP was rapid, the maximum peak in postmeal phase was observed after 30 min. On the other hand, the postprandial secretion of amylin increased slowly, the maximum peak in postmeal phase of amylin was observed after 120 min. The postmeal dynamics of GIP secretion was strong, however differences between patients with T2D and healthy controls were not as pronounced as in GLP. The lowest occurrence of postprandial changes was observed in secretion of PYY.

**Appetite hormones**

The concentrations of ghrelin and leptin differ significantly between patients with T2D and healthy controls during the whole meal test as shown in Figure 3 (F and G). In the fasting state,
plasma concentrations of ghrelin were lower in diabetic subjects by 56% and plasma concentrations of leptin were elevated by 150% compared to healthy controls. Plasma concentrations of ghrelin were significantly lower and those of leptin significantly higher in patients with T2D during the whole meal test. The physiological postprandial suppression of ghrelin secretion was not as much notable in diabetic subjects as in healthy controls (see Figure 3). Despite the significant increase of leptin concentrations in patients with T2D, the postprandial dynamics of leptin was slightly notable in both groups.

Oxidative stress parameters

In basal conditions (time 0) all of the measured oxidative stress markers were different in patients with T2D compared to control subjects: TBARS were increased by 67% while ascorbic acid, reduced glutathione and SOD activity were decreased in diabetic subjects by 5%, 13% and 48%, respectively (Figure 4). Lipid peroxidation measured as TBARS increased during the postprandial phase together with the changes in plasma glucose and triglycerides in diabetic patients (P<0.001). In healthy controls no postprandial dynamics of TBARS was observed (Figure 4). There were no significant changes in plasma concentrations of ascorbic acid during the postprandial phase in diabetic patients, while in healthy controls ascorbic acid increased (P<0.01; Figure 4). Plasma concentrations of reduced glutathione or superoxide dismutase activity did not change significantly either in diabetics or in healthy controls.

Correlations

Postprandial secretion of measured gastrointestinal hormones was increased in parallel with glucose and insulin concentrations in patients with T2D. As shown in table 2, a positive relationship was found between Δ GIP and Δ glucose and Δ IRI, between Δ PYY and Δ glucose, and between Δ amylin and Δ IRI and Δ C-peptide in patients with T2D. No significant relationship was observed between the changes in any gastrointestinal hormones and the changes in glucose or insulin in healthy controls (data not shown). Changes in triglycerides correlated negatively with Δ PP and Δ ghrelin and positively with Δ amylin. Changes in ascorbic acid correlated positively with Δ GIP and Δ PYY in diabetic patients.

Discussion

In the study in question the authors monitored postmeal response of gastrointestinal hormones and oxidative stress markers in diabetic patients and compared them with healthy controls. The postmeal phase is an important and independent predictor of macrovascular diabetic complications, more in females than in males [15]. Postprandial hyperglycemia is a stronger cardiovascular risk factor in women than in men, whereas other authors state that gender-related differences disappear after adjustment for the main cardiovascular risk factors [15]. In our study we observed the postprandial glycemic control in the general population and the proportion of women and men was equal.

Elevation of postmeal or postchallenge ghrelin supports the concept of “metabolic memory” [9] which is responsible for early diabetic complications and which is closely tied to oxidative stress, namely with increased mitochondrial superoxide production.

However, few studies were interested in postprandial phase after a meal test, which is more physiological as it contains all main nutrients than the usually used oral glucose tolerance test.

According to Alssema study [18], incretin effect could be diminished after OGTT and after a standard meal test. In this study GLP-1 secretion in diabetic patients was increased following oral glucose but not after the mixed meal [18]. Therefore, incretin secretion seems to depend on both the glucose and lipid metabolism as well.

The incretin effect is diminished secondarily in T2D as a consequence of metabolic and hormonal disturbances [16,17] while increased oxidative stress is directly involved in the pathogenesis of diabetes [26]. The authors focused on clarifying whether these parameters correlated with each other and whether they had mutual influence on each other.

Several studies have shown that the incretin effect is attenuated in T2D because of a severe defect in β-cell sensitivity to GIP [5,6], which has an insulinoergic effect [19]. It has also been suggested that changes in insulin secretion following a lifestyle intervention might be mediated via alterations in GIP secretion [20].

GIP, secreted strongly in response to fat ingestion, is involved in the translation of excessive amounts of dietary fat into adipocyte tissue stores [21]. Patients with T2D are resistant to the biological effects of GIP [22]. Specific GIP receptor antagonists improve glucose tolerance and β-cell function by amelioration of insulin resistance in ob/ob mice [23]. These effects are similar to improvements of metabolism after bariatric surgery in humans [24]. The blockade of GIP action appears promising as a new and potentially important approach to treat obesity-related diabetes [25].

PPY is released postprandially from gastrointestinal L-cells with GLP-1 and oxyntomodulin [28] and has anorexic effects [29]. In healthy humans stimulation of PYY and PP is dependent on fat digestion [30]. In obese subjects, the altered postprandial secretion of PYY is a consequence of a dysfunction of L cells, which become less sensitive to the positive feedback effect of lipids [31].

The positive correlation of changes in amylin, insulin and C-peptide observed by the authors is not surprising. Amylin is a peptide co-secreted with insulin. The role of amylin in the pathogenesis of T2D has been suggested by in vitro and in vivo studies indicating its effect to cause insulin resistance and/or inhibit insulin secretion [32]. It is worth noting that amylin interacts with numerous other gastrointestinal hormones to control eating and mediate the eating inhibitory effect of some of these hormones, most prominently peptide YY and GLP-1 [27]. These combinations lead to a stronger reduction of eating control than single hormones alone. Thus the diminished effect of amylin is possibly important for other gastrointestinal hormones. The positive correlation between postprandial changes in amylin and triglycerides is in accordance with a study which demonstrated a strong association of amylin with inflammatory markers and metabolic syndrome including triglycerides in healthy individuals [33].
On the other hand, postprandial changes in PP associated negatively with triglycerides changes and positively with FFA changes in patients with T2D. As suggested earlier, elevated plasma PP may be viewed as a negative marker and it has been demonstrated that after diet-induced weight loss, the decrease in PP correlated negatively with improvement in β-cell function [34].

To the best knowledge of the authors, the association between PP and postprandial lipids has not been published yet.

We observed lower fasting and postprandial plasma ghrelin and diminished postprandial suppression of ghrelin secretion in patients with T2D. That is in accordance with the previously demonstrated lower concentrations of ghrelin in response to weight gain, overfeeding and a high-fat diet [35]. Metformin
Table 2. Correlation between changes in gastrointestinal hormones, leptin and ghrelin and changes in oxidative stress and metabolic parameters in patients with T2D (n = 45).

<table>
<thead>
<tr>
<th></th>
<th>Δ glu</th>
<th>Δ GIP</th>
<th>Δ GLP</th>
<th>Δ PP</th>
<th>Δ PYY</th>
<th>Δ amylin</th>
<th>Δ ghrelin</th>
<th>Δ leptin</th>
<th>Δ GSH</th>
<th>Δ TBAES</th>
<th>Δ SOD</th>
<th>Δ TBARS</th>
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<tbody>
<tr>
<td>Δ IRI</td>
<td>-0.203</td>
<td>0.2702*</td>
<td>0.0943</td>
<td>-0.2735</td>
<td>-0.2877*</td>
<td>-0.2709**</td>
<td>-0.3203***</td>
<td>-0.3953</td>
<td>-0.3639</td>
<td>-0.2784</td>
<td>0.2553</td>
<td>-0.2524</td>
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<tr>
<td>Δ C-pep</td>
<td>0.3291</td>
<td>0.0693</td>
<td>0.0335</td>
<td>0.1239</td>
<td>0.0933</td>
<td>0.0093</td>
<td>-0.1929</td>
<td>0.0739</td>
<td>0.0817</td>
<td>0.0933</td>
<td>0.0933</td>
<td>0.0093</td>
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<tr>
<td>Δ Tg</td>
<td>-0.0215</td>
<td>0.0034</td>
<td>-0.0475</td>
<td>-0.0373</td>
<td>-0.0191</td>
<td>-0.0035</td>
<td>-0.1202</td>
<td>-0.0330</td>
<td>-0.0029</td>
<td>-0.0490</td>
<td>0.0146</td>
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<tr>
<td>Δ FFA</td>
<td>0.2005</td>
<td>0.0016</td>
<td>-0.2129</td>
<td>-0.2225</td>
<td>-0.2077*</td>
<td>-0.2529**</td>
<td>-0.1135</td>
<td>0.0037</td>
<td>0.0097</td>
<td>-0.0246</td>
<td>0.0097</td>
<td>-0.0097</td>
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<tr>
<td>Δ AA</td>
<td>0.5196***</td>
<td>0.5420***</td>
<td>0.2129</td>
<td>0.0943</td>
<td>0.0034</td>
<td>0.1239</td>
<td>0.0933</td>
<td>0.0668</td>
<td>0.0191</td>
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*denote p < 0.05,
** denote p < 0.01,
*** denote p < 0.001.
are, probably, two independent mechanisms in diabetes and it should be considered in therapeutic approach.

Supporting Information

Checklist S1 CONSORT Checklist. (DOC)

Protocol S1 Trial Protocol. (DOC)

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Acknowledgments

We thank Vojtech Klimt and Thomas O’Hearn for great help with text corrections and we thank the patients and volunteers for their long-standing collaboration in the course of this study.

Author Contributions

Conceived and designed the experiments: TP HK. Performed the experiments: HK LB TP. Analyzed the data: HM OO OT JV. Contributed reagents/materials/analysis tools: HM OO OT JV. Wrote the paper: HM HK LP TP.

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PLOS ONE | www.plosone.org 9 August 2014 | Volume 9 | Issue 8 | e103565
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Eating two larger meals a day (breakfast and lunch) is more effective than six smaller meals in a reduced-energy regimen for patients with type 2 diabetes: a randomised crossover study

Hana Kahleova · Lenka Belinova · Hana Malinska · Olena Oliyarnyk · Jaroslava Trnovska · Vojtech Skop · Ludmila Kazdova · Monika Dezortova · Milan Hajek · Andrea Tura · Martin Hill · Terezie Pelikanova

Received: 22 January 2014 / Accepted: 9 April 2014 / Published online: 18 May 2014
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Abstract
Aims/hypothesis The aim of the study was to compare the effect of six (A6 regimen) vs two meals a day, breakfast and lunch (B2 regimen), on body weight, hepatic fat content (HFC), insulin resistance and beta cell function.
Methods In a randomised, open, crossover, single-centre study (conducted in Prague, Czech Republic), we assigned 54 patients with type 2 diabetes treated with oral hypoglycaemic agents, both men and women, age 30–70 years, BMI 27–50 kg/m$^2$ and HbA$_1c$ 6–11.8% (42–105 mmol/mol), to follow two regimens of a hypoenergetic diet, A6 and B2, each for 12 weeks. Randomisation and allocation to trial groups ($n=27$ and $n=27$) were carried out by a central computer system. Individual calculations of energy requirements for both regimens were based on the formula: (resting energy expenditure×1.5)−2,092 kJ. The diet in both regimens had the same macronutrient and energy content. HFC was measured by proton magnetic resonance spectroscopy. Insulin sensitivity was measured by isoglycaemic–hyperinsulinaemic clamp and calculated by mathematical modelling as oral glucose insulin sensitivity (OGIS). Beta cell function was assessed during standard meal tests by C-peptide deconvolution and was quantified with a mathematical model. For statistical analysis, 2×2 crossover ANOVA was used.
Results The intention-to-treat analysis included all participants ($n=54$). Body weight decreased in both regimens ($p<0.001$), more for B2 ($−2.3$ kg; 95% CI $−2.7$, $−2.0$ kg for A6 vs $−3.7$ kg; 95% CI $−4.1$, $−3.4$ kg for B2; $p<0.001$). HFC decreased in response to both regimens ($p<0.001$), more for B2 ($−0.03%$; 95% CI $−0.033%$, $−0.027%$ for A6 vs $−0.04%$; 95% CI $−0.041%$, $−0.035%$ for B2; $p=0.009$). Fasting plasma glucose and C-peptide levels decreased in both regimens ($p<0.001$), more for B2 ($p=0.004$ and $p=0.04$, respectively). Fasting plasma glucagon decreased with the B2 regimen ($p<0.001$), whereas it increased ($p=0.04$) for the A6 regimen ($p<0.001$). OGIS increased in both regimens ($p<0.01$), more for B2 ($p=0.01$). No adverse events were observed for either regimen.
Conclusions/interpretation Eating only breakfast and lunch reduced body weight, HFC, fasting plasma glucose, C-peptide and glucagon, and increased OGIS, more than the same caloric restriction split into six meals. These results suggest that, for type 2 diabetic patients on a hypoenergetic diet, eating larger breakfasts and lunches may be more beneficial than six smaller meals during the day.
Trial registration ClinicalTrials.gov number, NCT01277471, completed.
Funding Grant NT/11238-4 from Ministry of Health, Prague, Czech Republic and the Agency of Charles University – GAUK No 702312.
Keywords Hepatic fat content · Insulin sensitivity · Two meals a day · Type 2 diabetes

Abbreviations
A6 Six meals a day regimen
B2 Two meals a day regimen
HFC Hepatic fat content
MCR Metabolic clearance rate of glucose
OGIS Oral glucose insulin sensitivity
REE Resting energy expenditure

Introduction

Frequency of meals is an important aspect of nutrition, with profound effects on human health and lifespan. Excessive energy intake is associated with an increased incidence of chronic diseases including diabetes and is a leading cause of disability and death in Western countries [1]. A hypoenergetic diet is crucial for both the prevention and treatment of type 2 diabetes. It is usually consumed as five or six small meals per day. Eating more frequently is presumed to reduce hunger and thus reduce energy intake and body weight. However, the effects of meal frequency on human health and longevity are unclear [2]. Reduced meal frequency can prevent the development of chronic diseases and extend the lifespan in laboratory animals due to lower oxidative damage and higher stress resistance [3, 4]. Mice under time-restricted feeding have an equivalent energy intake from a high-fat diet as those with ad libitum access yet are protected against obesity, hyperinsulinaemia and hepatic steatosis [5, 6]. Intermittent fasting leads to a prolonged lifespan and positively affects glucose tolerance, insulin sensitivity and incidence of type 2 diabetes in mice [3, 4]. There is also emerging literature demonstrating a relationship between the timing of feeding and weight regulation in animals.

Observational trials in humans indicate that eating more often than three times a day may play a role in overweight and obesity [7] and that frequent eating predisposes to a higher energy intake by increasing food stimuli and difficulty controlling energy balance [8]. In a randomised controlled study, more frequent eating was not related to a greater reduction in energy intake or body weight [9]. In type 2 diabetic patients it has been demonstrated that it may be more beneficial for glycaemic control to eat one larger instead of two smaller meals, provided the diet is rich in fibre [10].

It has been demonstrated that a large isocaloric mixed meal causes a greater postprandial thermogenic response than the same food consumed in six smaller portions [11]. Observational data suggest that eating meals later in the day may influence the success of weight-loss therapy, even in humans [12]. It has also been shown that fat storage increases during the day and is the greatest after an evening meal [13]. It has been observed that eating breakfast regularly may protect against weight gain, despite a higher total daily energy intake [14].

