

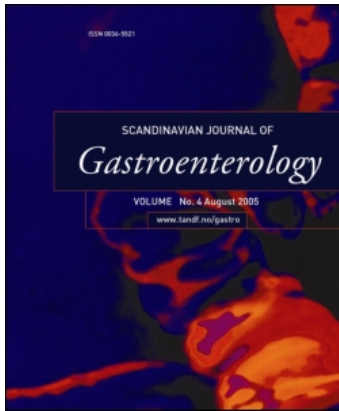
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ORIGINAL ARTICLE

## Thylakoids promote release of the satiety hormone cholecystokinin while reducing insulin in healthy humans

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### Abstract

**Objective.** The effects of a promising new appetite suppressor named “thylakoids” (membrane proteins derived from spinach leaves) were examined in a single meal in man. Thylakoids inhibit the lipase/colipase hydrolysis of triacylglycerols *in vitro* and suppress food intake, decrease body-weight gain and raise the satiety hormone cholecystokinin (CCK) in rats, but their effects in man remain unclear. The aim of this study was to investigate whether thylakoids, when added to a test meal, affect appetite regulation and blood parameters in healthy individuals. **Material and methods.** In an intervention crossover study, healthy individuals of normal weight ( $n = 11$ ) were offered a high-fat meal with and without the addition of thylakoids. Blood samples were taken 0 (prior to meal), 30, 60, 120, 180, 240, 300 and 360 min after the start of the meal. Blood samples were analysed for satiety and hunger hormones (CCK, leptin and ghrelin), insulin and blood metabolites (glucose and free fatty acids). **Results.** The CCK level increased, in particular between the 120 min time-point and onwards, the ghrelin level was reduced at 120 min and leptin level increased at 360 min after intake of the thylakoid-enriched meal. The insulin level was reduced, whereas glucose concentrations were unchanged. Free fatty acids were reduced between time-point 120 min and onwards after the thylakoid meal. **Conclusions.** The addition of thylakoids to energy-dense food promotes satiety signals and reduces insulin response during a single meal in man.

**Key Words:** Colipase, dietary protein, galactolipids, ghrelin, glucose, green plants, leptin, lipase

### Introduction

The prevalence of obesity is increasing at an alarming rate and has reached epidemic proportions [1,2]. The main reason for this obesity epidemic is the increased accessibility of palatable, energy-dense food products [3,4], which readily leads to over-consumption. Obesity greatly increases the risk of critical clinical conditions such as diabetes and cardiovascular disease making obesity a major cause of disease world-wide [5]. Treatment of obesity is challenging and today on the market there are three drugs for the treatment of obesity: orlistat, rimona-bant and sibutramine [6,7]. The effects of these

drugs are well documented but the pharmacological treatment has to be used together with long-term changes in lifestyle to reduce body-weight and cardiometabolic risk factors related to obesity [6].

Fibre and low glycaemic food is a concept to enhance satiety by slowing down intestinal digestion of carbohydrate [8]. Until now, there has been no such concept for satiety for fat. Since dietary fat readily promotes overeating through weak satiety mechanisms, [9] we have searched for mechanisms to strengthen the inherent satiety for fat.

We have thus identified thylakoids that induce satiety for fat in experimental animal studies [10].

The thylakoids we have identified to induce satiety consist of a biological membrane of the chloroplast, present in all photosynthetic plants. In our previous publication, the satiety-promoting effect was explained by an interaction of the thylakoids with the intestinal fat digestion. Owing to an interaction with both the fat droplets and the lipase/colipase complex, the thylakoids caused retardation of fat digestion [10]. Rats fed on a thylakoid-enriched diet showed a reduced food intake and body-weight as well as lowered blood lipids, lowered fasting glucose levels and increased levels of the satiety hormone, cholecystokinin (CCK) [10].

Having concluded that thylakoids promote satiety and reduce blood lipid levels in rats, the obvious question was whether this is also true in man. In this study we investigated whether thylakoids, when added to a test meal, affect appetite regulation and blood parameters in healthy individuals.

## Material and methods

### Subjects

Eleven healthy volunteers (5 M, 6 F) who had fasted overnight participated in the study. The study was approved by the Ethics Committee Lund, Sweden (org. nr: 361, date: 2006-08-03).

### Meals

*Control meal.* The subjects consumed a control meal consisting of a placebo pesto sandwich (Table I).

