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Intestinal regulation of hunger and reward

- Studies with thylakoids

Eva-Lena Stenblom

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Abstract:

Obesity is a worldwide epidemic that increases the risk for several serious diseases. Today, it is considered one of the five most important risk factors contributing to the global burden of disease, causing more deaths than underweight worldwide. Obesity is a multifactorial disease, caused by a combination of genetic predisposition and lifestyle. Contributing to the obesity epidemic is overeating of tasty energy dense foods. The problem is that our inherent appetite regulation system is not adapted to the abundance of food and food cues we are surrounded by today. Consequently, one way to counteract the development of obesity is to strengthen satiety signals and to suppress hunger signals and cravings for energy dense food.

Inside the chloroplasts of all green leaves there are biological membranes called thylakoids. Previous research has shown that thylakoids slow down fat digestion reversibly by binding to the lipase/colipase complex. Thylakoids also cover lipid droplets and the intestinal mucosa, reducing the absorption rate over the intestinal wall. In animals, ingestion of thylakoids leads to reduced food intake, reduced body weight gain, reduced body fat accumulation and reduced levels of blood lipids.

The overall aim of this thesis was to explore immediate and long-term effects of dietary thylakoids on secretion of appetite regulating hormones from the intestine, associated feelings of hunger, fullness and reward, as well as effects on body weight, body fat and metabolic parameters.

A key finding of this thesis is that supplementation of thylakoids with the diet leads to altered levels of appetite regulating hormones both immediately and after long-term treatment, as well as reduced feelings of hunger, increased satiety and reduced cravings for energy dense palatable food in between meals. Meal studies show reduced secretion of the hunger hormone ghrelin and increased levels of the satiety hormones cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1). Long-term supplementation with thylakoids show increased postprandial levels of GLP-1.

A second key finding is that daily supplementation with thylakoids promotes body weight loss in humans and body weight loss and body fat loss in rodents. In the latter case fat oxidative genes in the intestine were upregulated. Thylakoid ingestion is also associated with improved metabolic parameters, suggesting decreased risk for metabolic disease.

In conclusion, the results presented in this thesis show that supplementation of thylakoids to the diet is associated with effects that counteract overeating of palatable foods and ultimately promote body weight loss and improved metabolic health. This is important because there is a need for agents that can reinforce the natural appetite regulating system to counteract cravings, overeating and consequent body fat accumulation and the associated increased risk for disease.

Key words: Appetite regulation, Thylakoids, Hunger, Satiety, Hedonic hunger, Reward, Cravings, Overweight, Obesity, Fat metabolism, Food intake

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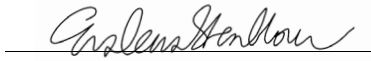
Signature  Date 2016-09-22

Intestinal regulation of hunger and reward

- Studies with thylakoids

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Abstract

Obesity is a worldwide epidemic that increases the risk for several serious diseases. Today, it is considered one of the five most important risk factors contributing to the global burden of disease, causing more deaths than underweight worldwide. Obesity is a multifactorial disease, caused by a combination of genetic predisposition and lifestyle. Contributing to the obesity epidemic is overeating of tasty energy dense foods. The problem is that our inherent appetite regulation system is not adapted to the abundance of food and food cues we are surrounded by today. Consequently, one way to counteract the development of obesity is to strengthen satiety signals and to suppress hunger signals and cravings for energy dense food.

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The overall aim of this thesis was to explore immediate and long-term effects of dietary thylakoids on secretion of appetite regulating hormones from the intestine, associated feelings of hunger, fullness and reward, as well as effects on body weight, body fat and metabolic parameters.

A key finding of this thesis is that supplementation of thylakoids with the diet leads to altered levels of appetite regulating hormones both immediately and after long-term treatment, as well as reduced feelings of hunger, increased satiety and reduced cravings for energy dense palatable food in between meals. Meal studies show reduced secretion of the hunger hormone ghrelin and increased levels of the satiety hormones cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1). Long-term supplementation with thylakoids show increased postprandial levels of GLP-1.