To the best of our knowledge, no interventional trials have investigated the relationship between eating frequency and weight change together with hepatic fat content (HFC), glucose tolerance and insulin resistance in humans, especially in patients with type 2 diabetes. The aim of our study was to compare the effect of six vs two meals a day (breakfast and lunch, as this regimen allows a reasonable fasting time, yet is sustainable in the long term) with the same caloric restriction on body weight, HFC, insulin resistance and beta cell function in individuals with type 2 diabetes. It was hypothesised that eating only breakfast and lunch would reduce body weight and HFC (and consequently, improve insulin resistance and beta cell function) more than six meals a day would.

Methods

Participants

Out of the 219 individuals screened, 54 patients with type 2 diabetes (with disease duration of more than 1 year) treated by oral hypoglycaemic agents (both men and women), age 30–70 years, BMI 27–50 kg/m² and HbA1c 6–11.8% (42–105 mmol/mol), met all the inclusion criteria, gave their written informed consent and underwent randomisation. Exclusion criteria comprised alcohol or drug abuse, pregnancy or lactation, unstable medication or weight in the last 3 months, a diagnosis of type 1 diabetes and the presence of a cardiostimulant.

Study design

We used a randomised crossover study design. The study protocol was approved by the Institutional Ethical Committee. In a single-centre study, after a 1 month run-in period (when the patients learned how to write their food diaries and use the pedometers and glucometers), the participants began a 12 week regimen of either six (A6) or two (B2) meals a day. The A6 regimen consisted of three main meals (breakfast, lunch and dinner), and three smaller snacks in between. The B2 regimen consisted of breakfast (eaten between 06:00 and 10:00 hours) and lunch (eaten between 12:00 and 16:00 hours). The regimens were switched for the subsequent 12 weeks. All measurements were performed at weeks 0 (baseline), 12 and 24 (Fig. 1 and Table 1).

Diet

The composition of the diet in both regimens followed the Study Group on Diabetes and Nutrition of the European
Association for the Study of Diabetes guidelines [15] with the same caloric restriction: a restriction of 2,092 kJ/day (500 kcal/day) based on the measurement of each individual’s resting energy expenditure (REE) by indirect calorimetry (metabolic monitor VMAX; SensorMedics, Anaheim, CA, USA) [16]. Individual calculations of energy requirements for both regimens were based on the formula: (REE×1.5)−2,092 kJ. The diet derived 50–55% of its total energy from carbohydrates, 20–25% from protein and less than 30% from fat (≤7% saturated fat, less than 200 mg/day of cholesterol), with 30–40 g/day of fibre. Alcoholic beverages were limited to one per day for women and two per day for men. Participants were asked not to alter their exercise habits during the study. Each regimen started with a 4 day tutorial where they learned in detail how to compose and prepare their diet, with follow-up 1 h weekly meetings with lectures and cooking classes throughout the whole study. All the meals during the entire 24 weeks of the study were provided for one half of the participants (randomised within each study arm with an equal number of participants) while the other half of the participants prepared their meals by themselves.

Compliance

At weeks 0, 12, and 24, a 3 day dietary record (2 weekdays and 1 weekend day) was completed by each participant. A registered dietitian analysed all these dietary records using a country-specific food-nutrient database NutriDan 1.2 (www.institut-danone.cz/cz/odborna-sekce/nutridan).

Physical activity

This was assessed with an Omron HJ-720IT pedometer (Omron, Kyoto, Japan; using a 1 month average step count for evaluation) and two questionnaires: the International Physical Activity Questionnaire [17] and the Baecke questionnaire [18] at weeks 0, 12, and 24.

Medication

Participants were asked to continue their pre-existing medication regimens, except when hypoglycaemia occurred repeatedly (fasting plasma glucose determined at the laboratory <4.4 mmol/l or a capillary glucose reading <3.4 mmol/l accompanied by hypoglycaemic symptoms). In such cases, medications were reduced by a study physician following the medication protocol. All participants were given an Accu-Chek Performa glucometer (Roche, Basel, Switzerland) and instructed how to use it.

Procedures

All measurements were performed on an outpatient basis at weeks 0, 12 and 24, after a 10–12 h overnight fasting with tap water ad libitum. Height and weight were measured using a
During a 45 min basal period before the clamp. Air flow and indirect calorimetry was recorded. 

Discarded, and the mean of the remaining two measurements were taken 2 min apart. The first measurement was after 5 min in a seated position at rest, using a digital M6 Comfort monitor (Omron, Kyoto, Japan). Three measurements were taken in expired and inspired air were periodically calibrated scale accurate to 0.1 kg. Waist circumference was measured with a tape placed at the midpoint between the lowest rib and the upper part of the iliac bone. Blood pressure and heart rate were measured during a 45 min basal period before the clamp. Air flow and O₂ and CO₂ concentrations in expired and inspired air were measured by a continuous open-circuit system (metabolic monitor VMAX; SensorMedics, Anaheim, CA, USA).

Meal tests Plasma concentrations of glucose, immunoreactive insulin and C-peptide were measured at 0, 30, 60, 120 and 180 min after a standard breakfast (1,895 kJ, 45% carbohydrates, 17% proteins, 38% lipids). Insulin secretion and whole-body insulin sensitivity were calculated by mathematical modelling (described below).

Indirect calorimetry Gas exchange measurements were taken during a 45 min basal period before the clamp. Air flow and O₂ and CO₂ concentrations in expired and inspired air were measured by a continuous open-circuit system (metabolic monitor VMAX; SensorMedics, Anaheim, CA, USA).

Table 1 Baseline characteristics of the study population

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Study group (n=54)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>59.4±7.0</td>
</tr>
<tr>
<td>Sex, n (%)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>29 (54)</td>
</tr>
<tr>
<td>Female</td>
<td>25 (46)</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>8.1±5.8</td>
</tr>
<tr>
<td>Smokers, n (%)</td>
<td>10 (19)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>94.1±15.5</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>32.6±4.9</td>
</tr>
<tr>
<td>HbA1c, (DCCT) (%)</td>
<td>7.2±3.3</td>
</tr>
<tr>
<td>HbA1c, (IFCC) (mmol/mol)</td>
<td>54.9±13.0</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>140±14</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>85±8</td>
</tr>
<tr>
<td>Resting heart rate (beats/min)</td>
<td>71±9</td>
</tr>
<tr>
<td>Oral hypoglycaemic agents, n (%)</td>
<td></td>
</tr>
<tr>
<td>Metformin</td>
<td>41 (76)</td>
</tr>
<tr>
<td>Sulfonylurea</td>
<td>16 (30)</td>
</tr>
<tr>
<td>Thiazolidinedione</td>
<td>3 (6)</td>
</tr>
<tr>
<td>Glinides</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Acarbose</td>
<td>1 (2)</td>
</tr>
<tr>
<td>DPP-4 inhibitors</td>
<td>19 (35)</td>
</tr>
<tr>
<td>Lipid-lowering therapy, n (%)</td>
<td>31 (57)</td>
</tr>
<tr>
<td>Antihypertensive therapy, n (%)</td>
<td>33 (61)</td>
</tr>
</tbody>
</table>

Data are means ± SD

DCCT, Diabetes Control and Complications Trial; IFCC, International Federation of Clinical Chemistry

Hyperinsulinaemic isoglycaemic clamp The hyperinsulinaemic (1 mU kg⁻¹ min⁻¹) isoglycaemic clamp, lasting 3 h, was conducted as previously described [19]. Insulin sensitivity was estimated as the metabolic clearance rate of glucose (MCR) [19].

Proton magnetic resonance spectroscopy HFC was measured by proton magnetic resonance spectroscopy on a 3 T MR scanner (Magnetom Trio, Siemens, Erlangen, Germany) with an eight-channel body array coil. This method has been validated at our institution [20]. The measurement protocol included conventional MRI using a localiser and HASTE sequence with breath-holding in the coronal and transversal planes. Spectra were obtained from three different segments of the right lobe of the liver—volume of interest, 30 ml each and evaluated using the LCModel (www.s-provencher.com/pages/lcmodel.shtml) and MestReC (Mestrelab Research, Santiago de Compostela, Spain) programs. The signal intensities of water and hepatic lipids were used to determine the fat to total signal peak area ratio and then converted to absolute concentrations expressed as a percentage of fat using equations validated by Longo et al [21]. Fourteen individuals did not undergo an HFC measurement due to the patient’s refusal, claustrophobia or the patient’s weight exceeding the limit of the equipment.

Calculations

Modelling analysis of beta cell function was performed during standard meal tests. Insulin secretory rates were calculated from plasma C-peptide levels by deconvolution [22] and expressed per square meter of estimated body surface area. The dependence of insulin secretory rates on glucose levels was modelled separately for each patient and each study day. The beta cell model used in the present study, describing the relationship between insulin secretion and glucose concentration, has previously been described in detail [23–25].

Briefly, insulin secretion consists of two components. The first component represents the dependence of insulin secretion on absolute glucose concentration at any time point and is characterised by a dose–response function. Characteristic variables of the dose–response are insulin secretion at a fixed glucose concentration and the mean slope in the observed glucose range. The dose–response was modulated by a potentiation factor that accounts for several agents (prolonged exposure to hyperglycaemia, non-glucose substrates, gastrointestinal hormones and neurotransmitters). The potentiation factor was set to be a positive function of time and to be an average of 1 during the experiment. It thus expresses a relative potentiation of the secretory response to glucose.

The second insulin secretion component represents a dynamic dependence of insulin secretion on the rate of change of glucose concentration. Termed the derivative component, it
is described by a single variable, rate sensitivity. This secretion component is related to early insulin release [23, 24].

The model variables (the variables of the dose–response, the rate sensitivity and the potentiation factor) were estimated from the glucose and C-peptide concentrations by regularised least squares, as previously described [23, 24]. Estimation of the individual model variables was performed blinded for the randomisation of the patients for treatment.

Whole-body insulin sensitivity was estimated in two ways: (1) as the MCR calculated during the last 20 min of the isoglycaemic hyperinsulinaemic clamp after correction for changes in glucose pool size [19], and (2) by a glucose–insulin model to derive an oral glucose insulin sensitivity (OGIS) index, validated against the clamp data [3].

Analytical methods

Serum glucose was analysed using the Beckman Analyser glucose-oxidase method (Beckman Instruments, Fullerton, CA, USA). Plasma immunoreactive insulin and C-peptide concentrations were determined using insulin and C-peptide IRMA kits (Immunotech, Prague, Czech Republic). HbA1c was measured by HPLC (Tosoh, Tokyo, Japan). Plasma concentrations of glucagon were measured using ELISA kits (BioVendor, Brno, Czech Republic). Plasma lipids concentrations were measured by enzymatic methods (Roche, Basel, Switzerland). HDL-cholesterol was measured after double precipitation with dextran and MgCl2. LDL-cholesterol was estimated using the Friedewald equation if the triacylglycerol concentration was <4.53 mmol/l.

Statistical analyses

The intention-to-treat analysis included all participants. We tested the distributions of the data. If the distribution was skewed, we used the Box-Cox transformation to attain data symmetry and homosedasticity [26]. Non-homogeneities in the data were detected using residual analysis as described elsewhere [27]. 2×2 crossover ANOVA was used for data evaluation. The model consisted of the between-subject factor ‘sequence’, the factor ‘subject’ and within-subject factors of ‘period’ and ‘treatment’. In a subsequent subanalysis, the factor for prepared meals that were collected by patients was added. The relationships between continuous variables were evaluated using Pearson’s correlation and BMI-adjusted partial correlations.

Results

The results are expressed as the changes in response to the A6 and B2 regimens, presented as means with 95% CIs (Fig. 2 and Table 2). The factors ‘period’ and ‘sequence’ (the order of the regimens) were not significant. No substantial unfavourable effects of the regimens were observed.

Dietary intake and physical activity

Reported dietary intake decreased (p<0.001) comparably under both regimens. Physical activity increased (p<0.05) slightly, but negligibly—by about 2,000 steps per month—in both regimens (see Table 2).

Body weight and HFC

Body weight and HFC decreased under both regimens (p<0.001), more with B2 (p<0.001; −2.3 kg; 95% CI −2.7, −2.0 kg with A6 vs −3.7 kg; 95% CI −4.1, −3.4 kg with B2; and p=0.009; −0.03%; 95% CI −0.033, −0.027% with A6 vs −0.04%; 95% CI −0.041, −0.035% with B2, respectively; Fig. 2a and b). Similarly, BMI and waist circumference decreased with both regimens (p<0.001), more with B2 (p<0.001; −0.82 kg/m2; 95% CI −0.94, −0.69 kg/m2 with
Table 2 Changes in anthropometric and laboratory variables in response to regimens of six (A6) and two (B2) meals a day

<table>
<thead>
<tr>
<th>Variables</th>
<th>Six meals a day (A6)</th>
<th>Two meals a day (B2)</th>
<th>p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dietary intake – energy (kJ/day)</td>
<td>−1,590 (−1,970 to −1,054)**</td>
<td>−1,757 (−2,105 to −1,201)**</td>
<td>0.731</td>
</tr>
<tr>
<td>Dietary intake – fat (g/day)</td>
<td>−35.0 (−43.7 to −27.9)***</td>
<td>−38.2 (−44.4 to −32.2)***</td>
<td>0.921</td>
</tr>
<tr>
<td>Dietary intake – carbohydrates (g/day)</td>
<td>−19.7 (−32.4 to −3.1)***</td>
<td>−23.2 (−37.8 to −8.5)***</td>
<td>0.873</td>
</tr>
<tr>
<td>Dietary intake – protein (g/day)</td>
<td>+0.9 (−3.7 to +5.6)</td>
<td>−4.6 (−8.7 to +1.1)</td>
<td>0.637</td>
</tr>
<tr>
<td>Step count (steps/month)</td>
<td>+2,092 (+879 to +3,493)*</td>
<td>+2,213 (+996 to +3,612)*</td>
<td>0.876</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>−0.82 (−0.94 to −0.69)***</td>
<td>−1.23 (−1.4 to −1.17)***</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>−1.37 (−2.01 to −0.73)***</td>
<td>−5.14 (−5.78 to −4.50)***</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/l)</td>
<td>−0.47 (−0.57 to −0.36)***</td>
<td>−0.78 (−0.89 to −0.68)***</td>
<td>0.004</td>
</tr>
<tr>
<td>Fasting immunoreactive insulin (pmol/l)</td>
<td>−0.69 (−1.18 to −0.21)*</td>
<td>−0.75 (−1.23 to −0.27)*</td>
<td>0.910</td>
</tr>
<tr>
<td>Triacylglycerols (mmol/l)</td>
<td>−0.28 (−0.39 to −0.17)**</td>
<td>−0.17 (−0.28 to −0.06)*</td>
<td>0.300</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>−0.05 (−0.13 to +0.04)</td>
<td>−0.07 (−0.15 to +0.01)</td>
<td>0.730</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>+0.016 (−0.006 to +0.038)</td>
<td>+0.003 (−0.019 to +0.025)</td>
<td>0.570</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>−0.08 (−0.15 to −0.01)***</td>
<td>−0.06 (−0.13 to −0.01)</td>
<td>0.823</td>
</tr>
<tr>
<td>Insulin secretion at reference level (pmol min⁻¹ m⁻²)</td>
<td>+22.9 (+11.7 to +34.0)*</td>
<td>+20.0 (+8.7 to +31.2)*</td>
<td>0.795</td>
</tr>
<tr>
<td>Glucose sensitivity (pmol min⁻¹ m⁻² mmol⁻¹ l⁻¹)</td>
<td>+5.8 (+2.3 to +9.5)*</td>
<td>+5.9 (+2.3 to +9.5)*</td>
<td>0.991</td>
</tr>
<tr>
<td>Rate sensitivity (pmol m⁻² mmol⁻¹ l⁻¹)</td>
<td>−141.9 (−248.2 to −36.8)*</td>
<td>−251.5 (−358.9 to −145.3)*</td>
<td>0.303</td>
</tr>
<tr>
<td>Potentiation factor (dimensionless)</td>
<td>−0.034 (−0.059 to −0.009)*</td>
<td>−0.038 (−0.062 to −0.013)*</td>
<td>0.890</td>
</tr>
</tbody>
</table>

Data are mean ± 95% CI

Listed p values are from 2×2 crossover ANOVA for the factor ‘treatment’

Significant changes during the regimens are indicated by: *p<0.05; **p<0.01; ***p<0.001

A6 vs −1.23 kg/m²; 95% CI −1.4, −1.17 kg/m² with B2; and −1.37 cm; 95% CI −2.01, −0.73 cm with A6 vs −5.14 cm; 95% CI −5.78, −4.50 cm in B2, respectively; Table 2).