The pesto sandwich had a total energy content of 544 kcal and energy percentage (E%) was 66 E% fat, 25 E% carbohydrates and 9 E% protein.

*Test meal 1.* The subjects consumed a meal consisting of a thylakoid-enriched pesto sandwich that was identical to a control pesto sandwich but with the addition of 50 g thylakoids to the pesto. The thylakoid pesto sandwich had a total energy content of 719 kcal and energy percentage was 56 E% fat, 25 E% carbohydrates and 19 E% protein.

*Test meal 2.* The same experimental protocol as described for the control meal was applied but with the addition of 25 g thylakoids. The thylakoid pesto sandwich had a total energy content of 631 kcal and the energy percentage was 60 E% fat, 25 E% carbohydrates and 15 E% protein.

*Test meal 3.* The same experimental protocol as described for the control meal was applied but with the addition of 25 g delipidated thylakoids. The thylakoid pesto sandwich had a total energy content of 614 kcal and the energy percentage was 59 E% fat, 25 E% carbohydrates and 16 E% protein.

Details of the composition of the meals are presented in Table I. On all six occasions the subjects were requested to finish the pesto sandwich within 15 min. The six different test meals took place at least one week apart.

Table I. Composition and energy content of the sandwiches.

Ingredients	Control (g)	Thyl 50 (g)	Thyl 25 (g)	Thyl 10 (g)	Thyl 5 (g)	Delipthyl 25 (g)
Cashew-nut	25	25	25	25	25	25
Rapeseed oil	26	26	26	26	26	26
Basil	15	15	15	15	15	15
Lime juice	15	15	15	15	15	15
Mustard powder	0.25	0.25	0.25	0.25	0.25	0.25
Salt	0.5	0.5	0.5	0.5	0.5	0.5
Black pepper	0.25	0.25	0.25	0.25	0.25	0.25
Tomato	70	70	70	70	70	70
Bread, whole grain	70	70	70	70	70	70
Thylakoid powder		50	25	10	5	
Thylakoid delipidated powder						25
Protein	12 (9E%)	35 (19E%)	23.5 (15E%)	16.6 (11E%)	14.3 (10E%)	23.75 (16E%)
Fat	40 (66E%)	44.5 (56E%)	42.25 (60%E)	40.9 (64E%)	40.45 (65 E%)	40.5 (59E%)
Carbohydrate	34 (25E%)	44.5 (25E%)	39.25 (25E%)	36.1 (25E%)	35.05 (25E%)	38.5 (25E%)
Kcal	544	718.5	631.25	578.9	561.45	613.5
Thylakoid energy content	Protein (g/100g)	Fat (g/100g)	Carbs (g/100g)	Energy (Kcal)		
Thylakoid powder	46	9	21	349		
Thylakoid delipidated powder	47	2	18	278		

*Dose-response curve for CCK release*

The same experimental protocol as described for the control meal was conducted with the addition of 5 g and 10 g thylakoids. The composition and energy percentages of these meals are recorded in Table I. The dose-response curve was established for the time-point 6 h postprandially.

*Blood sample collection*

Plasma and serum samples were taken at the following time-points: 0 (prior to meal), 30, 60, 120, 180, 240, 300 and 360 min after the start of eating.

*Plasma measurements*

Plasma glucose was measured using HemoCue Glucose 201<sup>+</sup> from HemoCue AB (Ångelholm, Sweden). Serum free fatty acids (FFAs) were measured using a NEFA kit from Wako Chemicals (Neuss, Germany). Serum leptin levels were determined using a commercially available human leptin ELISA kit from Phoenix Pharmaceuticals (Belmont, Calif., USA). Serum ghrelin was analysed with a RIA human kit, also from Phoenix Pharmaceuticals. Serum insulin was determined using the human insulin RIA kit from Linco Research (St Charles, Mich., USA). Serum CCK was measured with a highly specific antiserum (no. 92128) that does not bind any of the homologous gastrin peptides [11].

*Preparation of thylakoids*

The thylakoids (SwePharm AB, S. Sandby, Sweden) used in our study were prepared as follows: 1000 g spinach leaves were homogenized in a blender with 1250 ml water and filtered through four layers of Monodur polyester mesh (20 µm). The filtrate was diluted 10 times with distilled water and pH adjusted to 4.7 with HCl. The thylakoids flocculated and after standing in the cold (+4°C) for 4 h, a green sediment was obtained with a clear, slightly yellowish supernatant. The supernatant was discarded and the sediment washed in water; the sedimentation was repeated at the same pH. The final sediment was adjusted to pH 7.0 and then freeze-dried to a green powder.