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ingestion is also associated with improved metabolic parameters, suggesting decreased risk for metabolic disease.

In conclusion, the results presented in this thesis show that supplementation of thylakoids to the diet is associated with effects that counteract overeating of palatable foods and ultimately promote body weight loss and improved metabolic health. This is important because there is a need for agents that can reinforce the natural appetite regulating system to counteract cravings, overeating and consequent body fat accumulation and the associated increased risk for disease.

List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Eva-Lena Stenblom, Caroline Montelius, Karolina Östbring, Maria Håkansson, Sofia Nilsson, Jens F. Rehfeld and Charlotte Erlanson-Albertsson. **Supplementation by thylakoids to a high carbohydrate meal decreases feelings of hunger, elevates CCK levels and prevents postprandial hypoglycaemia in overweight women.** *Appetite* (2013) 68: 118-123, doi: 10.1016/j.appet.2013.04.022

II. Eva-Lena Stenblom, Caroline Montelius, Daniel Erlandsson, Line Skarping, Maria Fransson, Emil Egecioglu, Krzysztof Podgorski and Charlotte Erlanson-Albertsson. **Decreased urge for palatable food after a two-month dietary intervention with green-plant membranes in overweight women.** *Obesity & Weight Loss Therapy* (2014) 4(4): 238, doi: 10.4172/2165-7904.1000238

III. Caroline Montelius, Daniel Erlandsson, Egzona Vitija, Eva-Lena Stenblom, Emil Egecioglu and Charlotte Erlanson-Albertsson. **Body weight loss, reduced urge for palatable food and increased release of GLP-1 through daily supplementation with green-plant membranes for three months on overweight women.** *Appetite* (2014) 81: 295-304, doi: 10.1016/j.appet.2014.06.101

IV. Eva-Lena Stenblom, Emil Egecioglu, Mona Landin-Olsson and Charlotte Erlanson-Albertsson. **Consumption of thylakoid-rich spinach extract reduces hunger, increases satiety and reduces cravings for palatable food in overweight women.** *Appetite* (2015) 91: 209-219, doi: 10.1016/j.appet.2015.04.051

V. Eva-Lena Stenblom, Emil Egecioglu, Caroline Montelius, Deepti Ramachandran, Britta Bonn, Björn Weström, Abdelhak Mansouri, Wolfgang Langhans and Charlotte Erlanson-Albertsson. **Dietary thylakoids reduce visceral fat mass and increase expression of genes involved in intestinal fatty acid oxidation in high-fat fed rats.** *Am J Physiol Regul Integr Comp Physiol* (2016) 311: R618-R627.

Papers not included in the thesis

Stenblom E-L, Weström B, Linninge C, Bonn P, Farrell M, Rehfeld JF, Montelius C. **Dietary green-plant thylakoids decrease gastric emptying and gut transit, promote changes in the gut microbial flora, but does not cause steatorrhea.** *Manuscript submitted to J Nutr Metab, currently under revision.*

Erlanson-Albertsson C, Stenblom E-L, Montelius C, Egecioglu E, Stenkula KG. **Thylakoids reduce food intake, body fat and adipose cell size, while increasing fecal fat in high-fat fed mice.** *Manuscript in preparation.*

Montelius C, Stenblom E-L, Landin–Olsson M, Erlanson–Albertsson C. **Thylakoid supplementation reduces blood–cholesterol, waist circumference and body weight in a randomized trial with overweight to obese middle–aged men.** *Manuscript in preparation.*

Abbreviations

ACADL/LCAD	Acyl-CoA-dehydrogenase, long chain
ANOVA	Analysis of variance
ApoB1	Apolipoprotein B1
AUC	Area under the curve
BCA	Body composition analysis
BIA	Bioelectric impedance analyser
BHB	Beta-hydroxybutyrate
BMI	Body mass index (kg/m^2)
BMC	Biomedical Centre
BW	Body weight
CCK	Cholecystokinin
CPT1a	Carnitine palmitoyltransferase 1a
CYP	Cytochrome P450
DSM-5	The diagnostic and statistical manual of mental disorders, fifth edition
E%	Energy percent (distribution of macronutrients)
EMA	European Medicines Agency
FABP2	Fatty acid binding protein 2
FAT/CD36	Fatty acid translocase
fMRI	Functional magnetic resonance imaging
GI	Gastro-intestinal
GLP-1	Glucagon-like peptide 1
HDL	High density lipoprotein
HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2