Glycaemic control

Fasting plasma glucose decreased under both regimens (p<0.001), more with B2 (p=0.004; −0.47 mmol/l; 95% CI −0.57, −0.36 mmol/l with A6 vs −0.78 mmol/l; 95% CI −0.89, −0.68 mmol/l with B2; Fig. 2c). Fasting C-peptide decreased in both regimens, more with B2 (p=0.04; −0.049 mmol/l; 95% CI −0.091, −0.006 mmol/l with A6 vs p<0.001; −0.14 mmol/l 95% CI −0.181, −0.099 mmol/l with B2; p=0.04; Fig. 2d). Fasting immunoreactive insulin decreased (p<0.04) comparably with both regimens (−0.69 pmol/l; 95% CI −1.18, −0.21 pmol/l with A6 vs −0.75 pmol/l; 95% CI −1.23, −0.27 pmol/l with B2; p=0.9; Table 2). Fasting glucagon decreased with B2 (p<0.001; −343 pg/ml; 95% CI −375, −311 pg/ml), whereas it increased (p=0.04; +53 pg/ml; 95% CI +22, +84 pg/ml) with A6 (p<0.001; Fig. 2e). HbA1c decreased (p<0.001) comparably with both regimens (−0.23%; 95% CI −0.27, −0.19% with A6 vs −0.25%; 95% CI −0.29, −0.20% with B2; p=0.08; Fig. 2f).

Whole-body insulin sensitivity

MCR increased (p<0.001) comparably with both regimens (+0.45 ml kg⁻¹ min⁻¹; 95% CI +0.24, +0.67 ml kg⁻¹ min⁻¹ with A6 vs +0.52 ml kg⁻¹ min⁻¹; 95% CI +0.30, +0.74 ml kg⁻¹ min⁻¹ with B2; p=0.8; Fig. 2g). OGIS increased in both regimens (p<0.01), more with B2 (p=0.01; +8.2 ml min⁻¹ m⁻²; 95% CI +3.4, +13.1 ml min⁻¹ m⁻² with A6 vs +21 ml min⁻¹ m⁻²; 95% CI +16.1, +26.0 ml min⁻¹ m⁻² with B2; Fig. 2h).

Beta cell function

Insulin secretion at the reference level and glucose sensitivity increased (p<0.05) comparably with both regimens. Rate sensitivity and potentiation factor also decreased (p<0.05) comparably (Table 2).

Plasma lipids

Triacylglycerols and LDL-cholesterol decreased comparably under both regimens. No significant change in total or HDL-cholesterol was observed in either regimen (Table 2).

REE

REE decreased under both regimens (p<0.001), with a trend toward a greater decrease with A6 (~453.1 kJ/day; 95% CI −524.3, −382.8 kJ/day with A6 vs −379.9 kJ/day; 95% CI −449.8, −310.9 kJ/day with B2; p=0.3; Fig. 2i).
Correlations

The decrease in HFC showed a strong positive correlation with the decrease in fasting plasma glucose \((r=+0.56; p<0.001)\). This association remained significant even after adjustment for changes in BMI \((r=+0.28; p=0.05)\).

Changes in glucose sensitivity and OGIS correlated negatively with changes in HFC \((r=-0.28; p=0.02\) and \(r=-0.47; p<0.001\), respectively). After adjustment for changes in BMI, the correlations were no longer significant.

Discussion

Principal findings

This randomised crossover 24 week study examined the effect of frequency of meals on body weight, HFC, insulin resistance and beta cell function in type 2 diabetic patients. A comparison of the effect of six vs two meals (breakfast and lunch) with the same daily energy restriction (\(-2,092\) KJ/day) and macronutrient content, each regimen lasting 12 weeks, demonstrated a superior effect of B2 on body weight, HFC, fasting plasma glucose, C-peptide, glucagon and OGIS. The effect of meal frequency on MCR and beta cell function was not significant.

Findings in relation to other research

The superior effect of B2 on most of the variables studied supports our hypothesis and previous lines of evidence from animal models, observational studies and randomised trials [5, 7, 8, 12, 14]. Our results are in strong agreement with animal studies, which have demonstrated the glucose-lowering effects of intermittent fasting regimens (such as every other day fasting or fasting for 2 days a week). These regimens reduce blood glucose and insulin concentrations, improve glucose tolerance [4, 28], mainly due to increased insulin sensitivity [29], and extend the lifespan of laboratory animals [30].

Our data contradict the widely held opinion that eating more frequently is healthier than eating less frequent larger meals. Some studies have suggested that people who consume more snacks are less likely to be obese [31], but other large prospective studies have demonstrated that frequent snacking may lead to weight gain [32] and an increased risk of type 2 diabetes [33, 34] because of the higher energy intake, mainly from added sugars [35]. Furthermore, the reported benefits of more frequent meals are usually associated with meal frequencies exceeding those which might be translated into practical recommendations [36]. In fact, some of the early experiments with frequency of eating [37, 38] only underline the importance of fibre and low glycaemic index foods in the diet.

Studies involving individuals with type 2 diabetes are rather limited in both length and sample size. A longer term study, comparing the effect of three and nine meals daily (each period lasting 4 weeks), in 13 patients with type 2 diabetes, did not confirm the beneficial effects of increased meal frequency [39]. It has recently been demonstrated that, for glycaemic control, eating fewer larger meals rich in fibre instead of more smaller ones may be more beneficial for type 2 diabetic patients [10].

Ours is the first study into the effect of meal frequency on insulin resistance and HFC. MCR increased in response to both regimens, with a trend toward a greater increase with B2. The difference between the two regimens was not significant, probably due to a limited number of participants. OGIS increased in response to both regimens, more for B2. The increased insulin sensitivity positively influenced the decrease in fasting plasma glucose and HFC (although the HbA1c level decreased comparably in both regimens) or, conversely, decreased HFC may have led to increased insulin sensitivity, because HFC is typically associated with insulin resistance (independent of BMI) [40], metabolic syndrome, type 2 diabetes and subclinical atherosclerosis [41]. In this context, the greater reduction in HFC in the B2 regimen is one of the most important results of our study.

We are also the first to observe an effect on fasting plasma concentrations of glucagon with a B2 regimen. Inappropriately elevated plasma glucagon concentrations play a role in dysregulated hepatic glucose production and abnormal glucose homeostasis in type 2 diabetes [42]. There is no treatment that specifically decreases glucagon levels. However, incretin-based agents reduce plasma glucagon, which contributes to their action to lower blood glucose [43]. In this regard, a decrease of glucagon with B2 in our study is a very positive finding.

Possible mechanisms

The mechanisms responsible for a greater weight loss with the B2 regimen may be the trend toward a smaller decrease in REE with B2 that we measured (although the difference between regimens did not reach the threshold for statistical significance), together with a greater thermogenic response of larger meals, as documented by others [11].

Furthermore, some of the positive effects of intermittent fasting on glucose homeostasis may be mediated by the nervous system, mainly by the increased production of brain-derived neurotrophic factor, which increases the resistance of neurones to dysfunction and degeneration in animal models [3]. It is possible that overnight fasting between lunch and breakfast the next day elicited some of these effects.

Periods of fasting between meals may be even more important than the composition of the diet. In one experimental study, the timing of meals led to increased insulin sensitivity
and decreased body weight in spite of the high fat content of the consumed diet [6]. The difference in weight loss success was not explained by differences in caloric intake, macronutrient distribution or energy expenditure [12]. A potential mechanism explaining this difference is that the timing of food intake can influence the circadian system [44]. The circadian system must continuously adapt to and synchronise our physiology with the environment [45]. A genetic variance in clock genes may be important in meal timing, possibly in part by changes in the recently demonstrated circadian control of hunger and appetite [46]. Another recent study demonstrated that a high carbohydrate and protein breakfast may prevent weight regain by reducing diet-induced compensatory changes in hunger, cravings and ghrelin suppression [47]. Although the mechanisms linking the timing of meals and the regulation of body weight are unknown, satiety hormones, such as leptin or ghrelin, may be involved [48]. Changes in the levels of these hormones by circadian misalignment could influence energy intake and expenditure [49].

Weaknesses

The short duration of our study and provision of food precludes a generalisation of our study to free-living conditions. We provided all the meals during the whole study for one half of the participants in order to ensure the best possible compliance, yet we have to admit the possibility of a reduced energy intake with the B2 regimen, even though the energy intake reported by our participants was similar in both regimens. The dropout rates were also comparable for both regimens. The sample of diabetic patients in our study was not representative: the duration of diabetes in our patients was quite short; all the participants were being treated by oral hypoglycaemic agents, with motivation not to initiate insulin therapy, and they were willing to make substantial changes in their lifestyle.

Conclusions and research needs

In conclusion, a hypoenergetic diet (a restriction of 2,092 kJ/day) consumed as breakfast and lunch reduced body weight, HFC, fasting plasma glucose, C-peptide and glucagon, and increased calculated insulin sensitivity (OGIS), more than the same hypoenergetic diet (a similar restriction of 2,092 kJ/day) divided into six, more frequent, meals. MCR and beta cell function improved comparably in both regimens. These results suggest that eating two larger meals a day (breakfast and lunch) may be more beneficial for patients with type 2 diabetes than six smaller meals during the day. Novel therapeutic strategies should incorporate not only the energy and macronutrient content but also the frequency and timing of food. Further larger scale, long-term studies are essential before offering recommendations in terms of meal frequency.

Acknowledgements We thank the 54 participants for their cooperation, as well as our staff from the Diabetes Centre, Institute for Clinical and Experimental Medicine, Prague, Czech Republic: the registered dietitians (V. Havlova and R. Milatova) for providing both group and individual nutrition counselling and study nurses (D. Lapesova, D. Sisakova, D. Kobrova, J. Purrova and B. Vodicova) for conducting the procedures. We thank J. Sievenpiper from the Clinical Nutrition and Risk Factor Modification Centre, St Michael’s Hospital, Toronto, Canada for great help with text corrections. Some of the data were presented as an abstract at the 72nd and 73rd Scientific Sessions of the ADA and 48th and 49th EASD Annual Meetings in 2012 and 2013.

Funding This study was supported by grant NT/11238-4 from Ministry of Health, Prague, Czech Republic and by the Grant Agency of Charles University - GAUK No 702312.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement HK, LB and TP designed the study, wrote the grant application, recruited the patients, collected the data and wrote the manuscript. HM, OO, JT, VS, IK, MD, MHs and AF were involved in the acquisition and analysis of the data. MHi carried out the statistical analyses and interpretation of data. All authors had full access to data and revised and approved the manuscript for publication. The guarantor is TP.

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References

The effect of meal frequency in a reduced-energy regimen on the gastrointestinal and appetite hormones in patients with type 2 diabetes: A randomised crossover study

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Abstract

Background

Appetite and gastrointestinal hormones (GIHs) participate in energy homeostasis, feeding behavior and regulation of body weight. We demonstrated previously the superior effect of a hypocaloric diet regimen with lower meal frequency (B2) on body weight, hepatic fat content, insulin sensitivity and feelings of hunger compared to the same diet divided into six smaller meals a day (A6). Studies with isoenergetic diet regimens indicate that lower meal frequency should also have an effect on fasting and postprandial responses of GIHs. The aim of this secondary analysis was to explore the effect of two hypocaloric diet regimens on fasting levels of appetite and GIHs and on their postprandial responses after a standard meal. It was hypothesized that lower meal frequency in a reduced-energy regimen leading to greater body weight reduction and reduced hunger would be associated with decreased plasma concentrations of GIHs: gastric inhibitory peptide (GIP), glucagon-like peptide-1(GLP-1), peptide YY(PYY), pancreatic polypeptide (PP) and leptin and increased plasma concentration of ghrelin. The postprandial response of satiety hormones (GLP-1, PYY and PP) and postprandial suppression of ghrelin will be improved.

Methods

In a randomized crossover study, 54 patients suffering from type 2 diabetes (T2D) underwent both regimens. The concentrations of GLP-1, GIP, PP, PYY, amylin, leptin and ghrelin were determined using multiplex immunoanalyses.

Results

Fasting leptin and GIP decreased in response to both regimens with no difference between the treatments (p = 0.37 and p = 0.83, respectively). Fasting ghrelin decreased in A6 and

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**Editor:** Dorit Samocha-Bonet, Garvan Institute of Medical Research, AUSTRALIA

**Received:** December 6, 2016

**Accepted:** March 14, 2017

**Published:** April 3, 2017

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**Data Availability Statement:** Our data are available from the Dryad Digital Repository: [http://dx.doi.org/10.5061/dryad.2sj7q](http://dx.doi.org/10.5061/dryad.2sj7q).

**Funding:** This work was supported by the project grant AZV15-27431A from Ministry of Health, Prague, Czech Republic and Institutional Support MZCR 00023001 (IKEM, Prague, Czech Republic) and by the Grant Agency of Charles University - GAUK No 702312. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
increased in B2 (with difference between regimens $p = 0.023$). Fasting PP increased in B2 with no significant difference between regimens ($p = 0.17$). Neither GLP-1 nor PYY did change in either regimen. The decrease in body weight correlated negatively with changes in fasting ghrelin ($r = -0.4, p < 0.043$) and the postprandial reduction of ghrelin correlated positively with its fasting level ($r = 0.9, p < 0.001$). The postprandial responses of GIHs and appetite hormones were similar after both diet regimens.

Conclusions
Both hypocaloric diet regimens reduced fasting leptin and GIP and postprandial response of GIP comparably. The postprandial responses of GIHs and appetite hormones were similar after both diet regimens. Eating only breakfast and lunch increased fasting plasma ghrelin more than the same caloric restriction split into six meals. The changes in fasting ghrelin correlated negatively with the decrease in body weight. These results suggest that for type 2 diabetic patients on a hypocaloric diet, eating larger breakfast and lunch may be more efficient than six smaller meals during the day.