*Preparation of delipidated thylakoids*

Delipidated thylakoids were prepared as described above with the addition that the final sediment was washed with 96% ethanol for 2 h. The remaining sediment was then freeze-dried to a grey powder (the green-coloured chlorophyll was discarded).

*Statistics*

Non-parametric statistics were used since the material was not normally distributed. Friedman analysis for repeated measurements of paired samples was used to test for differences in blood samples between the control and thylakoid groups, and the Wilcoxon signed-rank test was used to test paired differences. Differences were considered significant if the *p*-value was less than 0.05. All statistical data were analysed with the StatView software program.

**Results***Effects of thylakoid enrichment on serum CCK, ghrelin and leptin*

A single meal of a thylakoid-enriched sandwich resulted in an increased CCK response compared to the control (Figures 1A–C). This effect was seen at the time-points 240 min and 360 min, which were the only time-points where a significant difference was observed. The zero values were not statistically different, nor the early time-points of the meal (0–120 min). A dose-response curve for the effect of a thylakoid-enriched meal on CCK response is depicted in Figure 1D. As can be seen, increasing doses of thylakoids caused an increased CCK response, the response being optimal at 25 g thylakoids. Ghrelin concentrations were also measured and found to be reduced at time-point 120 min, returning to baseline values at time-point 360 min both for the control and the thylakoid meals. We chose the 120 min value since this was the time-point at which the lowest ghrelin level was observed (data with other time points not shown). A significantly reduced ghrelin signal was observed for the 50 g thylakoid meal (Figure 2A), the 25 g thylakoid meal (Figure 2B) as well as the 25 g delipidated meal (Figure 2C). The figures are given as percentage control, since there were large individual differences in ghrelin values, the ghrelin plasma level being related to gender, age and physical activity [12]. Based on previous observations, postprandial leptin concentrations were measured only at time-point 360 min [12]. At this time-point, leptin concentrations were significantly increased with the 50 g and 25 g thylakoid-enriched meal compared to the control meal (Figures 3A and 3B), whereas there was no effect of delipidated thylakoids on the leptin concentrations at time-point 360 min (Figure 3C).

*Effects of thylakoid enrichment on serum insulin, glucose and FFAs*

Thylakoid enrichment showed no differences in overall serum glucose levels (Figures 4A–C).