LCAD	Acyl-CoA-dehydrogenase, long chain
LDL	Low density lipoprotein
MPA	Medical Products Agency - Sweden
NEFA	Non-esterified fatty acids
NFA	National food agency Sweden
NNR	Nordic Nutrition Recommendations
p-	Plasma
PCR	Polymerase chain reaction
PPAR-α/PPARA	Peroxisome proliferator receptor activator-alpha
q-RT-PCR	Quantitative real-time polymerase chain reaction
RDI	Recommended daily intake
RNA	Ribonucleic acid
RQ	Respiratory quotient
RT	Reverse transcription
SCFA	Short chain fatty acids
SD	Standard deviation
SEM	Standard error of the mean
SUS	Skåne University Hospital
TAG/TG	Triacylglycerol
tAUC	Total area under the curve
TFEQ-R18V2	Three Factor Eating Questionnaire Revised 18-item Version 2
TNF-α	Tumor necrosis factor-alpha
Two-way RM ANOVA	Two-way repeated measures analysis of variance
VAS	Visual analogue scale
WHO	World Health Organisation
YFAS	Yale food addiction scale

Populärvetenskaplig sammanfattning

Mat är ett av livets stora glädjeämnen. Förutom för att tillgodogöra oss energi äter vi av många andra anledningar: för att det är gott, för att umgås och vara sociala, för att fira, belöna prestationer, trösta vid motgångar, för att man är uttråkad, eller för att lindra stress och ångest. I populärkulturen har det blivit vanligt att dela på sorger och motgångar över en skopa glass. På grund av att det idag nästan överallt går att få tag på mat under dygnets alla timmar har det blivit lätt att äta för mycket. Dessutom blir vi konstant påminda och uppmuntrade att äta via marknadsföring i tv, radio och tidningar samt reklamskyltar ute i samhället. Till saken hör att den moderna maten är tillverkad så att den ska vara så smakrik, tilltalande och billig som möjligt för att vi ska lockas att äta i tid och otid. Inte sällan är den mycket energirika och välsmakande maten samtidigt fattig på vitaminer och mineraler som vi behöver för att kroppen ska fungera.

Resultatet blir att vi intar ett överskott av energi som med tiden lagras som kroppsfett, vilket kan leda till övervikt och fetma. Övervikt och fetma definieras som tillstånd med onormal ansamling av kroppsfett som ökar risken för ohälsa. Fetma i sig klassificeras också som en sjukdom. Följdsjukdomarna till fetma är många, till exempel diabetes typ 2, hjärtkärlsjukdom och olika typer av cancer. Därför leder fetma idag till fler dödsfall än undervikt gör.

Anledningen till att vi kan äta för mycket är att vi har ett nedärvt aptitregleringssystem som inte passar ihop med dagens överflöds- och konsumtionsamhälle. Vår aptitreglering utvecklades i tider av nöd när aktivitetsnivån samtidigt var högre än idag, därför prioriterades hungersignaler och belöningssignaler framför mättnad, lagring av energi framför förbrukning av energi. Ett sätt att motverka överätande, övervikt och fetma skulle vara att dämpa hungersignalerna och suget efter belönande mat samt förstärka mättnadssignalerna. Det finns idag få behandlingsalternativ vid fetma, därför är det av största samhällsintresse att det forskas mera på detta område.