Introduction
Appetite and gastrointestinal peptides play an important role in the regulation of energy intake, appetite and overall energy homeostasis in humans [1, 2]. Their fasting plasma levels could be increased and postprandial responses attenuated by the presence of obesity [3], impaired glucose tolerance [4] and type 2 diabetes mellitus (T2D) [5, 6]. Caloric restriction and body weight reduction are supposed to decrease fasting plasma levels [7, 8] and increase meal responses of gastrointestinal hormones (GIH) with effects on satiety: for instance, glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) [9, 10] and reduce postprandial response of gastric inhibitory polypeptide (GIP) [11]. Studies with hypocaloric diet regimens indicate that lower meal frequency should also have an effect on GIH’s postprandial responses: ghrelin and GLP-1 [12]. To the best of our knowledge, the effects of different hypocaloric diet regimens on other GIHs and their postprandial responses have not been published yet.

It has been postulated that increasing eating frequency regimens could help to maintain appetite control, improve glucose metabolism, and therefore reduce body fat storage and body weight [13]. Contrary to this, a very recent meta-analysis has showed that eating more frequently during the day (i.e., “grazing”) may not assist with reducing energy intake or improving weight status [14]. It is suggested that eating more often than three times a day may lead to obesity [15], by increasing food stimuli and appetite [16]. However, there are also studies, which do not support any associations between body weight and eating frequency at all [17, 18].

We have shown previously that in patients with type 2 diabetes less frequent eating (breakfast and lunch consumption) reduced body weight, hepatic fat content, insulin resistance [19] and hunger [20] more than a diet with the same caloric restriction divided into six more frequent meals. The aim of this secondary analysis was to explore the effect of these two hypocaloric diet regimens on the fasting levels of appetite and GIHs and on their postprandial responses after a standard meal. The resulting positive effects following the hypocaloric diet divided into less frequent meals could be due, at least in part, to an alteration in the circulating levels and physiology of certain gastrointestinal and appetite hormones.
Appetite hormones, ghrelin and leptin, are well known to play a prominent role in regulation of energy homeostasis [2]. In obese subjects [21] and patients with T2D, ghrelin secretion is down-regulated and the decline in plasma ghrelin after a meal is blunted [22, 23]. Hypocaloric diet regimen is postulated to improve ghrelin sensitivity in these individuals [24, 25] and improves the postprandial ghrelin response independently on diet composition [26].

Peptide YY (PYY) is co-secreted predominantly from the endocrine L cells in the ileum together with glucagon-like peptide-1 (GLP-1). Pancreatic polypeptide (PP) is secreted from endocrine cells in the pancreas. GLP-1, PYY and PP are supposed to induce satiety and to reduce appetite and energy intake in healthy humans [27–29].

The aim of our study was to compare the effects of two different hypocaloric regimens, six vs. two meals a day (breakfast and lunch; as this regimen allows reasonable fasting time, yet is sustainable in the long term), with the same caloric restriction on fasting levels of appetite and GI hormones and their postprandial responses after a standard meal.

It was hypothesized that lower meal frequency in a reduced-energy regimen leading to greater body weight reduction and reduced hunger would be associated with decreased plasma concentrations of GIHs (GIP, GLP-1, PYY, PP) and leptin and increased plasma concentration of ghrelin. The postprandial response of satiety hormones (GLP-1, PYY and PP) and postprandial suppression of ghrelin will be improved.

### Methods and materials

#### Study design and participants

The protocol for this trial and supporting CONSORT checklist are available as supporting information; see S1–S3 Files. We included a group of 54 patients suffering from T2D (with disease duration of more than 1 year) treated by oral hypoglycaemic agents (both men and women), age 30–70 years, BMI 27–50 kg/m2 and HbA1c 6–11.8% (42–105 mmol/mol). Exclusion criteria comprised alcohol or drug abuse, pregnancy or lactation, unstable medication or weight in the last 3 months, a diagnosis of type 1 diabetes and the presence of a cardiomediator. Written informed consent was obtained from all participants prior to enrollment in the study; the study protocol (Protocol S3), informed consent, and patient information were reviewed and approved by the Ethics Committee of the Thomayer Hospital and Institute for Clinical and Experimental Medicine in Prague, Czech Republic on December 18, 2009. On December 1, 2010 we started the telephone screening of potential participants. After approval of financial support, subjects were screened in person and enrolled into the study on January 10, and February 28, 2011. The first patient entered the intervention phase on March 14, 2011 and the last completed on October 20, 2011. In this single-center study the samples were collected in the Laboratory of Clinical Pathophysiology in Institute for Clinical and Experimental Medicine in Prague. There was no follow-up phase. The study flowchart is presented in Fig 1.

Of the 219 patients, who were screened, 54 participants were included. Randomisation and allocation to trial groups (n = 27 and n = 27) were carried out by a central computer system. Registration on ClinicalTrials.gov was initiated on January 6, 2011, after the telephone enrollment of participants started (Identifier: NCT01277471). The authors confirm that all ongoing and related trials for this intervention are registered. Characteristics of included participants, who underwent randomisation within a 24-week-crossover study, are presented in Table 1. After a 1-month-run-in-period, the participants started with a regimen of either six (A6) or two (B2) meals a day that was followed for 12 weeks. The A6 regimen consisted of three main meals (breakfast, lunch and dinner), and 3 smaller snacks in between. The B2 regimen consisted of breakfast (eaten between 6–10 a.m.) and lunch (eaten between noon and 4 p.m.). Then the participants switched regimens for a subsequent 12 weeks. All measurements were
Fig 1. CONSORT flow diagram.

https://doi.org/10.1371/journal.pone.0174820.g001
performed at weeks 0 (baseline), 12 and 24. The study protocol was approved by the Institutional Ethics Committee. After a 1-month-run-in-period the participants began a 12-week regimen of either six (A6) or two (B2) meals a day. The A6 regimen consisted of three main meals (breakfast, lunch and dinner), and three smaller snacks in between. The B2 regimen consisted of breakfast (eaten between 6–10 a.m.) and lunch (eaten between noon and 4 p.m.).

**Diet**

The diet composition in both regimens followed the DNSG (Study Group on Diabetes and Nutrition of the European Association for the Study of Diabetes) guidelines [30], and had the same caloric restriction of -500 kcal/day based on measurements of the resting energy expenditure of each subject by indirect calorimetry (metabolic monitor VMAX; Sensor Medics, Anaheim, CA, USA) [31]. The diet contained 50–55% of total energy from carbohydrates, 20–25% protein, less than 30% fat (≤7% saturated fat, less than 200 mg/day of cholesterol/day) and 30–40 g/day of fibre.

**Study procedure**

All measurements were performed on an outpatient basis after overnight fasting with only tap water allowed ad libitum. The patients did not take any of their diabetes medication the evening or the morning before the assessments. Plasma concentrations of glucose, immunoreactive insulin, C-peptide, appetite and gastrointestinal (GI) hormones were measured at 0, 30, 60,

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**Table 1. Baseline characteristics of the study population.**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Study group (n = 54)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age—years</td>
<td>59.4±7.0</td>
</tr>
<tr>
<td>Sex—no. (%)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>29 (54)</td>
</tr>
<tr>
<td>Female</td>
<td>25 (46)</td>
</tr>
<tr>
<td>Duration of diabetes—years</td>
<td>8.1±5.8</td>
</tr>
<tr>
<td>Smokers—no. (%)</td>
<td>10 (19)</td>
</tr>
<tr>
<td>Weight—kg</td>
<td>94.1±15.5</td>
</tr>
<tr>
<td>BMI—kg·m⁻²</td>
<td>32.6±4.9</td>
</tr>
<tr>
<td>HbA1c (DCCT)—%</td>
<td>7.2±3.3</td>
</tr>
<tr>
<td>HbA1c (IFCC)—mmol/mol</td>
<td>54.9±13.0</td>
</tr>
<tr>
<td>Systolic blood pressure—mm Hg</td>
<td>140±14</td>
</tr>
<tr>
<td>Diastolic blood pressure—mm Hg</td>
<td>85±8</td>
</tr>
<tr>
<td>Resting heart rate—beats.min⁻¹</td>
<td>71±9</td>
</tr>
<tr>
<td>Oral hypoglycemic agents—no. (%)</td>
<td></td>
</tr>
<tr>
<td>Metformin</td>
<td>41 (76)</td>
</tr>
<tr>
<td>Sulfonylurea</td>
<td>16 (30)</td>
</tr>
<tr>
<td>Thiazolidinedione</td>
<td>3 (6)</td>
</tr>
<tr>
<td>Gilinides</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Acarbose</td>
<td>1 (2)</td>
</tr>
<tr>
<td>DPP-4 inhibitors</td>
<td>19 (35)</td>
</tr>
<tr>
<td>Lipid-lowering therapy—no. (%)</td>
<td>31 (57)</td>
</tr>
<tr>
<td>Antihypertensive therapy—no. (%)</td>
<td>33 (61)</td>
</tr>
</tbody>
</table>

Data are means ± SD.

https://doi.org/10.1371/journal.pone.0174820.t001
120, and 180 min after standard breakfast (453 kcal, 45% carbohydrates, 17% proteins, 38% lipids).

Compliance with both regimens was maximized by an initial 4-day tutorial at the beginning of each regimen where the patients learned in detail how to compose and divide their diet, with follow-up weekly meetings with lectures, cooking classes and food diary consultation with a registered dietician. Registered dieticians analysed 3-day dietary records (2 weekdays and 1 weekend day) completed by each participant at weeks 0, 12, and 24. Participants were asked not to alter their exercise habits during the study. Physical activity was monitored with a pedometer (Omron HJ-720IT, Omron, Kyoto, Japan), using the average 1-month step count for evaluation. The International Physical Activity Questionnaire [32] and the Baecke Questionnaire [33] were completed by each participant at weeks 0, 12, and 24. As described in detail previously [19].

Analytical methods

The concentrations of GLP-1, GIP, PP, PYY, amylin, leptin and ghrelin were determined via multiplex immunoanalyses based on xMAP technology using a MILLIPLEX MAP Human Gut Hormone Panel (Millipore, Billerica, MA, USA) and a Luminex 100 IS analyzer (Luminex Corporation, Austin, TX, USA)[34]. Protease and dipeptidyl peptidase-4 inhibitors were added to two samples at each time point. The assay sensitivities, expressed as the minimum detectable concentrations reported in the instructions for use by the manufacturer, are (in pg/mL): amylin (total) 3.2; ghrelin 1.8; leptin 157.2; GIP 0.2; GLP-1 5.2; PP 2.4; and PYY 8.4. For our measurements, the sensitivity was set as the value of the lowest standard concentration.

Statistical analyses

The power analysis was completed to estimate the number of subjects for the experiment with assumed weight loss (as the most important variable) for treated and untreated subjects 3 kg and 2 kg, respectively (assumed estimated difference between weight losses was 1 kg). The square root of the within mean square error was $sw = 1$ kg and effect size $1$. The required probability of the positive probability of a false positive null hypothesis was $\alpha = 0.05$ and the required number of subjects under investigation was 30, 24, 22, and 18 for power 0.96, 0.91, 0.88, and 0.8, respectively. Based on these data we considered the total number of 45 subjects as sufficient for the experiment (statistic software PASS 2005; Number Cruncher Statistical Systems, Kaysville, UT, USA). The intention-to-treat analysis included all participants. We tested the distributions of the data. If the distribution was skewed, we used the Box-Cox transformation to attain data symmetry and homoscedasticity [35]. 2x2 cross-over ANOVA (Statistical software Statgraphics Centurion XV version 15.2.06 from Statpoint Technologies, Inc., Herndon, VA, USA) was used for data evaluation. The model consisted of between-subject factors “sequence” and “gender”, the factor “subject”, and within-subject factors of “period and treatment”. We have checked the carry-over effect using the model including the factors “period” (order of diet regimens) and “sequence” and we have not found any significance of these factors for any dependent variables.

In a subsequent subanalysis, to estimate the postprandial changes, repeated-measures ANOVA was performed. The factors of “subject”, “sequence”, “diet” and “time” were included in the analyses. In each “sequence” the interactions between “diet” and “time” (“diet” x “time”) were calculated for each variable. The data are presented as the means with 95% confidence intervals. We have checked the carry-over effect using the model including the factor “sequence” and we have not found any significance of this factor for any dependent variables.
Spearman’s correlations were calculated for the relationship between changes in concentrations of investigated parameters. They were compared in 4 periods (0–30, 30–60, 60–120, 120–180 minutes after ingestion of standard breakfast).

Results
The results are expressed as the changes in response to the A6 and B2 diet regimens. They are presented as means with 95% confidence intervals in Fig 2.

The postprandial profiles of appetite and gastrointestinal hormones measured at 0, 30, 60, 120, and 180 min after the meal at basal state and after the A6 and B2 diet regimens are illustrated in Fig 3.

Body weight
Body weight decreased under both regimens more with B2 ($p = 0.0005$; $-2.4$ kg; 95% CI $-2.8$, $-2.0$ kg with A6 vs. $-3.8$ kg; 95% CI $-4.1$, $-3.4$ kg with B2) as already described previously [19]. As far as gender differences are concerned, the body weight at baseline in men vs. women ($p = 0.99$) and also the body weight reduction after the diet regimens ($p = 0.87$; $-3.0$ kg; 95% CI $-3.9$, $-2.2$ kg in women vs. $-3.2$ kg; 95% CI $-3.9$, $-2.4$ kg in men) were similar.

Appetite hormones
Fasting leptin decreased comparably in both regimens ($p = 0.37$; $-1308$ pg/ml; 95% CI: $-1941$, $-693$ pg/ml with A6 vs. $-1862$; 95% CI: $-2513$, $-1231$ pg/ml with B2). In women, the fasting concentrations of leptin were significantly higher than in men ($p = 0.000003$). If we consider the factor “gender” in the statistical model the hypocaloric diet reduced significantly leptin but only in females. In males the change in fasting leptin was not significant ($p = 0.02$; $-3000$ pg/ml; 95% CI: $-4423$, $-1672$ pg/ml in women vs. $-255$ pg/ml; 95% CI: $-1174$, $626$ pg/ml in men). Fasting plasma levels of ghrelin decreased in A6 and increased in B2 with significant difference between both diet regimens ($p = 0.023$; $-5.42$ pg/ml; 95% CI: $-10.1$, $-0.1$ pg/ml with A6 vs. $4.5$ pg/ml; 95% CI: $0.37$, $8.5$ pg/ml with B2). In a subsequent subanalysis, the decrease in body weight correlated negatively with changes in fasting ghrelin ($r = -0.4$, $p<0.043$). The postprandial drop in ghrelin levels correlated positively with its fasting level ($r = 0.9$, $p<0.001$). Reduced feelings of hunger after the hypocaloric diet regimen with B2 were previously described in detail elsewhere. In the added subanalysis, fasting plasma ghrelin levels did not correlate with changes in hunger ($p = 0.7$). The changes in fasting concentrations of ghrelin were similar in men vs. women ($p = 0.85$; $-1.36$ pg/ml; 95% CI: $-13.29$, $9.8$ pg/ml in women vs. $0.59$ pg/ml; 95% CI: $-8.5$, $9.2$ pg/ml in men). The two different diet regimens induced relatively similar postprandial responses of the both appetite hormones, leptin and ghrelin, when the time-course (interaction diet x time) is considered ($p = 0.8241$ and $p = 0.503$, respectively) (Fig 3E and 3F). The plasma concentrations of ghrelin were significantly higher at fasting state and after 180 minutes after meal ingestion after the diet regimen B2.