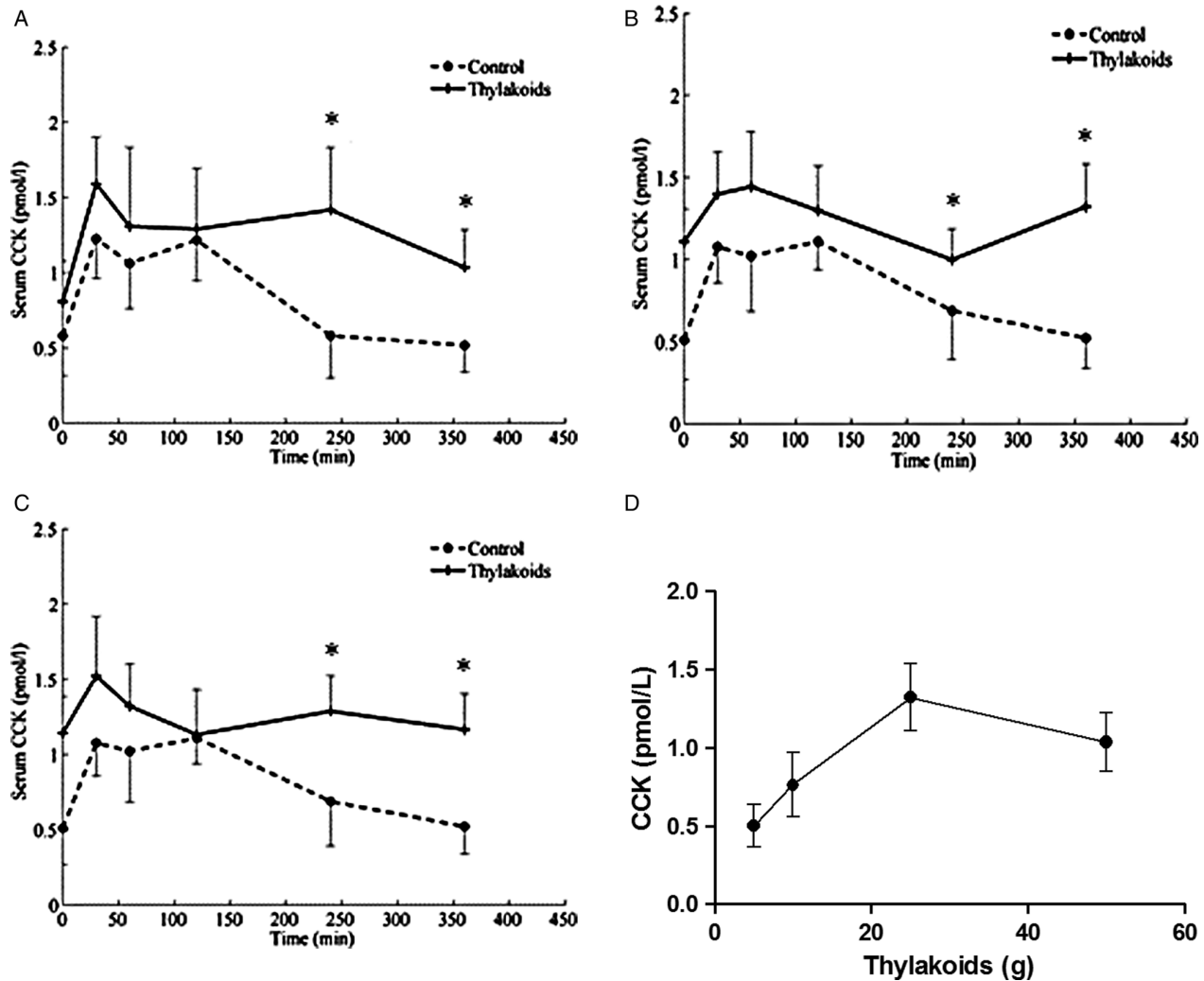


Figure 1. Serum cholecystikinin (CCK) levels in humans after ingestion of a control meal and thylakoid-enriched meals. A. 50 g thylakoid-enriched meal; B. 25 g thylakoid-enriched meal; C. 25 g delipidated thylakoid-enriched meal; D. Dose-response curve for the thylakoid-enriched meal at time-point 6 h postprandially. Data are given as mean values ( $\pm$ SEM) for 11 subjects.

However, the insulin concentrations were reduced by the thylakoid supplement (Figures 4D–F). Serum FFA levels were decreased after intake of 50 g thylakoids. This effect was seen at time-point 240 min, 300 min and 360 min (Figure 5A). However, no effect of 25 g thylakoids and 25 g delipidated thylakoids was observed (Figures 5B and 5C).

## Discussion

In this study we have shown that thylakoids from chloroplast membranes in green leaves, when added to a meal, promote the release of the satiety hormone CCK (Figure 1), at the same time reducing insulin levels in healthy, normal-weight humans (Figure 4). The release of CCK was particularly evident in the late phase postprandially (240–360 min) suggesting that thylakoids acted to retard fat digestion, as demonstrated in previous studies [10]. In the late

phase of the meal, the satiety hormone leptin was released at a significantly higher level compared to the control (Figure 3). The release of leptin occurred after both 50 g and 25 g thylakoids, whereas thylakoids that were delipidated had no effect on leptin release (Figure 3C). In addition to promotion of satiety signalling, the thylakoids suppressed the hunger hormone, ghrelin (Figure 2), which occurred in the early phase postprandially (120 min). The suppression of ghrelin in the early phase of the meal together with the elevation of CCK in the late phase suggests that the thylakoids act to promote satiety signals in an efficient way.

CCK is a satiety hormone which is secreted from the small intestine by the presence of fatty acids as well as by amino acids [13–15]. The addition of thylakoids to the test meals resulted in elevated CCK levels in all test meals and at all concentrations of thylakoids (50 g, 25 g thylakoids and 25 g delipidated