Thylakoider är membran som finns inuti kloroplasterna i alla gröna bladväxter. Om man äter thylakoider som tillskott till maten så kommer thylakoiderna att binda till enzymerna som normalt bryter ner fettet i maten och därmed göra fettnedbrytningen långsammare. Thylakoiderna lägger sig också som ett tunt lager utmed tarmväggens insida vilket gör så att upptaget av näringsämnen från tarmen till blodet går långsammare. Detta leder till att frisättningen av mättnadshormon

från tarmen ökar och hungerhormon minskar. När djur har fått äta mat med thylakoider en längre tid så har de gått upp mindre i vikt och samlat på sig mindre kroppsfett jämfört med djur som inte fått thylakoider i maten. Blodfetterna har också minskat.

Avhandlingens syfte har varit att undersöka effekten av thylakoider på utsöndring av aptitreglerande hormoner från tarmen, känslor av hunger, mättnad och sug efter belönande mat samt effekterna på kroppssammansättning och riskfaktorer för sjukdomar som förknippas med övervikt och fetma - dels omedelbara effekter, dels efter dagligt intag under en längre period i djur och människa.

Resultaten visar att intag av thylakoider med maten ökar utsöndringen av mättnadshormoner och minskar utsöndringen av hunger-hormoner mellan måltiderna. Blodsockernivåerna tenderar dessutom att svänga mindre efter thylakoid-intag. Deltagarna kände sig mindre hungriga och upplevde att suget efter smakrika energirika produkter som bakverk, choklad, godis och snacks var mindre – även efter viktnedgång då hunger och sötsug normalt sett brukar öka. I viktnedgångsstudien med valfri kost gick thylakoid-gruppen ner mer i vikt jämfört med kontrollgruppen. I viktnedgångsstudien med energireducerad diet förbättrade de thylakoidbehandlade deltagarna sina metabola värden mer jämfört med kontrollgruppen. Långtidsförsök i råtta visade att thylakoider tillsammans med en diet med hög fetthalt ökar fettförbränningen, och att detta delvis kan ske i tarmen. Thylakoid-gruppen visade minskad ansamling av kroppsfett, särskilt bukfett, och mindre viktuppgång.

Sammanfattningsvis påverkar intag av thylakoider med kosten aptitregleringssystemet så att mättnadssignalerna ökar och hungersignalerna minskar vilket är precis det som efterfrågas i dagens samhälle. Deltagarna kände mindre hunger, mer mättnad och mindre sug efter mellanmål, godis och snacks mellan måltiderna. Detta kan förhoppningsvis leda till ett minskat födointag över tid. Vi inte mätt födointag över tid i våra studier, det är försök som återstår att göra. Däremot har vi visat större viktminskning i thylakoidbehandlade individer vilket skulle kunna vara ett tecken på att de ätit mindre och/eller rört sig mer. Intag av thylakoider ökar också fettförbränningen och minskar bukfettet, vilket vi visat i råttstudier. Detta är ett viktigt fynd eftersom minskad fettförbränning är en känd riskfaktor för viktuppgång. Ansamling av bukfett medför dessutom ökad risk för metabola sjukdomar. Eftersom thylakoidtillskott inte har några påvisade biverkningar kan tillskott av thylakoider tillsammans med en hälsosam livstil med daglig aktivitet och utan energirika mellanmål förebygga oplanerat överätande och fetma.

General introduction

Eating is a great pleasure in life. Today, food is available at any time of the day in most countries. We are constantly reminded of this fact by advertisements telling us that there is a fast food place open day and night only one minute away or that we must not forget to buy tacos, potato chips and soft drinks for Friday[1]. To further attract our attention, foods are processed to become as palatable as possible, attractive to look at as well as inexpensive to buy, making them hard to resist even when we are not hungry, which can lead to overeating[2,3]. Furthermore, portion sizes of many foods have increased since the 1970s[4,5]. Due to this, the consumption of energy-dense palatable foods, soft drinks and high-fructose corn sweeteners has increased during the last 50 years[6,7]. Our total energy intake has also increased and our activity levels have decreased due to the gradual shift from an agriculture based to a consumer society[8,9]. All these changes have occurred in parallel with a dramatic increase in the prevalence of obesity[10]. Between 2008 and 2014 obesity more than doubled worldwide and it continues to increase[11]. Recent studies in children and adolescents have suggested a plateau in the obesity trend[12]. However, the inequality between socio-economic groups is widening, and individuals with a lower socio-economic position are more likely to be overweight and obese. Furthermore, obesity and undernutrition can coexist in a household since energy-dense foods rich in fat, sugar and salt, tend to be lower in cost but also lower in nutrient quality and micronutrient poor[11].