Gastrointestinal hormones
Fasting GIP decreased comparably in both regimens ($p = 0.83$; $-3.36$; 95% CI: $-4.8$, $-1.96$ pg/ml with A6 vs. $-3.1$; 95% CI: $-4.4$, $-1.73$ pg/ml with B2) and also comparably in men and women ($p = 0.39$). During the postprandial phase, there was a considerable difference in the GIP response at basal state and after both diet regimens ($p<0.0001$) (Fig 3B). There was a significantly higher postprandial GIP response at baseline in first 30 minutes after the meal ingestion than after both hypocaloric diet regimens, where the postprandial responses were similar. The
Meal frequency and gastrointestinal and appetite hormones

postprandial curves of GIP have the same shapes in time in all three measurements (interaction diet × time: \( p = 0.7093 \)). Fasting PP increased in B2 and did not significantly change in A6 with no significant difference between both regimens (\( p = 0.17; \) 4.64 pg/ml; 95% CI: -6.9, 16.6 pg/ml with A6 vs. 21.0 pg/ml; 95% CI: 8.87, 33.6 pg/ml with B2). Gender differences in changes of fasting levels of plasma PP concentrations were also not significant (\( p = 0.76 \)).

When the time-course is considered, the postprandial responses of PP were similar with rapid increase in the first 30 minutes and then slower fall (interaction diet × time: \( p = 0.34 \)) (Fig 3D).

GLP-1 did not change after any regimen (\( p = 0.65; \) -1.571 pg/ml; 95% CI: -7.02, 3.88 pg/ml with A6 vs. -3.91 pg/ml; 95% CI: -9.1, 1.28 pg/ml with B2), with no difference in gender (\( p = 0.39 \)). The postprandial responses of GLP-1 were similar in all three meal tests (interaction diet × time: \( p = 0.998 \)) (Fig 3A).

The fasting plasma levels of PYY did not change (\( p = 0.21; \) 2.14; 95% CI: -1.03, 5.26 pg/ml with A6 vs. -1.71; 95% CI: -4.84, 1.37 pg/ml with B2), with no difference in men vs. women (\( p = 0.79 \)). The postprandial responses of PYY were also similar as illustrated in Fig 3C.

Fig 2. Changes in anthropometric and laboratory parameters. Data are shown as changes from baseline in response to the regimen of six (A6) and two meals a day (B2). Data are means with 95% CI. Significance of the factor treatment (assessed by 2x2 crossover ANOVA) is indicated by * for \( p<0.05 \), ** for \( p<0.01 \), *** for \( p<0.001 \) and NS for non-significant. a: Δ Weight, n = 54, b: Δ Fasting ghrelin, n = 54, c: Δ Fasting leptin, n = 54, d: Δ Fasting GLP-1, n = 54, e: Δ Fasting PYY, n = 54, f: Δ Fasting GIP, n = 54, g: Δ Fasting PP, n = 54, h: Δ Fasting amylin, n = 54.
Fig 3. Postprandial changes in plasma concentrations of gastrointestinal and appetite hormones after standard meal ingestion. At basal state (triangle, dotted line), after the diet regimen with six meals a day, A6 (rhombus, dashed line) and after the diet regimen with 2 meals a day, B2 (circle, dashed line). Data are expressed as means with 95%CI. A: GLP-1: diet: F = 0.4, p = 0.6828; time: F = 28.5, p < 0.0001; subject: F = 37.5, p < 0.0001; interaction diet × time: F = 0.1, p = 0.9984. B: GIP: diet: F = 17.3, p < 0.0001; time: F = 291.7, p < 0.0001; subject: F = 17, p < 0.0001; interaction diet × time: F = 0.7, p = 0.7093. C: PYY: diet: F = 1.7, p = 0.1897; time: F = 20.7, p < 0.0001; subject: F = 29.1, p < 0.0001; diet × time: F = 0.6,
Fasting amylin decreased after B2 and did not significantly change after A6 with no significant difference between both diet regimens (p = 0.1; 1.77pg/ml; 95% CI: -1.18, 4.76pg/ml in A6 vs. -3.04; 95% CI: -5.91, -0.12pg/ml in B2). Gender differences in changes of fasting levels of amylin plasma concentrations were also not significant (p = 0.9). The postprandial responses of amylin were similar in all three meal tests (interaction diet x time: p = 0.97) (Fig 3G).

Correlations of postprandial concentrations of Δ glucose, Δ IRI and Δ c-peptide, Δ GIHs and Δ appetite hormones

Postprandial secretion of measured gastrointestinal hormones was increased in parallel with glucose and insulin concentrations as shown in Table 2. In the first 30 minutes after meal ingestion (0–30´), a positive relationship was found between Δ PYY and Δ glucose, Δ Immunoreactive insulin (IRI) and Δ c-peptide. Changes in PYY concentrations were also correlated with changes in leptin, GIP (p = 0.035, p = 0.016, respectively) and strongly correlated with changes in GLP-1, amylin and PP (p<0.001 all). Changes in amylin concentrations correlated with changes in glucose (p = 0.003), leptin (p = 0.002) and all measured GH1’s. Between 30–60 minutes after meal ingestion a positive relationship was found between Δ GLP-1 and Δ glucose (p = 0.004) and Δ amylin and Δ glucose (p = 0.044). Δ leptin correlated with ΔGIP (p = 0.029). Changes in GLP-1 concentrations correlated with changes in GIP (p = 0.015), amylin, PP (p = 0.007, p = 0.001, respectively) and PYY (p<0.001) concentrations. Between 60–120 minutes after meal ingestion changes in amylin correlated positively with ΔPYY and GLP-1 (p = 0.001, p<0.001, respectively). Changes in amylin concentrations correlated strongly with changes in glucose, IRI and c-peptide (p<0.001 all). The correlations between Δ GLP-1, Δ GIP, Δ PYY and Δ PP remained significant. Between 120–180 minutes after meal ingestion there was an inverse correlation between Δ ghrelin and Δ glucose (p = 0.026). A positive relationship was found between Δ IRI, Δ GLP-1 and ΔGIP (p = 0.008, p = 0.005, respectively). Changes in GIP concentrations strongly correlated with changes in Δ GLP-1, amylin, PP and PYY (p<0.001 all).

Discussion

Appetite hormones

In this secondary analysis, we tested the effect of two hypocaloric diet regimens with different meal frequency on fasting and postprandial levels of appetite and GI hormones. We have shown previously that in patients with type 2 diabetes breakfast and lunch consumption reduced feelings of hunger [20] more than a diet with the same caloric restriction divided into six more frequent meals. Our results indicate that lower meal frequency in a reduced-energy regimen, leading to greater reduction in body weight, is also associated with an increase in fasting plasma levels of ghrelin, that negatively correlate with changes in body mass index. This is a very good outcome given the fact that it may increase hunger after waking up, encouraging the patients to eat more calories for breakfast. Obese and T2D individuals usually have low hunger sensation at wake up, making difficult to eat breakfast. Moreover, the breakfast omission is associated with increased risk of poor glycaemic control and visceral adiposity despite the same daily energy intake [36–38]. The rise in ghrelin plasma levels in B2 group is also likely
There is some evidence that the increase of fasting plasma concentration of ghrelin appear to be the result of longer fasting period between both meals, that will be in line with the results of a study suggesting that the preprandial increase in ghrelin is associated with inter-meal interval [39]. The variations of body weight lead to compensatory responses of fasting ghrelin levels. Ghrelin is known to be reduced in obese subjects and increased after diet-induced weight loss [25]. It is supposed to stimulate appetite and food intake, enhancing fat mass deposition and weight regain. However, the physiological importance of ghrelin as a regulator of energy homeostasis is still unclear, because there are also studies, which do not confirm the association between weight loss following the hypocaloric diet and the increase in fasting ghrelin [40]. There is some evidence that the increase of fasting plasma concentration of ghrelin appear to be correlated with the increase [41, 42], but also the decrease in hunger [43]. In our study the changes in fasting plasma ghrelin levels did not correlate with changes in hunger. In one

### Table 2. Correlations of postprandial concentrations of Δ glucose, Δ IRI and Δ c-peptide, Δ GIH’s and Δ appetite hormones, n = 54.

<table>
<thead>
<tr>
<th>Δ (0–30)</th>
<th>Δ glucose p</th>
<th>Δ IRI p</th>
<th>Δ c-peptide p</th>
<th>Δ GIP p</th>
<th>Δ GLP-1 p</th>
<th>Δ amylin p</th>
<th>Δ PP p</th>
<th>Δ PYY p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ ghrelin</td>
<td>-0.209</td>
<td>-0.104</td>
<td>-0.080</td>
<td>0.079</td>
<td>0.082</td>
<td>0.020</td>
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<td>0.127</td>
<td>0.339</td>
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<tr>
<td>Δ GIP</td>
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<td>0.104</td>
<td>0.190</td>
<td>0.331</td>
<td>**</td>
<td>0.340</td>
<td>**</td>
<td>0.214</td>
</tr>
<tr>
<td>Δ GLP-1</td>
<td>0.104</td>
<td>0.261</td>
<td>*</td>
<td>0.160</td>
<td>0.331</td>
<td>**</td>
<td>0.381</td>
<td>**</td>
</tr>
<tr>
<td>Δ amylin</td>
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<td>**</td>
<td>0.209</td>
<td>0.176</td>
<td>0.340</td>
<td>**</td>
<td>0.381</td>
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</tr>
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<td>0.269</td>
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<td>Δ GLP-1 p</td>
<td>Δ amylin p</td>
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<td>Δ c-peptide p</td>
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<td>Δ GLP-1 p</td>
<td>Δ amylin p</td>
<td>Δ PP p</td>
<td>Δ PYY p</td>
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<td>-0.234</td>
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<td>0.007</td>
<td>0.103</td>
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<tr>
<td>Δ GIP</td>
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<td>*</td>
<td>0.094</td>
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<td>0.269</td>
<td>0.338</td>
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<td>-0.070</td>
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<td>0.159</td>
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<tr>
<td>Δ PP</td>
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<td>0.119</td>
<td>0.116</td>
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<td>0.066</td>
<td>0.445</td>
<td>***</td>
<td>0.581</td>
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* denote p < 0.05, ** denote p < 0.01, *** denote p < 0.001.

https://doi.org/10.1371/journal.pone.0174820.t002
randomized controlled study, less frequent eating was related to increased satiety and appetite control during the day [12]. Decreased hunger and increased satiety in response to lower meal frequency has already been demonstrated earlier [44]. One probable explanation of this finding is that larger meals impart the sense of fullness and satiety and decreased postprandial ghrelin levels [12], making periods of fasting more bearable than being hungry all day, from eating more smaller meals without getting full and being in the postprandial phase for the majority of the day. In lean healthy individuals, the ghrelin plasma concentrations increase during fasting and are suppressed by meal intake [45]. In obese subjects and patients with T2D, ghrelin secretion is down-regulated and the decline in plasma ghrelin after a meal is diminished [22, 23]. Weight loss induced by food restriction increases ghrelin plasma levels [24, 25] and improves the postprandial ghrelin response independently of diet composition [26]. Postprandial decreases in ghrelin were also positively correlated with inter-meal interval in healthy normal weight subjects. It was shown in one study that meal request after energy restriction was preceded by an increase in ghrelin and was positively correlated with inter-meal interval most profound after energy restriction, not during energy balance diet [39]. Periods of fasting between meals may be even more important than the composition of the diet. As far as the postprandial state is concerned, the plasma concentrations of ghrelin were significantly higher at fasting state and after 180 minutes after meal ingestion after the diet regimen B2. We described the positive association between the baseline level of ghrelin and its decrease after meal ingestion. It means that the higher the baseline level of ghrelin, the larger the postprandial drop. Furthermore, between 120–180 minutes postprandially, there was an inverse correlation observed between changes in plasma ghrelin levels and changes in plasma glucose levels. The increase of ghrelin postprandially might be dependent on the return of plasma glucose to fasting concentrations. The failure of returning postprandial glucose plasma levels to normal levels causes a lack of a preprandial increase in ghrelin concentration. That is in accordance with previous findings [39].

In our study, fasting leptin decreased in response to both regimens with no difference between the treatments. Leptin, an adipokine secreted from white adipose tissue, is known for its effects on decreasing appetite and food intake. Obesity and T2D are associated with increased leptin levels and resistance to leptin action [46]. Increased leptin levels, probably reflecting leptin resistance, were strongly related to insulin resistance [47]. The two different diet regimens induced expected similar postprandial responses of leptin, which acts rather as a long-term adiposity signal than a gastrointestinal signal that regulates postprandial state [1].

The fasting concentrations of leptin at baseline were significantly higher in women than in men. According to previous findings, plasma leptin concentration is directly related to the degree of obesity and is higher in women than in men of the same body mass index [48, 49]. Furthermore, according to our results, the hypocaloric diet reduced significantly leptin but only in females. In males the leptin concentrations remained unchanged. The weight at baseline in men vs. women and also the body weight reduction after the diet regimens were similar. Also in a study with 6-month weight loss intervention, relative decline in circulating leptin was greater in women than in men, although the percentage of fat mass loss was similar [50]. Whereas that leptin is believed to increase energy expenditure [51], this sex asymmetry is supposed to represent an evolutionary paradigm for females to resist the loss of energy stores [52].

**Amylin**

Fasting amylin decreased in the hypocaloric diet regimen with lower frequency of meals. It is known to regulate both glucose and energy homeostasis [53]. Proportional changes in amylin and insulin concentrations were highly correlated, because amylin is a peptide co-secreted
with insulin upon glucose stimulation [54]. It also suppresses gastrointestinal motility and food intake [55]. In obese humans, the amylin analog pramlintide elicited sustained reductions in food intake and body weight [56]. Amylin seems to be particularly effective when combined with other hormones such as leptin. According to our study, the postprandial responses of amylin were similar in all three meal tests. Postprandial changes in amylin concentrations correlated with changes in concentrations of all measured GIHs, and there was a strong correlation with postprandial changes of leptin. Leptin is known as a long-term adiposity signal, whereas gastrointestinal hormones, such as amylin are regarded as short-term satiety signals. There might be a potential interaction between them and according to some evidence amylin agonism could even restore leptin responsiveness in diet-induced obesity [57]. It is worth noting that amylin interacts with numerous gastrointestinal hormones to control eating and mediate the eating inhibitory effect of some of these hormones, most prominently peptide YY and GLP-1 [58]. These combinations lead to a stronger reduction of eating control than single hormones alone.

Glucagon-like peptide-1, peptide YY and pancreatic polypeptide

In the present study, fasting plasma levels of neither GLP-1 nor PYY and also their postprandial responses did change after neither hypocaloric diet regimens. Despite we have expected lower fasting plasma levels and higher postprandial responses after both diet regimens more pronounced with B2, because of bigger weight loss. It was demonstrated that both peptides exert anorexigenic effects and that their satiating effects should be even additive [28]. Caloric restriction and body weight reduction are supposed to decrease fasting plasma levels [7, 8, 10] and increase meal responses of PYY and GLP-1 [9]. Studies with hypocaloric diet regimens indicate that lower meal frequency should also have positive effect on GLP-1s postprandial response [12]. From some studies in obese people after gastric bypass surgery is evident, that postprandial response of both peptides is significantly higher with larger weight loss [59]. On the contrary, another study suggests that it is not the weight loss, but rather the surgical procedure, which increases GLP-1 levels [60]. In humans, intravenous administration of GLP-1 increases satiety, inhibit gastric emptying and decreases weight [61]. Obese individuals and patients with T2D have been reported to have delayed postprandial release of GLP-1 with improvement after weight loss [62] and that successful weight loss maintenance includes long-term increase meal responses of GLP-1 and PYY [9]. Correlation of changes in GLP-1 and insulin during the whole postprandial phase observed by the authors is not surprising due to its well-documented incretin effect.