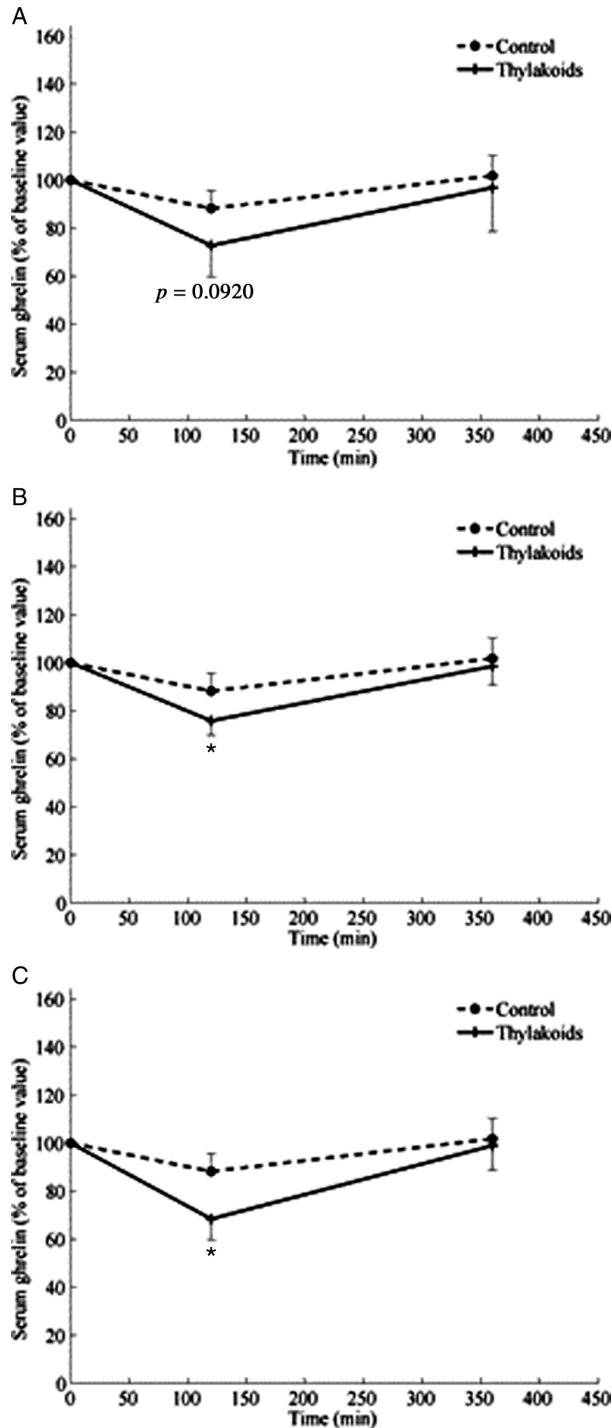


Figure 2. Serum ghrelin levels in humans after ingestion of a control meal and thylakoid-enriched meals. A. 50 g thylakoid-enriched meal; B. 25 g thylakoid-enriched meal; C. 25 g delipidated thylakoid-enriched meal. Data are given as mean values ( $\pm$  SEM) for 11 subjects.

thylakoids) (Figure 1), suggesting that thylakoids promote satiety in humans. Thus, the elevation of CCK levels in humans is similar to previous observations in rat [10]. The satiety-promoting effect of thylakoids can be explained by an augmented time