Obesity is a multifactorial disease, which means that both genotype and lifestyle matters for the development, but ultimately when energy intake exceeds energy expenditure there is a positive energy balance and the surplus will be stored as fat. The World Health Organisation (WHO) defines overweight and obesity as conditions with abnormal or excessive accumulation of body fat that can lead to impaired health[11]. The list of comorbidities includes diabetes type 2, cardiovascular disease, liver disease, breast cancer, colon cancer, musculoskeletal disorders such as osteoarthritis, sleep apnea and psychiatric disorders[11,13], making excess body weight one of the five most important risk factors contributing to the global burden of disease[14]. Medical costs increase progressively with increasing BMI[15]. According to the WHO, obesity causes 8% of all health care costs in Europe and 10% of all deaths. In fact, today overweight and obesity are linked to more deaths worldwide than underweight[11].

Let us remember that overweight and obesity are preventable conditions, but to curb the obesity epidemic the overeating has to stop[11]. The problem is that over the thousands of years during which our bodies developed a system to regulate the energy-balance, there was a shortage of food while at the same time activity levels were high[16,17]. Consequently, hunger signals and reward signals were, and still are, prioritized to ensure stable stores of fat and to make us search for sugar, needed for the neurons in the brain. Today, when the food and activity-situation has reversed, this is a wrong priority. Our inherent appetite regulating system is not built to cope with an abundance of food[18,17]. Therefore, we have to find ways to strengthen our inherent satiety signals and dampen our hunger/reward signals.

Background

Appetite regulation

Physiological control of appetite and food intake is mediated by a combination of long-term and short-term signals that interact synergistically to influence energy intake and expenditure[19]. The metabolic, hormonal and neuronal signals affect feelings of hunger before a meal, satiation during a meal and satiety until next meal starts (Figure 1)[20,21,22,199].

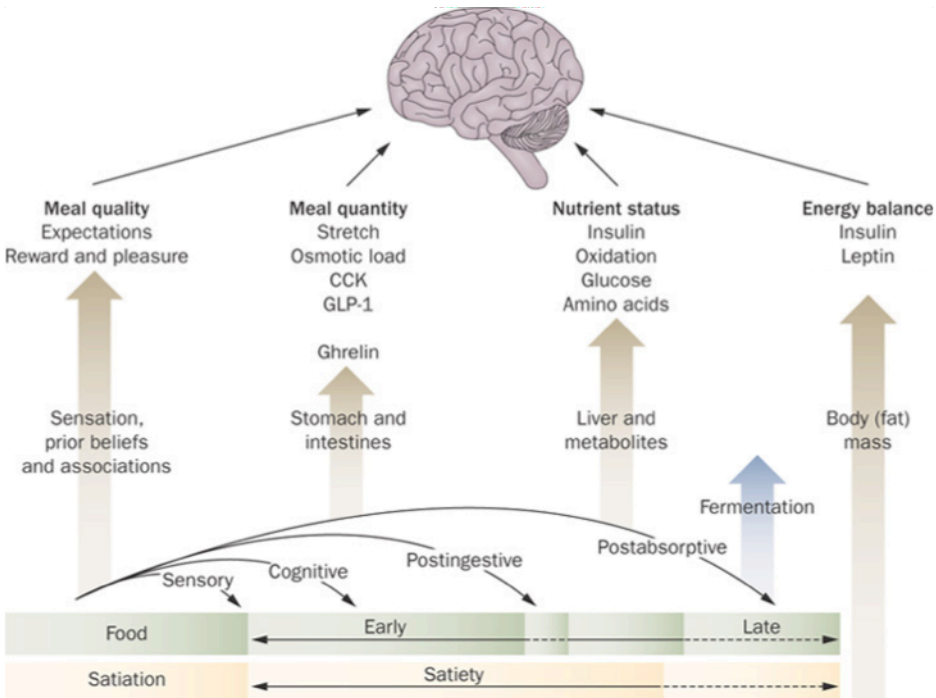

Figure 1. Effects of eating – satiation and satiety.
After consumption of a meal, sensations of appetite are influenced by both psychological and physiological stimuli that arise as a consequence of that meal. Satiety cascade from J. Blundell modified by D. Mela and Stenblom. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Endocrinology, Blundell J. © 2010.