PYY plays an important regulatory role in GIT function [63]. It is produced in all segments of the intestine and co-secreted predominantly from the endocrine L cells in the ileum together with GLP-1. The role of PYY in feeding regulation has attracted considerable attention. In humans it has been reported, that it is an anorectic hormone, because it decreases food intake and appetite when injected peripherally [3]. The level of circulating PYY may also be increased by exercise [64]. It was demonstrated that PYY levels are decreased by complete fasting for more than two days in lean subjects and it was not regulated by leptin [10]. In obese state, the fasting and postprandial PYY levels are often lower [65], but not all studies have confirmed this to be true [66]. In contrast to reduced sensitivity to leptin among obese and diabetic patients, the sensitivity to the anorectic effects of PYY remained significant [67]. Postprandially, we have not found any effects of both hypocaloric diet regimens on the response after the standard meal. According to previous studies, the initial postprandial response of PYY is supposed to be attenuated in patients with T2D [6], especially after a fatty meal [68]. The increased PYY3–36 response represents an improved capacity to regulate
satiety and potentially body weight in insulin-resistant patients [11]. We were expecting an increase in meal-stimulated PYY response after B2.

We have found a positive relationship between postprandial changes in PYY and insulin. To the best knowledge of the authors, the association between PYY and postprandial insulin levels have not been published yet.

As far as PP is concerned, we have detected the increase in fasting PP after B2 with no significant difference between both diet regimens. Its role in patients with T2D is uncertain. Some studies have shown higher fasting levels with blunted postprandial response, similar to PYY [68]. According to previous studies, peripheral administration of PP reduced food intake in lean healthy individuals [27]. We have not found any effects of both hypocaloric diet regimens on the postprandial response of PP. According to a recent overview, the effects of a hypocaloric regimen or changes in body weight on fasting or postprandial levels of PP were not proven [29].

**GIP**

We detected that fasting plasma levels of GIP decreased comparably after both hypocaloric diet regimens. The reduction in fasting plasma concentration following a hypocaloric diet is a positive finding, because increased GIP levels directly promote fat storage and energy deposition [69]. It is released postprandially in response to feeding, especially with high fat diet. It produces a glucose-dependent stimulation of insulin secretion consistent with its role as an incretin hormone [70]. We detected the positive correlation of changes in GIP and insulin between 120–180 minutes after meal ingestion. It may be interpreted that beta-cells in T2D are insensitive to meal and stimulation by incretin hormone GIP in the early postprandial period. This is in accordance with previous findings [71].

Postprandially, there was a significantly higher GIP response at baseline in first 30 minutes after the meal ingestion than after both hypocaloric diet regimens, where the postprandial responses were similar. We have expected lower postprandial response in the regimen B2 due to a bigger weight loss, which is supposed to mediate this effect [72]. In one intervention study hypocaloric diet following a weight loss was associated with reduction in GIP response to ingested glucose [11]. Diet regimens with calorie reduction that lower the postprandial response of the anabolic hormone GIP could be beneficial and effective for weight management [73]. It has been suggested that changes in insulin secretion following a lifestyle intervention might be mediated via alterations in GIP secretion [74].

Several studies have shown that an increase in meal size increases diet-induced thermogenesis (DIT). It has been demonstrated that a large isocaloric mixed meal causes a greater postprandial thermogenic response than the same food consumed in six smaller portions [75]. It was also demonstrated, that lower postprandial response of GIP leads to greater postprandial thermogenesis and that GIP response could be a negative regulator of postprandial thermogenic efficiency [73]. In current study we have not investigated postprandial thermogenesis and possible correlations with gastrointestinal hormone release or the effect of caloric restriction or meal frequency on DIT. This mechanism could explain the greater weight loss with the B2 regimen.

The study has a number of strengths. First of all, the cross-over design increased our power to detect differences in response to both regimens. Second, we used a physiological approach to measure the concentrations of GIHs in both fasting and postprandial state after the ingestion of a standardized meal.

The main limitation of our study is the relatively short duration, which precludes a generalisation of our findings. Also, the duration of diabetes in our patients was quite short. All
patients were treated by oral hypoglycemic agents, so the sample of diabetic patients in our study was not representative of the whole T2D population. Lower caloric intake leading to bigger weight loss in B2 could be a confounding factor of the results. We have to admit the possibility of a reduced energy intake with the B2 regimen, even though the energy intake reported by our participants was similar in both regimens. Reported dietary intake and also physical activity were comparable in both regimens. Another limitation is the number of statistical tests and factors in the statistical model, which may cause inaccurate findings. A further limitation is that the present study revealed interrelationships, not causal relationships and we cannot therefore address the mechanisms underlying the correlations.

Conclusions
Both hypocaloric diet regimens decreased fasting leptin and GIP and reduced the postprandial response of GIP similarly. Eating only breakfast and lunch increased fasting plasma ghrelin more than the same caloric restriction split into six meals. The changes in fasting ghrelin correlated negatively with the decrease in body weight. These results suggest that for type 2 diabetic patients on a hypocaloric diet, eating larger breakfast and lunch may be more efficient and therefore beneficial than six smaller meals during the day.

Supporting information
S1 File. Checklist.
(DOC)
S2 File. Study protocol in English.
(DOC)
S3 File. Study protocol in Czech.
(DOC)

Acknowledgments
We thank the 54 participants for their cooperation, as well as our staff from the Diabetes Centre, Institute for Clinical and Experimental Medicine, Prague, Czech Republic: the registered dieticians (Vladka Havlova and Ruzena Milatova) for providing both group and individual nutrition counselling and study nurses (Danuse Lapesova, Dagmar Sisakova, Dana Kobrova, Jitka Purrova and Blazena Vodickova) for conducting the procedures.

Author Contributions
Conceptualization: LB HK TP.
Formal analysis: LB MH.
Funding acquisition: TP LB.
Investigation: LB HK HM.
Methodology: LB HK MH.
Project administration: TP LB HK.
Resources: LK OO HM JW OT.
Software: MH.
Supervision: TP.
Validation: MH.
Visualization: LB HK HM.
Writing – original draft: LB.
Writing – review & editing: HK TP HM OO JW OT LK MH.

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Meal frequency and gastrointestinal and appetite hormones


ORIGINAL ARTICLES

Length and weight of very low birth weight infants in Germany at 2 years of age: does it matter at what age they start complementary food?

Functional cobalamin (vitamin B12) deficiency: role of advanced age and disorders associated with increased oxidative stress

The role of acetic acid on glucose uptake and blood flow rates in the skeletal muscle in humans with impaired glucose tolerance
LETTER TO THE EDITOR

Do patients with type 2 diabetes still need to eat snacks?

European Journal of Clinical Nutrition advance online publication, 1 April 2015; doi:10.1038/ejcn.2015.46

The size and frequency of meals are important elements of nutrition, with considerable effects on the human health. A hypocaloric diet is a key component in both prevention and treatment of type 2 diabetes and it is usually apportioned into three main meals and two or three snacks in between.

Eating snacks is often advocated as a means of controlling body weight and glycaemic control, but studies endorsing this practice are lacking. In fact, it has been demonstrated that snacking leads to weight gain and increased risk of type 2 diabetes. Snacking encourages higher energy intake by increasing food stimuli, hunger and the desire to eat, which is absolutely counter-productive to the treatment of type 2 diabetes.

On the other hand, diets incorporating intermittent fasting seem to be efficient for weight loss and type 2 diabetes risk reduction in overweight and obese populations. We have shown previously that breakfast and lunch consumption reduced body weight, hepatic fat content, fasting plasma glucose, C-peptide and glucagon, and increased insulin sensitivity more than a diet with the same caloric restriction divided into six more frequent meals in patients with type 2 diabetes. Also for glycaemic control it may be more beneficial to eat larger meals rich in fibre instead of dividing them into smaller portions.

However, for a long-term effectiveness of any dietary approach not only metabolic benefits are important, but also the effects on quality of life and eating behaviour. To the best of our knowledge, no study has been published as yet on the effect of meal frequency on quality of life, depressive symptoms and eating behaviour in patients with type 2 diabetes.

In this secondary analysis of our previously published data, during a 24-week crossover trial, we studied quality of life, Beck depression score and eating behaviour in response to a hypocaloric diet consumed as either six (A6) or two meals, breakfast and lunch, per day (B2). The characteristics of the sample and the methods are described in detail elsewhere. Briefly, 54 patients with type 2 diabetes treated by oral hypoglycaemic agents (both men and women, aged 30–70 years, body mass index 27–50 kg/m² and glycated haemoglobin 6–11.8%, that is, 42–105 mmol/mol) were randomized within a 24-week crossover single-centre study (conducted in Prague, the Czech Republic).

After a 1-month run-in period, the participants began a 12-week regimen of either six (A6) or two (B2) meals a day. The A6 regimen consisted of three main meals (breakfast, lunch and dinner) and three smaller snacks in between. The B2 regimen consisted of breakfast (eaten between 0600 and 1000 hours) and lunch (eaten between 1200 and 1600 hours). Then the regimens were switched between 1200 and 1600 hours). Changes from baseline in response to a hypocaloric diet consumed as either six (A6) or two meals, breakfast and lunch, per day (B2). The characteristics of the sample and the methods are described in detail elsewhere. Briefly, 54 patients with type 2 diabetes treated by oral hypoglycaemic agents (both men and women, aged 30–70 years, body mass index 27–50 kg/m² and glycated haemoglobin 6–11.8%, that is, 42–105 mmol/mol) were randomized within a 24-week crossover single-centre study (conducted in Prague, the Czech Republic).

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The diet composition under both regimens followed the Study Group on Diabetes and Nutrition of the European Association for

Figure 1. Changes in quality of life, Beck Depression Inventory and Three-Factor Eating Questionnaire. Changes from baseline in response to the regimen of six (A6) and two meals a day (B2), means ± 95% confidence intervals. Significance of the factor treatment (assessed by 2 × 2 crossover analysis of variance) is indicated by *P < 0.05, ***P < 0.001 and NS for nonsignificant. (a) ΔOWLQoL, n = 48; (b) ΔWRSM, n = 48; (c) ΔBeck depression score, n = 47; (d) Δdietary restraint, n = 47, (e) Δdisinhibition, n = 47; (f) Δhunger, n = 47.
the Study of Diabetes guidelines, with the same caloric restriction of −500 kcal/day, based on the measurement of resting energy expenditure of each subject by indirect calorimetry (metabolic monitor VMAX; Sensor Medics, Anaheim, CA, USA). The diet derived 50–55% of total energy from carbohydrates, 20–25% protein, < 30% fat (<7% saturated fat, < 200 mg/day of cholesterol), and 30–40 g/day of fibre. Participants were asked not to alter their exercise habits during the study.

Quality of life was assessed using two questionnaires: Obesity and Weight-Loss Quality of Life (OWLQoL) and Weight-Related Symptoms (WRSMS)—see Supplementary Appendix 1 and 2. We used the Three-Factor Eating Questionnaire17 to monitor changes in eating behaviour and the Beck Depression Inventory to screen for depressive symptoms—see Supplementary Appendix 3. For statistical analysis we used a 2 × 2 crossover analysis of variance.

The variables of quality of life, Beck Depression Inventory and Three-Factor Eating Questionnaire are shown in Figure 1. Quality of life (the OWLQoL score) increased (P < 0.01) comparably under both regimens (Figure 1a). The decrease in negative WRSMS was borderline significant in A6 (P = 0.05) and not statistically significant in B2 (P = 0.06), with no significant difference between treatments (P = 0.6; Figure 1b). Beck depression inventory score decreased (P < 0.05) in response to both regimens, more with B2 (P = 0.04; Figure 1c). Dietary restraint (Figure 1d) increased and disinhibition (Figure 1e) decreased comparably under both regimens (P < 0.01 each). Feelings of hunger remained unchanged in A6 but decreased significantly in B2 (P < 0.001; difference between treatments: P < 0.001; Figure 1f). No substantial unfavourable effects of the regimens were observed.

Both regimens elicited a positive effect on the quality of life, Beck score of depression and eating behaviour. The positive effects on depressive symptoms and feelings of hunger were greater with B2.

The mechanism by which B2 can reduce depressive symptoms may be change in concentrations of various neuropeptides including increased production of brain-derived neurotrophic factor in response to intermittent fasting, which has been shown to increase the resistance of brain neurons to dysfunction and degeneration in animal models.15

The three-factor eating questionnaire revealed that dietary restraint increased under both regimens, suggesting better voluntary control over food intake, aimed at reducing body weight. The decrease in disinhibition in both regimens means that the participants were less likely to overeat in, for example, stressful situations. A greater decrease in reported feelings of hunger in B2 suggests easier adherence to the regimen of breakfast and lunch compared with A6, in the long term. Decreased hunger in response to lower meal frequency has already been demonstrated.5,13 One probable explanation of this finding is that larger meals impart the sense of fullness and satiety and inhibit ghrelin,13 making periods of fasting more bearable than being hungry all the day long, from eating more smaller meals without getting full.

In conclusion, both regimens elicited a positive effect on the quality of life, Beck score of depression and eating behaviour. The positive effects on depressive symptoms and feelings of hunger were greater with B2. These results, together with the metabolic data (that is, greater positive effects of B2 on body weight, hepatic fat content, fasting plasma glucose and increased insulin sensitivity) suggest that for long-term adherence of type 2 diabetic patients to a hypocaloric diet rich in fibre, eating large breakfasts and lunches may be more beneficial than the usual snacking model. Further larger-scale, long-term studies are essential before offering recommendations in terms of meal frequency.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGEMENTS
This work was supported by the project grant IGA MZCR NT/14250-3 from Ministry of Health, Prague, Czech Republic and Institutional Support MZCR 00023001 (IKEM, Prague, Czech Republic).

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Supplementary Information accompanies this paper on European Journal of Clinical Nutrition website (http://www.nature.com/ejcn)
The Effect of Meal Frequency on the Fatty Acid Composition of Serum Phospholipids in Patients with Type 2 Diabetes

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To cite this article: Hana Kahleova MD, PhD, Hana Malinska PhD, Ludmila Kazdova PhD, Lenka Belinova MD, Andrea Tura PhD, Martin Hill PhD & Terezie Pelikanova MD, PhD (2015): The Effect of Meal Frequency on the Fatty Acid Composition of Serum Phospholipids in Patients with Type 2 Diabetes, Journal of the American College of Nutrition, DOI: 10.1080/07315724.2015.1046197

To link to this article: http://dx.doi.org/10.1080/07315724.2015.1046197

Published online: 23 Dec 2015.
Original Research

The Effect of Meal Frequency on the Fatty Acid Composition of Serum Phospholipids in Patients with Type 2 Diabetes

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Key words: meal frequency, phospholipid fatty acid composition, type 2 diabetes, nutrition, insulin sensitivity

Objective:
Fatty acids are important cellular constituents that can affect many metabolic processes relevant for the development of diabetes and its complications. We previously demonstrated a positive effect of eating just 2 meals a day, breakfast and lunch, compared to 6 small meals. The aim of this secondary analysis was to explore the effect of meal frequency on the fatty acid composition of serum phospholipids in subjects with type 2 diabetes (T2D).