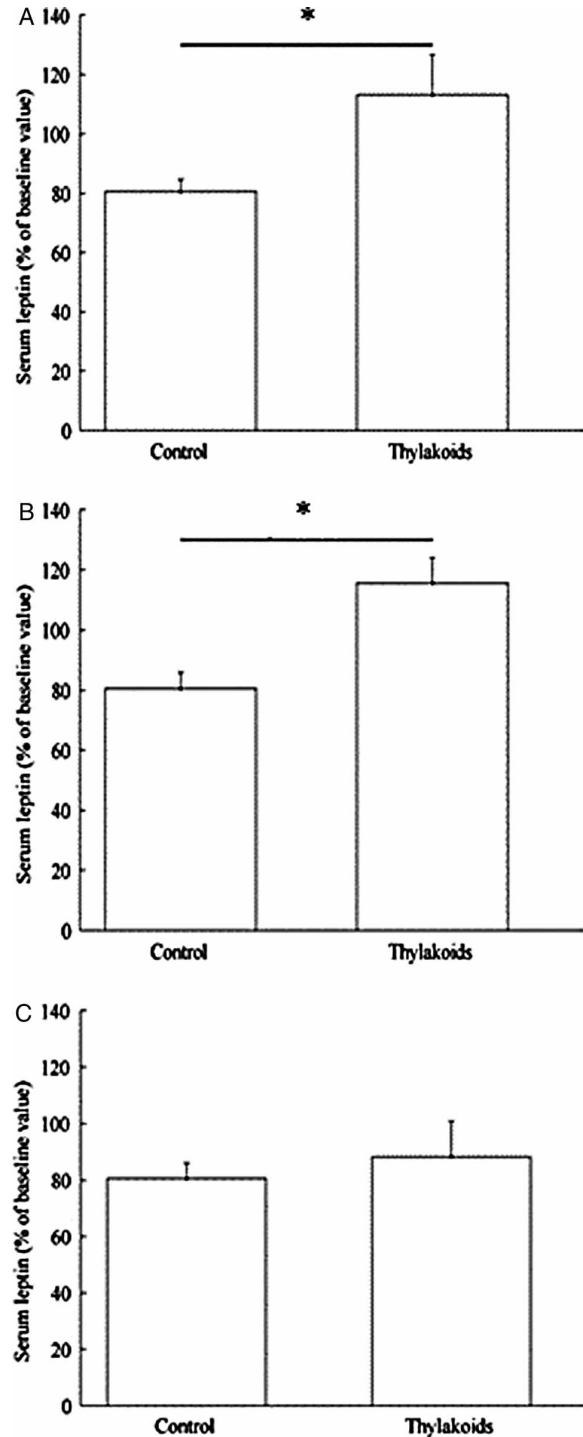


Figure 3. Serum leptin levels in humans after ingestion of a control meal and thylakoid-enriched meals. A. 50 g thylakoid-enriched meal; B. 25 g thylakoid-enriched meal; C. 25 g delipidated thylakoid-enriched meal. Data are given as mean values ( $\pm$  SEM) for 11 subjects.

period for fat digestion, the fatty acids released during triacylglycerol hydrolysis being able to stimulate the secretion of CCK. This kind of mechanism is supported by Beglinger et al. [13] who reported that the satiety through intestinal fat acts via a CCK pathway