Long-term signals from fat stores, so-called adiposity signals, (leptin, insulin) inform the brain about energy reserves while short-term hunger signals (ghrelin) and satiation signals (cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1) and more) depend on the consumption of food[21]. The long-term adiposity signals convey information about energy balance over time and thus provide a background to the short-term signals. When an individual's weight changes, the adiposity signals change and this affects the sensitivity of the brain to the short-term signals[23]. Hepatic sensing of glycogen stores has also been suggested to affect hunger and satiety[24].

The gastrointestinal tract includes all associated structures between the mouth and the anus. It is the largest endocrine organ in the body, expressing more than 30 gut hormone genes and producing over a 100 bioactive peptides[25,26,27]. The secretion of these peptides is stimulated by anticipation of a meal and the presence of food in the stomach and the small intestine, eliciting mechanical and chemical stimuli. For example, when food enters the stomach, distention of the stomach activates stretch receptors and mechanoreceptors that transmit satiety signals to the brain[28]. The intestine can sense the volume, composition and satiating effect of the nutrients present[29]. Enteroendocrine cells can also sense and respond to non-nutrient signals, such as bile acids and inflammatory cytokines[27].

Different macronutrients have different effects on satiety. Protein has been shown to be the most satiating nutrient[30,31,32,33]. The effect of carbohydrates on satiety is also high and can be modulated by dietary fiber, which prolongs the satiating effect[30]. Fat, as the least satiating nutrient, has even been shown to stimulate appetite, leading to high energy intake[24,34,35]. It is still controversial whether energy from fluids is as satiating as energy from solid foods[36]. Here, temporal aspects seem to matter since volume exerts its effects on satiety in short-term while nutrients and energy content are important in the longer term.

The sensory vagus nerve serves as a link between the gut and the brain, conveying satiety signals from the gastrointestinal tract to the brainstem[37]. The central nervous system also continuously receives information about the appearance, aroma and taste of potential foods as well as the presence of inflammatory or toxic substances in the blood. All these signals are integrated with information from pleasure and reward pathways and higher cognitive functions such an awareness of social context as well as external signals and cues in the environment[9,29]. How we react to the external cues is determined by our traits and emotional states.

The hypothalamus (nucleus arcuatus and other areas) and the brain stem (mainly nucleus tractus solitarius) are central for the metabolic aspects of appetite regulation and energy homeostasis, while many areas are associated with the rewarding aspects of food, mainly the ventral tegmental area, nucleus accumbens

and the forebrain[37,38]. The midbrain rewarding system is involved in the control of hedonic feeding, i.e. the intake of palatable foods[37].

Long-term signals

Leptin

Leptin is an adiposity signal mainly secreted from subcutaneous white adipose tissue in direct proportion to the amount of fat stored in the body[23]. Leptin levels affect several biological mechanisms, through its strong influence on several endocrine axes[39], including initiation of human puberty[40]. Leptin may act as the link between adipose tissue and the reproductive system, since adequate energy stores are needed for normal reproductive function[39]. Leptin is also a mediator of long-term regulation of energy balance, suppressing food intake and inducing weight loss. Leptin can pass from the blood circulation, through the blood-brain barrier, into the brain, reach the nucleus arcuatus of the hypothalamus and there influence energy homeostasis[19,40]. By binding to its receptors, leptin affects the expression of several orexigenic and anorexigenic neuropeptides in different parts of the hypothalamus as well as suppresses hedonic activation in reward areas in response to food intake[38]. Consequently, increased influx of leptin reduces food intake, but only in lean individuals[19], because obese individuals develop leptin resistance[23]. The obese also have a disturbed diurnal variation in leptin levels that normally display diurnal variation with higher nocturnal levels, suggested to suppress appetite while sleeping[41].