Methods:
In a randomized, crossover study, we assigned 54 patients with T2D to follow one of 2 regimens of a hypocaloric diet (~500 kcal/day), each for 12 weeks: 6 meals (A6) or 2 meals a day, breakfast and lunch (B2). The diet in both regimens had the same macronutrient and energy content. The fatty acid composition of serum phospholipids was measured at weeks 0, 12, and 24, using gas liquid chromatography. Insulin sensitivity was derived as an oral glucose insulin sensitivity (OGIS) index.

Results:
Saturated fatty acids (mainly myristic and palmitic acids) decreased ($p < 0.001$) and n6 polyunsaturated fatty acids increased ($p < 0.001$) in response to both regimens but more with B2 ($p < 0.001$ for both). Monounsaturated fatty acids decreased ($p < 0.05$) and n3 polyunsaturated fatty acids increased ($p < 0.001$) in response to both regimens, with no difference between the regimens. An increase in OGIS correlated positively with changes in the proportion of linoleic acid in B2. This correlation remained significant even after adjustment for changes in body mass index (BMI; $r = -0.38$; $p = 0.012$).

Conclusions:
We demonstrated that meal frequency affects the fatty acid composition of serum phospholipids. The B2 regimen had more marked positive effects, with saturated fatty acids and the ratio of saturated to unsaturated fatty acids decreasing more. The increase in linoleic acid could partly explain the insulin-sensitizing effect of B2 in T2D.

INTRODUCTION

The fatty acid composition of membrane phospholipids can affect the biophysical properties of cell membranes, modulating both insulin binding and action [1,2]. Diet-induced alterations in membrane composition may provide a mechanism for improving the cellular response to insulin [3,4]. In addition, metabolomic studies have demonstrated that fatty acids are metabolites associated strongly with metabolic syndrome, impaired glucose tolerance and type 2 diabetes. As such, they form a considerable part of the so called metabolic signature [5,6].

The composition of serum fatty acids predicts the long-term development of metabolic syndrome [7,8]. Insulin
Meal Frequency and Fatty Acid Profile in T2D

resistance is often associated with a particular serum fatty acid pattern, characterized by an increased proportion of palmitic, palmitoleic, and a low proportion of linoleic acids, with the distribution of other fatty acids indicating an increased index of Δ9 and Δ6-desaturase and decreased Δ5-desaturase index [9–11]. This corroborates the notion that there may be a causal relationship between the type of fat in the diet and insulin action [3].

Differences in obesity levels and lifestyle variables are associated with typical changes in fatty acid profiles [12]. The fatty acid composition in serum phospholipids reflects both the dietary fatty acid intake and endogenous fatty acid metabolism [13]. It has been shown that the fatty acid compositions of both serum phospholipids and adipose tissue lipids are valid biomarkers of dietary intake in men [1,14]. In addition, the fatty acid composition of serum phospholipids correlates with the composition of tissue lipids [1]. The content of polyunsaturated fatty acids in the diet has been shown to correlate with the fatty acid composition in adipose tissue and all blood lipid fractions [15], with weaker correlations for monounsaturated and saturated fatty acids [1]. In line with this, nutrition and dietary habits (together with physical activity) are the key components in the management of type 2 diabetes (T2D) [16]. Intervention studies have shown that the plasma fatty acid pattern changes in response to dietary interventions, and these are associated with changes in insulin sensitivity [17].

We have previously demonstrated a negative correlation between the content of saturated fatty acids in plasma phospholipids and insulin sensitivity and a positive association between insulin action and the proportion of linoleic acid in healthy individuals [15,18]. We further demonstrated that a vegetarian diet–induced increase in linoleic acid in serum phospholipids is associated with improved insulin sensitivity in patients with T2D [19]. We also demonstrated the superior effect of eating 2 meals a day, breakfast and lunch (B2), on body weight, hepatic fat content, fasting plasma glucose, C-peptide, glucagon, and insulin sensitivity, compared to the same diet (with the same caloric restriction and macronutrient composition) divided into 6 smaller meals a day (A6) [20]. However, to the best of our knowledge, the effect of meal frequency on fatty acid composition has not yet been studied. The aim of this secondary analysis of our previously published data was to compare the effect of A6 vs B2 with the same caloric restriction and macronutrient composition on the fatty acid composition in serum phospholipids and to test whether these changes are related to the insulin-sensitizing effect of B2 in patients with T2D. Our hypothesis was that increased insulin sensitivity induced by B2 would be related to changes in the pattern of serum phospholipid fatty acids, namely, to the decrease in saturated fatty acids (and subsequent decrease in the ratio of saturated to unsaturated fatty acids) and to the increase in linoleic acid.

MATERIALS AND METHODS

The characteristics of the subjects and methods are described in detail elsewhere [20]. Briefly, 54 patients with T2D (both men and women) treated only by oral hypoglycaemic agents (not insulin), mean age 59.4 ± 7.0 (from 30 to 70) years, mean BMI 32.6 ± 4.9 (from 27 to 50) kg.m², and mean HbA1c (International Federation of Clinical Chemistry and Laboratory Medicine [IFCC]) 54.9 ± 13.0 (from 42 to 105) mmol/L, met all inclusion criteria, gave a written informed consent, and underwent randomization within a 24-week crossover study. After a 1-month run-in period, the participants started with a regimen of either 6 (A6) or 2 (B2) meals a day that was followed for 12 weeks. The A6 regimen consisted of 5 main meals (breakfast, lunch, and dinner), and 3 smaller snacks in between. The B2 regimen consisted of breakfast (eaten between 6 and 10 AM) and lunch (eaten between noon and 4 PM). Then the participants switched regimens for a subsequent 12 weeks. The study protocol was approved by the Institutional Ethics Committee.

The diet composition in both regimens followed the Study Group on Diabetes and Nutrition of the European Association for the Study of Diabetes guidelines [21] and had the same caloric restriction of −500 kcal/day based on measurements of the resting energy expenditure of each subject by indirect calorimetry (metabolic monitor VMAX; Sensor Medics, Anaheim, CA) [22]. The diet contained 50%–55% of total energy from carbohydrates, 20%–25% protein, less than 30% fat (<7% saturated fat, less than 200 mg/day of cholesterol/day), and 30–40 g/day of fiber. Participants were asked not to alter their exercise habits during the study. Whole-body insulin sensitivity was estimated by a glucose–insulin model to derive an oral glucose insulin sensitivity (OGIS) index, validated against clamp data [23]. OGIS was calculated from glucose and insulin concentrations after the standard meal test at 0, 30, 60, 120, and 180 minutes [20, 24].

Compliance with both regimens was maximized by an initial 4-day tutorial at the beginning of each regimen where the patients learned in detail how to compose and divide their diet, with follow-up weekly meetings with lectures, cooking classes, and food diary consultation with a registered dietician. Registered dieticians analyzed 3-day dietary records (2 weekdays and 1 weekend day) completed by each participant at weeks 0, 12, and 24. The International Physical Activity Questionnaire [25] and the Baecke Questionnaire [26] were completed by each participant at weeks 0, 12, and 24.

Analytic Methods

Blood samples for biochemical analyses were drawn after 10- to 12-hour overnight fasting with only tap water allowed ad libitum.
Phospholipid Fatty Acid Composition

The methods are described in detail elsewhere [27]. Briefly, serum total lipids were extracted by chloroform : methanol (2:1). Phospholipids were isolated by thin-layer chromatography using hexane–diethyl ether–acetic acid (80:20:3, v/v) as a solvent system. Fatty acids in serum phospholipids were converted to methyl esters using a 1% solution of Na in methanol, and the fatty acid methyl esters were eluted with hexane. Gas chromatography of the fatty acid methyl esters was performed on a GS 5890A (Hewlett Packard, Palo Alto, CA) equipped with a flame ionization detector, using a carbowax-fused silica capillary column (25m × 0.25 mm i.d.). The column temperature ranged from 150 to 225°C (2°C/min) and hydrogen was used as the carrier gas [28]. Individual peaks of fatty acid methyl esters were identified by comparing retention times with those of authenticated standards (Sigma, Prague, Czech Republic). The external mix of standard fatty acids (Restek) was used. The composition of serum fatty acids (a spectrum of the 18 main fatty acids of interest) was analyzed. The product/

Statistical Analyses

The intention-to-treat analysis included all participants. We tested the distributions of the data, and if the distribution was skewed we used the Box-Cox transformation to obtain data symmetry and homoscedasticity [29]. Nonhomogeneities in the data were detected using residual analysis as described elsewhere [30]. Two by two crossover analysis of variance was used for data evaluation. The model consisted of the between-subject factor sequence (i.e., the order in which participants underwent the diet regimens), the factor subject (the identity of the patient), and the within-subject factors period (12 weeks) and treatment (A2 vs B6). The sequence factor was used to test for any potential carry-over effects. Data are presented as means with 95% confidential intervals. The relationships between continuous variables were evaluated using Pearson’s correlation and BMI-adjusted partial correlations.

RESULTS

All results are expressed as changes in response to the regimens of 6 (A6) and 2 meals a day (breakfast and lunch, B2). The factors period and sequence were not significant. Changes in the relative contents of measured fatty acids in serum phospholipids for both regimens are shown in Table 1 and Fig. 1.

Fatty Acid Composition of the Diet

The fatty acid composition of the diet was comparable in both regimens. The intake of saturated fatty acids was 19 g/day in B2 (95% confidence interval [CI], 16.9 to 21.4 g/day) and 21.6 g/day in A6 (17.6 to 26.2 g/day). The intake of monounsaturated fatty acids was 21.5 g/day in B2 (95% CI, 18.9 to 24.6 g/day) and 21.3 g/day in A6 (19.1 to 24.0 g/day). The intake of polyunsaturated fatty acids was 18.8 g/day in B2 (95% CI, 16.1 to 22.2 g/day) and 17.7 g/day in A6 (16.4 to 19.5 g/day). The ratio of n6 to n3 polyunsaturated fatty acids was about 8:1 in both regimens.

Saturated Fatty Acids in Serum Phospholipids

Saturated fatty acids decreased (p < 0.001) in response to both regimens but more with B2 (p < 0.001; Fig. 1A). This decrease of saturated fatty acids was mainly due to a decrease in myristic and palmitic acids (p < 0.001), which was more marked with B2 (p < 0.001; Figs. 1B and 1C). On the other hand, stearic acid increased (p < 0.01) in response to both regimens but more with B2 (p < 0.001; Fig. 1D).

Mono- and Polyunsaturated Fatty Acids in Serum Phospholipids

Monounsaturated fatty acids decreased slightly (p < 0.05) in response to both regimens, with no difference between the regimens (Fig. 1E). The proportion of n3 polyunsaturated fatty acids in phospholipids increased (p < 0.001) in response to both regimens, with no difference between the regimens (Fig. 1F). On the other hand, the proportion of n6 polyunsaturated fatty acids in phospholipids increased (p < 0.001) in response to both regimens but more with B2 (p < 0.001; Fig. 1G). The difference between both regimens was most marked in linoleic acid, which decreased in response to A6 (p < 0.05) compared to a trend toward increase with B2 (p < 0.05; Fig. 1H) and in arachidonic acid, which increased (p < 0.01) in response to both regimens but more with B2 (p < 0.05).

The ratios of saturated to polyunsaturated and of n6 to n3 polyunsaturated fatty acids decreased (p < 0.001) in response to both regimens. The ratio of saturated to polyunsaturated fatty acids decreased more with B2 (p < 0.001; Fig. 1I).

Desaturase, Elongase, and Fatty Acid Ratios

The index of Δ9-desaturase decreased (p < 0.01) and the index of Δ6-desaturase slightly increased (p < 0.05) in response to both regimens, with no difference between the regimens. The index of elongase increased (p < 0.01) in response to both regimens but more with B2 (p < 0.001).
Table 1 Changes in Fatty Acids in Serum Phospholipids (mol %)