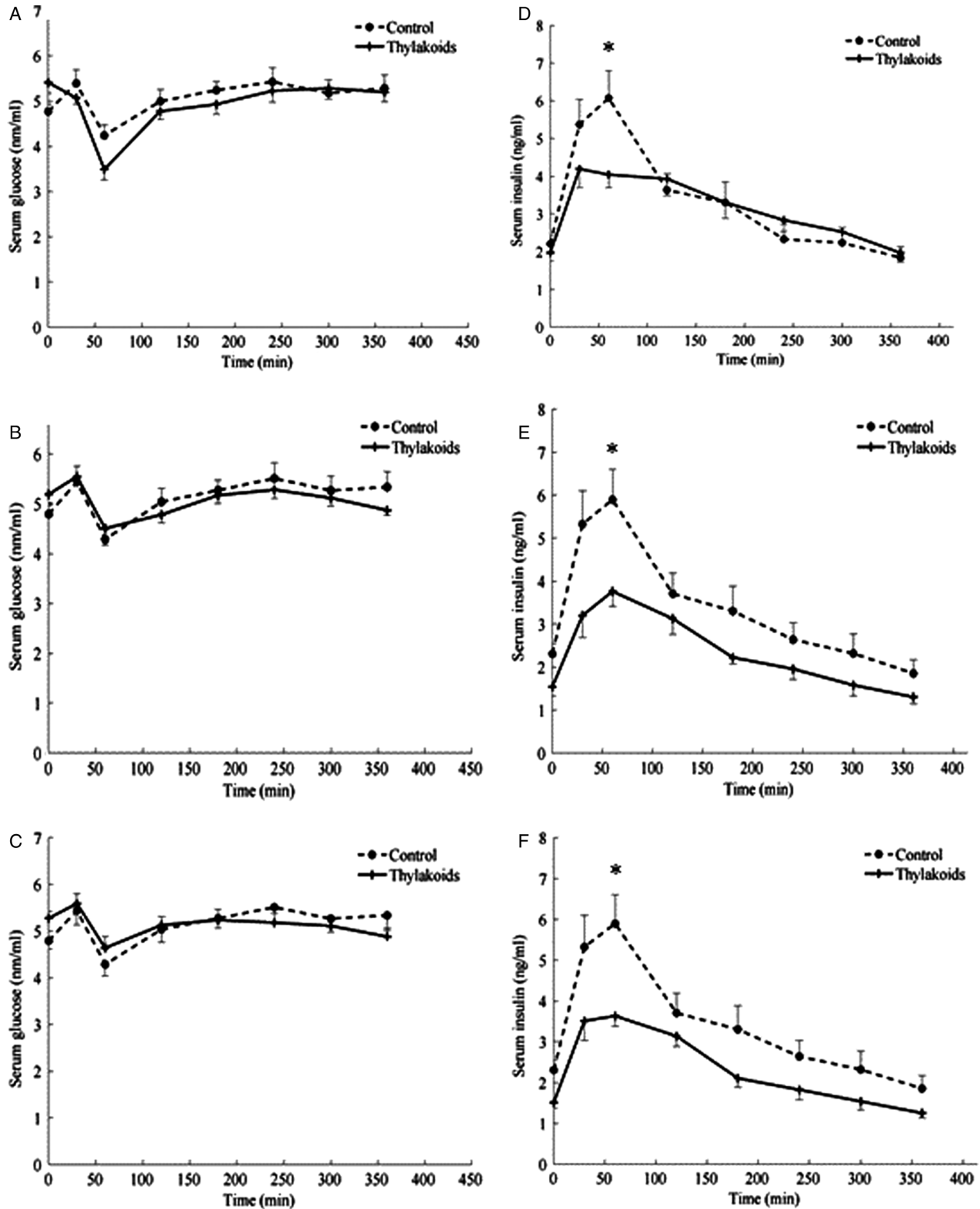


Figure 4. Serum insulin and glucose levels in humans after ingestion of a control meal and thylakoid-enriched meals. 50 g thylakoid-enriched meal (A, D); 25 g thylakoid-enriched meal (B, E); 25 g delipidated thylakoid-enriched meal (C, F). Data are given as mean values ( $\pm$ SEM) for 11 subjects.

in humans. Since the food was supplemented with the thylakoids, it could be argued that the satiety signals are due to the added proteins inherent in the

thylakoids, thylakoids being a mixture of membrane proteins and galactolipids. In the analysis of CCK release by dietary protein, it has been demonstrated

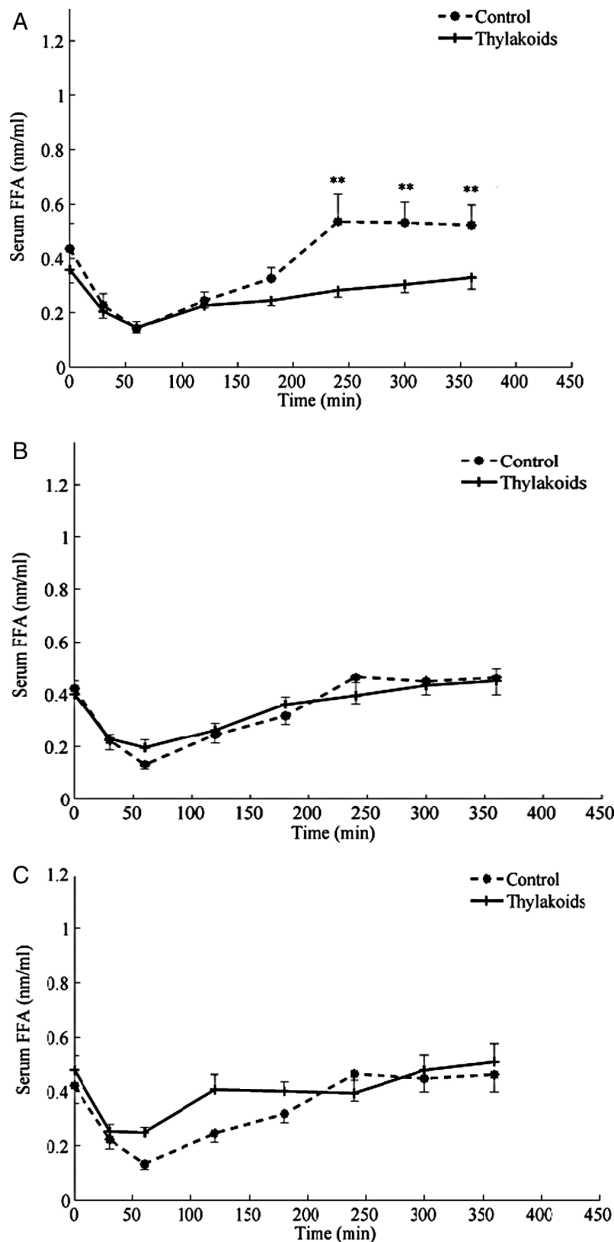


Figure 5. Serum free fatty acid (FFA) levels in humans after ingestion of a control meal and thylakoid-enriched meals. A. 50 g thylakoid-enriched meal; B. 25 g thylakoid-enriched meal; C. 25 g delipidated thylakoid-enriched meal. Data are given as mean values ( $\pm$ SEM) for 11 subjects.

that the CCK response is released after 30–120 min of protein consumption [16–18], thus an early event compared to the thylakoid-enriched meal, the CCK release being at time-points 240–360 min (Figure 1). We thus believe that the release of CCK is actually inherent in the thylakoid component and its processing in the intestine, as demonstrated in Figure 1D, where a dose–response curve for CCK response and thylakoid meal was demonstrated.