Weight-loss causes a reduction in leptin levels, making the brain more sensitive to environmental food cues, such as smells[17]. Congenital leptin deficiency, which is a very rare condition, is associated with reduced satiety, over-eating and severe obesity[23].

Insulin

Insulin is an adiposity signal secreted from pancreatic β-cells in response to increases in circulating glucose after a meal and in direct proportion to the amount of fat stored in white adipose tissue throughout the body[23,42]. Eating increases the concentration of insulin in the brain, stimulating anorexigenic pathways leading to reduced food intake in the lean[19,43]. Fasting decreases the insulin concentration. There is also a continuous background secretion of insulin, basal insulin, between meals. Insulin acts throughout the body to reduce circulating energy and to increase energy storage. Insulin is best known for its role of reducing circulating glucose, acting on insulin-sensitive tissues to increase their uptake of glucose, use it as fuel or else store it as glycogen[42], but it also stimulates the uptake of lipids into adipocytes and amino acids into skeletal muscle cells. Just like leptin, insulin

can pass from the blood, through the blood-brain barrier, into the brain and affect energy homeostasis. Obesity is associated with insulin resistance, therefore the brain and peripheral tissues do not respond to the signals properly.

Short-term signals

Metabolic signals – nutrients

The sight, smell and taste of food as well as sensory impulses generated by mastication and swallowing send excitatory signals to the gastrointestinal tract to be prepared[44]. When the nutrients reach the intestine, they are digested and absorbed and enteroendocrine cells sense the digestion products in the absorption process[44,45]. In response, hormones are secreted that signal to neurons within the intestine and to neurons in the brain.

The hypothalamus can sense changes in circulating adiposity hormones, gastric hormones and nutrients[46,47]. Multiple nutrient-related signals converge and integrate in the hypothalamus as it also recieves information from other nutrients sensors, mainly within the brainstem. Within specific hypothalamic nuclei, nutrient-sensing neurons detect glucose, fatty acids, amino acids and other fuel-related stimuli, and respond by regulating energy intake, the release of stored nutrients and nutrient utilization. For example, neurons require a continuous flow of energy from the blood to remain functional, mainly derived from blood glucose[42]. If hypoglycaemia occurs, receptors in the brain detect it and trigger reflexes to increase glucose secretion from the liver while simultaneously stimulating food intake[42,48,49].

Ghrelin

Ghrelin, first recognized for its stimulating effect on growth hormone secretion[50], is an appetite-stimulating hormone, mainly secreted from the stomach and duodenum depending on the nutritional state[40,51]. Ghrelin stimulates gastric motility, acid secretion and pancreatic exocrine secretion in anticipation of meals, preparing the gastrointestinal tract to process food[52]. Being the only hunger hormone, ghrelin stimulates food intake and body weight gain[37,53,54]. Ghrelin also interacts with the brain reward pathways, increasing the mesolimbic dopamine secretion, to enhance food reward and alter food preference in favour of calorie-dense foods, leading to an increased wanting and food seeking[55,56].

Given that ghrelin levels are elevated during fast, rise before each meal and decrease rapidly after eating, ghrelin has been suggested to be responsible for meal initiation[51]. Accordingly, preprandial increases in ghrelin correlate with hunger scores in humans[40,51]. After a meal, ghrelin levels are suppressed within minutes[51]. How fast ghrelin levels decline after a meal, as well as the depth and the duration

of the suppression, is determined by the caloric load, the size, the frequency and the composition of the meals[40,51]. Carbohydrates and proteins suppress ghrelin levels more effectively than do dietary lipids[52], and glucose-sweetened beverages are better at suppressing ghrelin levels compared to isocaloric fructose-sweetened beverages. Ghrelin levels are also influenced by age, gender, BMI, growth hormone, glucose and insulin.