<table>
<thead>
<tr>
<th>Fatty acids in serum phospholipids (mol %)</th>
<th>Baseline (median ± interquartile range)</th>
<th>Baseline (means ± 95% CI)</th>
<th>Δ A6 (means ± 95% CI)</th>
<th>Δ B2 (means ± 95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myristic acid (14:00)</td>
<td>0.24 (0.18, 0.28)</td>
<td>0.16 (0.15 to 0.18)</td>
<td>-0.11 (-0.12 to -0.1)</td>
<td>-0.14 (-0.15 to -0.15)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Palmitic acid (16:00)</td>
<td>30.8 (28.3, 32.2)</td>
<td>16.2 (13.4 to 18.9)</td>
<td>-5.33 (-5.93 to -4.73)</td>
<td>-7.99 (-8.62 to -7.37)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Stearic acid (18:00)</td>
<td>14.1 (13.5, 15.1)</td>
<td>16.5 (15.9 to 17.1)</td>
<td>+0.68 (+0.51 to +0.84)</td>
<td>+1.30 (+1.14 to +1.46)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Arachidic acid (20:00)</td>
<td>0.14 (0.12, 0.17)</td>
<td>0.16 (0.11 to 0.25)</td>
<td>-0.018 (-0.015 to +0.022)</td>
<td>+0.019 (+0.015 to +0.023)</td>
<td>&gt;0.850</td>
</tr>
<tr>
<td>Monounsaturated fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitoleic acid (16:1n7)</td>
<td>1.17 (0.915, 1.45)</td>
<td>0.16 (0.11 to 0.21)</td>
<td>-0.51 (-0.56 to -0.47)</td>
<td>-0.54 (-0.59 to -0.50)</td>
<td>0.493</td>
</tr>
<tr>
<td>Oleic acid (18:1n9)</td>
<td>10.7 (9.81, 11.4)</td>
<td>10.3 (9.68 to 10.8)</td>
<td>+0.13 (+0.08 to +0.34)</td>
<td>+0.07 (+0.14 to +0.28)</td>
<td>0.782</td>
</tr>
<tr>
<td>Vaccenic acid (18:1n7)</td>
<td>1.5 (1.33, 1.67)</td>
<td>1.4 (1.3 to 1.5)</td>
<td>-0.003 (-0.008 to -0.02)</td>
<td>+0.06 (+0.01 to +0.11)</td>
<td>0.079</td>
</tr>
<tr>
<td>Gondoic acid (20:1n9)</td>
<td>0.063 (0.027, 0.081)</td>
<td>0.16 (0.11 to 0.21)</td>
<td>+0.06 (+0.05 to +0.07)</td>
<td>+0.06 (+0.05 to +0.07)</td>
<td>0.889</td>
</tr>
<tr>
<td>N3 polyunsaturated fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-linolenic acid (18:3n3)</td>
<td>0.09 (0.05, 0.14)</td>
<td>0.22 (0.12 to 0.31)</td>
<td>-0.05 (+0.02 to +0.09)</td>
<td>+0.08 (+0.05 to +0.11)</td>
<td>0.474</td>
</tr>
<tr>
<td>Eicospentaenoic acid (20:5n3)</td>
<td>0.96 (0.77, 1.28)</td>
<td>1.0 (0.73 to 1.3)</td>
<td>+0.25 (+0.15 to +0.35)</td>
<td>+0.24 (+0.14 to +0.34)</td>
<td>0.899</td>
</tr>
<tr>
<td>Docosapentaenoic acid (22:5n3)</td>
<td>0.72 (0.59, 0.89)</td>
<td>1.6 (1.3 to 1.9)</td>
<td>+0.32 (+0.25 to +0.40)</td>
<td>+0.37 (+0.29 to +0.44)</td>
<td>0.576</td>
</tr>
<tr>
<td>Docosahexaenoic acid (22:6n3)</td>
<td>2.28 (1.92, 2.9)</td>
<td>6.0 (4.9 to 7.2)</td>
<td>+1.08 (+0.89 to +1.28)</td>
<td>+1.23 (+1.03 to +1.45)</td>
<td>0.459</td>
</tr>
<tr>
<td>N6 polyunsaturated fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linoleic acid (18:2n6)</td>
<td>22.2 (20.2, 23.9)</td>
<td>21.7 (20.7 to 22.5)</td>
<td>-0.57 (-0.90 to -0.23)</td>
<td>+0.31 (-0.04 to -0.68)</td>
<td>0.014</td>
</tr>
<tr>
<td>γ-linolenic acid (18:3n6)</td>
<td>0.175 (0.129, 0.284)</td>
<td>0.2 (0.17 to 0.25)</td>
<td>+0.018 (+0.013 to +0.039)</td>
<td>+0.027 (+0.014 to +0.04)</td>
<td>0.945</td>
</tr>
<tr>
<td>Eicosadienoic acid (20:2n6)</td>
<td>0.46 (0.40, 0.55)</td>
<td>0.61 (0.54 to 0.69)</td>
<td>+0.14 (+0.11 to +0.17)</td>
<td>+0.15 (+0.12 to +0.18)</td>
<td>0.605</td>
</tr>
<tr>
<td>Dihomo-γ-linolenic acid (20:3n6)</td>
<td>3.27 (2.85, 3.68)</td>
<td>5.3 (4.8 to 5.9)</td>
<td>+0.026 (+0.013 to +0.039)</td>
<td>+0.027 (+0.014 to +0.04)</td>
<td>0.945</td>
</tr>
<tr>
<td>Arachidonic acid (20:4n6)</td>
<td>10.6 (8.92, 12)</td>
<td>16.3 (15.0 to 17.6)</td>
<td>+2.35 (+1.98 to +2.73)</td>
<td>+3.15 (+2.77 to +3.54)</td>
<td>0.041</td>
</tr>
<tr>
<td>Eicosatetraenoic acid (22:4n6)</td>
<td>0.14 (0.13, 0.17)</td>
<td>0.3 (0.25 to 0.36)</td>
<td>+0.083 (+0.072 to +0.095)</td>
<td>+0.096 (+0.084 to +0.11)</td>
<td>0.284</td>
</tr>
<tr>
<td>The ratio of n6 to n3</td>
<td>8.89 (7.12, 10.6)</td>
<td>11.5 (11.0 to 12.1)</td>
<td>-2.23 (-2.58 to -1.87)</td>
<td>-2.30 (-2.65 to -1.95)</td>
<td>0.836</td>
</tr>
<tr>
<td>The ratio of saturated to unsaturated FA</td>
<td>1.1 (1.04, 1.2)</td>
<td>0.51 (0.46 to 0.56)</td>
<td>-0.23 (-0.25 to -0.21)</td>
<td>-0.31 (-0.33 to -0.28)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Elongase (the ratio of 18:00 to 16:00)</td>
<td>0.474 (0.412, 0.545)</td>
<td>7.0 (6.8 to 7.3)</td>
<td>+0.12 (+0.10 to +0.14)</td>
<td>+0.21 (+0.18 to +0.23)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Δ6-desaturase (the ratio of 18:3n6 to 18:2n6)</td>
<td>3.45 (2.73, 3.93)</td>
<td>3.1 (2.9 to 3.4)</td>
<td>+0.001 (+0.006 to +0.002)</td>
<td>+0.0008 (+0.0003 to +0.001)</td>
<td>0.581</td>
</tr>
<tr>
<td>Δ5-desaturase (the ratio of 20:4n6 to 20:3n6)</td>
<td>0.008 (0.007, 0.012)</td>
<td>0.009 (0.008 to 0.01)</td>
<td>-0.005 (-0.019 to -0.09)</td>
<td>-0.08 (-0.22 to -0.0)</td>
<td>0.843</td>
</tr>
<tr>
<td>Δ9-desaturase (the ratio of 16:1n7 to 16:00)</td>
<td>0.04 (0.03, 0.04)</td>
<td>0.52 (0.51 to 0.53)</td>
<td>-0.012 (-0.014 to -0.01)</td>
<td>-0.01 (-0.012 to -0.008)</td>
<td>&lt;0.20</td>
</tr>
</tbody>
</table>

Data are means ± 95% confidence intervals. The results are expressed as changes in response to the regimens of 6 (Δ A6) and 2 meals a day (breakfast and lunch, Δ B2). Listed p-values are from 2 × 2 crossover analysis of variance for factor treatment, indicating the difference between A6 and B2. Significant changes in response to both regimens are indicated by

* for p < 0.05,
** for p < 0.01, and
*** for p < 0.001.
Insulin Sensitivity

As reported earlier [20], insulin sensitivity (OGIS) increased in response to both regimens ($p < 0.01$) but more with B2 ($p = 0.01$; +8.2 ml.min$^{-1}$.m$^{-2}$; 95% CI, +3.4, +13.1 ml.min$^{-1}$.m$^{-2}$ with A6 vs +21 ml.min$^{-1}$.m$^{-2}$; 95% CI, +16.1, +26.0 ml.min$^{-1}$.m$^{-2}$ with B2).

**Fig. 1.** Changes in phospholipid fatty acid composition for the main fatty acids of interest. Data are shown as changes from baseline in response to the regimens of 6 (A6) and 2 meals a day (B2). Data are means ± 95% confidence intervals. Significance of the factor treatment (assessed by $2 \times 2$ cross-over analysis of variance) is indicated by * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$ and ns for nonsignificant. SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids.
Meal Frequency and Fatty Acid Profile in T2D

Correlations

The increase in insulin sensitivity (OGIS) correlated positively with changes in the proportion of linoleic acid in B2. This correlation remained significant even after adjustment for changes in BMI ($r = +0.38; p = 0.012$). The correlations of the changes in all measured fatty acids in serum phospholipids with changes in OGIS are listed in Supplementary Table 1.

DISCUSSION

In this secondary analysis, we tested the effect of meal frequency on the fatty acid composition of serum phospholipids, and we explored whether these changes are related to the insulin-sensitizing effect of B2. We verified that the fatty acid composition of serum phospholipids is affected by meal frequency (independent of diet composition, because the diet in both regimens had the same macronutrient and energy content). To the best of our knowledge, ours is the first study to show that not only the composition of the diet but also the frequency of meals affects the fatty acid composition of serum phospholipids.

The mechanisms responsible for changes in the fatty acid composition of serum phospholipids in response to meal frequency include not only dietary fatty acid intake but also endogenous fatty acid metabolism, including their synthesis, β-oxidation, desaturation, elongation, and lipoperoxidation. Other possible mechanisms involve the uptake and transport of fatty acids [13].

Saturated fatty acids (mainly myristic and palmitic acids) decreased and n6 polyunsaturated fatty acids increased in response to both regimens, though more with B2. The increase in insulin sensitivity (OGIS) was associated positively with changes in the proportion of linoleic acid in B2. This correlation remained significant even after adjustment for changes in BMI ($r = +0.38; p = 0.012$).

Our results are in strong accordance with previous findings that the fatty acid pattern associated with insulin resistance is characterised by a high proportion of palmitic, palmitoleic, and dihomo-γ-linoleic acids and a low proportion of linoleic acid [31].

The precise mechanism of how the fatty acid composition of serum phospholipids influences insulin action is still unclear. However, it is well known that the cell membrane fatty acid composition affects cell membrane function [32]. Membrane fatty acid composition can affect several cellular functions, such as the translocation of glucose transporters, the alteration of membrane fluidity, flexibility, and ion permeability and thus affects insulin receptors [1,2].

The decrease in saturated fatty acids, more markedly expressed in B2, is a positive finding. We previously demonstrated a negative correlation between the content of saturated fat in plasma phospholipids and insulin sensitivity in healthy individuals [15]. The decrease of saturated fatty acids was due to a decrease in myristic and palmitic acids. Saturated fatty acids impair insulin action and a high proportion of saturated fatty acids is related to insulin resistance and type 2 diabetes. Nevertheless, each saturated fatty acid does not have the same importance. Vessby et al. [31] proposed that an especially high proportion of palmitic acid in membrane phospholipids may promote insulin resistance and that a major role of Δ9 desaturase may be to reduce the availability of palmitic acid by converting it to palmitooleic acid. Some authors suppose that palmitic acid might be regulated more rigorously in the body than other saturated fatty acids, possibly due to its potentially adverse effects on cellular glucose transport, ceramide synthesis, cellular signaling, apoptosis, and lipogenesis [33,34].

Stearic acid, on the other hand, increased in response to both regimens but more in B2. This could be due to an adaptation mechanism to keep the fatty acid proportion in membrane phospholipids stable. However, an increase in stearic acid is not associated with insulin resistance and/or diabetes [35].

The ratio of saturated to unsaturated fatty acids decreased in response to both regimens, though more with B2. This is one of the main positive findings of our study, because this ratio is crucial for membrane fluidity and flexibility as well as metabolic processes including insulin binding and action [2].

Palmitoleic acid and Δ9-desaturase decreased in response to both hypocaloric diet regimens, which is a positive finding even though we did not observe any impact of meal frequency on these metabolites. High levels of Δ9-desaturase and subsequent high levels of palmitoleic acid are significantly associated with insulin resistance and metabolic syndrome in other studies, although we did not confirm this in our study.

Concerning serum phospholipids, high concentrations of palmitic, palmitoleic, and dihomo-γ-linoleic acids have been observed in people with insulin resistance and metabolic syndrome [9–11]. Palmitoleic acid is positively correlated with triglycerides and high-sensitivity C-reactive protein. Increased palmitoleic acid and Δ9-desaturase are associated with an early alteration of fasting glycemic status [10]. On the other hand, the plasma level of circulating palmitoleolate (as an adipokine secreted by the adipose tissue) seems to be positively associated with insulin sensitivity in humans [11,36], but there is no strong evidence from human studies.

Changes in the elongase and desaturase activities suggest that fatty acid synthesis and metabolism is altered in diabetes [37]. Reduced elongase and Δ5-desaturase activities may explain the decreased levels of polyunsaturated fatty acids among patients with metabolic syndrome and T2D. High Δ9-desaturase and low Δ5-desaturase activities have been found to be associated with insulin resistance [38,39]. The biosynthesis of polyunsaturated fatty acids is modulated mainly by Δ5- and Δ6-desaturase. These enzymes are regulated and modulated by dietary and hormonal factors, including insulin, and circadian rhythms [40].
The ratio of n6 to n3 polyunsaturated fatty acids decreased comparably in both regimens. It has been proposed that a high n6/n3 ratio is related to insulin resistance [41]. Then again, a controlled study on the effects of the n6/n3 ratio on insulin sensitivity (OPTILIP) found no effect of decreasing the n6/n3 ratio [42].

An interesting finding in our study is the positive association between an increase in insulin sensitivity and changes in linoleic acid in the B2 regimen. The different response of linoleic acid between both regimens may be due to oxidative stress or the different conversion rate of linoleic acid to arachidonic acid as a precursor for eicosanoid synthesis. Linoleic acid in membrane phospholipids is probably more susceptible to oxidative stress compared to n3 polyunsaturated fatty acids [43,44]. Incorporation of n3 polyunsaturated fatty acids into membrane phospholipids leads to conformational changes and lower availability of double bonds for lipoperoxidation. Peroxyl radical derived from n3 polyunsaturated fatty acids are more hydrophilic than those generated from linoleic acid. They diffuse more readily through the lipid layer of membranes and radical reaction can be terminated more rapidly. Therefore, a lower proportion of linoleic acid in patients with T2D is also most probably associated with increased oxidative stress. Because oxidative stress is increased postprandially, especially in patients with T2D [45], the A6 regimen most probably resulted in increased oxidative stress, and we postulate this to be the mechanism of the different response in A6. However, some in vitro and in vivo studies are controversial, showing differences in the susceptibility of n3 and n6 polyunsaturated fatty acids to oxidation [46].

We previously demonstrated a positive association between insulin action and the proportions of linoleic and arachidonic acid in plasma phospholipids and insulin sensitivity in healthy individuals [15]. We have also demonstrated that a vegetarian diet–induced increase in linoleic acid in serum phospholipids is associated with improved insulin sensitivity in patients with T2D [19]. Metabolomic research has identified linoleic acid as a potential biomarker for diabetes mellitus [47]. In a prospective cohort study, men with a high proportion of linoleic acid in plasma fatty acids, indicating a high dietary intake of linoleic acid, had a lower risk of developing diabetes. This is comparable with earlier findings [48] and is also in line with dietary epidemiology [49], which has indicated that individuals with a low proportion of linoleic acid in their diet have an increased risk of developing type 2 diabetes.

The strengths of our study include the crossover design, allowing a direct comparison between A6 and B2. The study duration was long enough to allow sufficient time for adaptation to both dietary regimens. The patients started both regimens in groups, which was important for maximizing their compliance. The study investigated several metabolic variables, with results applicable in free-living conditions.

We are aware of several limitations in our study. First of all, the sample of diabetic patients in our study was not representative: all participants were being treated by oral hypoglycemic agents, with motivation not to initiate insulin therapy, and they were willing to make substantial changes in their lifestyle, a situation rather not typical for most patients with T2D. In addition, all blood drawings were performed in the morning, which means that patients in the B2 regimen were fasting for longer than those in A6. Despite these limitations, however, we feel that the results bring important insights into the role of meal frequency on the fatty acid composition of serum phospholipids.

CONCLUSION

In conclusion, we demonstrated that B2 elicited more marked positive effects on the fatty acid composition of serum phospholipids. To the best of our knowledge, ours is the first study to show that not only the composition of the diet but also the frequency of meals affects the fatty acid composition of serum phospholipids. Saturated fatty acids and the ratio of saturated to unsaturated fatty acids decreased more in B2. We did not find any association between changes in insulin sensitivity and changes in saturated fatty acid or in the ratio of saturated to unsaturated fatty acids. The increase in linoleic acid could partly explain the insulin-sensitizing effect of B2. This is in accordance with the consensus that changes in the fatty acid composition may play a role in the modulation of insulin action in peripheral tissues. We also confirmed our previous findings that an increase in insulin sensitivity is positively associated with changes in linoleic acid.

Our results suggest that, with the exception of dietary lipids and fat quality, the fatty acid composition of serum phospholipids is also affected by meal frequency. Further studies are needed to elucidate the potential mechanisms of this association.

AUTHOR CONTRIBUTIONS

H.K., L.B., and T.P. designed the study, wrote the grant application, recruited the patients, collected the data, and wrote the article. H.M., L.K., and A.T. were involved in the acquisition and analyses of data. M.H. carried out the statistical analyses and interpretation of data. All authors had full access to the data and revised and approved the article for publication. The guarantor is T.P.

ACKNOWLEDGMENTS

We thank the 54 participants for their cooperation.
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FUNDING

This work was supported by the project grants NT/14250-3 and NT/14325-3 from the Ministry of Health, Prague, Czech Republic

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Received March 9, 2015; accepted April 25, 2015.