We found lowered ghrelin levels at all different doses of thylakoids at time-point 120 min compared

to the control meal (Figure 2). Ghrelin is a well-established feeding initiator [19] and promoter of adipose tissue growth. A recent study indicates that ghrelin suppression is mediated by intestinal fat via CCK signalling [20]. The synergistic effect of CCK and ghrelin could be one reason for the satiety-promoting effect of thylakoids.

We also found a significantly higher leptin concentration in blood 6 h after consumption of the thylakoid-enriched meal compared to the control meal (Figure 3). Leptin is a satiety hormone that plays a key role in regulating energy intake between meals and over longer periods [12]. We observed that leptin levels were raised after both the 50 g and the 25 g thylakoid-enriched meal, whereas no effect was observed after the 25 g delipidated thylakoid meal (Figure 3C). The absence of any leptin effect after thylakoid delipidation, suggests that the leptin-releasing effect of thylakoids is inherent in the lipid phase of the thylakoids or the combined protein/lipid phase of the thylakoids. The main lipid components in the thylakoids membrane are galactolipids, digalactosyldiglyceride, monogalactosyldiglyceride and sulphoquinovosyldiglyceride. Thus it might be important to investigate the effect of galactolipids on short-term leptin release. Other lipid components that are lost upon delipidation of thylakoids are the lipid-soluble pigments such as the chlorophyll and the carotenoids as well as antioxidants such as tocopherols [10]. These may also be important for leptin release and/or satiety through interaction with gastric lipolysis [21,22]. Another explanation for the lower response by delipidated thylakoids may be that these are more readily degraded by proteases.

Surprisingly, the insulin response was lower after ingestion of the thylakoid-enriched meals compared to the control meal (Figures 4A–C). This is a robust and completely novel finding. Insulin is secreted from pancreatic  $\beta$ -cells mainly in response to high blood glucose levels as well as some amino acids. The thylakoid-enriched meals contain more carbohydrates and protein (Table I), and should theoretically give a higher insulin response. However, this was not the case; rather, the opposite effect was observed. One possible explanation is the release of an inhibitory gut hormone, e.g. somatostatin. Another possibility is inhibition of amylase activity by thylakoids, thereby prolonging the digestion and absorption of carbohydrates. Future studies in our laboratory will address these possibilities.

We also found lower levels of FFAs with the 50 g thylakoid meal compared to the control meal (Figure 5A); this was specifically obvious at time-points 240, 300 and 360 min. The effect was not observed with the 25 g thylakoid dose or with the delipidated thylakoids. The lower level of FFAs in the 50 g

thylakoid-enriched diet can be explained by a prolonged intraluminal fat digestion, the uptake of fatty acids from the intestine occurring later than in the control. Bickerton et al. [23] reported an initial fall in plasma FFA levels postprandially, followed by a rise after 2 h, and a maximal rise after 6 h. This pattern of serum FFAs is similar to that of the control meal in our experiment (Figure 5). The initial fall in serum FFA levels postprandially may be due to diminished FFA release from the adipose tissue, whereas the late postprandial rise indicates increased circulating levels of FFAs for oxidation, mainly in skeletal muscle [23]. With 50 g thylakoids, we observed an initial dip in FFAs, indicating a blockade of release of FFAs from the adipose tissue. However, during the late postprandial phase there was no rise in FFAs (Figure 5A), indicating that there was a reduced level of FFAs in the circulation. A reduction in FFA levels is important in preventing cardiovascular disease [24].

In conclusion, we report that supplementation of thylakoids (proteins derived from spinach leaves) to high-fat meals resulted in higher serum levels of CCK and leptin, and lower levels of ghrelin, FFA and insulin. Thylakoids are thus able to promote satiety signals and reduce insulin response, which are necessary factors in the treatment of obesity and/or obesity-related disorders such as type 2 diabetes.

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**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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