Besides being released from the stomach and other tissues, following the bloodstream to cross the blood brain barrier to the hypothalamus, there are two other ways for ghrelin to affect appetite: via signals in the vagal nerve to the nucleus tractus solitarius in the brainstem or through local production of ghrelin in the hypothalamus. Even though ghrelin is commonly described as a short-term signal, it has been argued that it can also be considered a long-term signal, since it correlates inversely to adiposity and is important for body weight regulation[52]. Accordingly, obese individuals have lower ghrelin levels compared to lean individuals[37]. In addition, the ghrelin levels are suppressed less well by meals in obese compared to lean individuals[52]. Ghrelin levels are normalised upon diet-induced weight loss. Elevated ghrelin levels occur with weight loss resulting from caloric restriction, anorexia nervosa and bulimia nervosa as well as from chronic exercise without hypophagia[52].

Cholecystokinin (CCK)

CCK is a satiation hormone, released from the gastrointestinal tract after consumption of a meal, which helps to terminate food intake[38,57]. When nutrients reach the small intestine, the digestion products of fat and protein and, to a lesser extent glucose, stimulate secretion of CCK from I-cells of the duodenal and jejunal mucosa[27,58]. Concentrations increase within 15 minutes after a meal and then slowly decrease over the next three to five hours[58]. CCK has many physiological effects: it stimulates gallbladder contraction, bile and pancreatic secretion, slows gastric emptying, modulates gastrointestinal motility, increases the perception of fullness, decreases hunger and reduces food intake in a dose-dependent way[23]. CCK is also a neurotransmitter present in enteric vagal afferent neurons as well as in several locations in the brain. The slowing effects of CCK on gastric emptying and antral, pyloric and duodenal motility may be relevant to its appetite suppressant properties[58]. Gastrointestinal infection is associated with elevated levels of CCK, which may explain the reduced appetite associated with the condition[27]. Anorexia nervosa has also been associated with increased levels of CCK[59].

GLP-1

The satiety hormone GLP-1 functions both as a hormone and a brainstem neurotransmitter and excerts various effects in peripheral tissues[56,60]. After a meal,

GLP-1 is released from enteroendocrine L-cells in the jejunum, ileum and colon, secreted in proportion to energy intake, both stimulated by nutrients and nerve signals from the enteric nerve system[29,44]. GLP-1 release is strongly stimulated by glucose and fat[45], but protein also triggers secretion of GLP-1, as do bile acids and short chain fatty acids produced through gut bacterial fermentation[61]. A preprandial ghrelin surge enhances the GLP-1 secretion[62].

Elevated levels of GLP-1 can be detected within 10-15 minutes of eating and persists for several hours, depending on the nutritional composition of the meal[61]. Therefore, GLP-1 is thought to regulate inter-meal intervals[38]. Being an incretin hormone, GLP-1 increases glucose-dependent secretion of insulin postprandially. It also reduces the secretion of glucagon, delays gastric emptying and inhibits food intake. GLP-1 also suppresses food reward behaviour and reduces food palatability[56].

Studies have shown that GLP-1 secretion is usually impaired in obesity or type 2 diabetes compared with health[44,60]. Consequently, GLP-1 mimetics and inhibitors of GLP-1 degradation have been used successfully for the treatment of type 2 diabetes[45]. Recently, GLP-1 analogues were also approved by the European Medicines Agency (EMA) for the treatment of obesity in Europe.

Eating for pleasure

Hedonic hunger

The phenomenon of eating for pleasure is called hedonic hunger – in contrast to homeostatic or metabolic hunger, which is eating to correct an energy deficit[63]. The hedonic experience can be divided into two entities, “wanting” and “liking”, where wanting represents the anticipatory phase, the desire and motivation to engage in eating and to receive a reward, while liking represents the affective response to the oro-sensory stimulation during consumption[64]. Learning to make associations and predict a pleasureable hedonic reaction is another component of the reward process[17]. Wanting/desire for food is a function of innate and learned liking, internal state and environmental cues (Figure 2)[65,66].

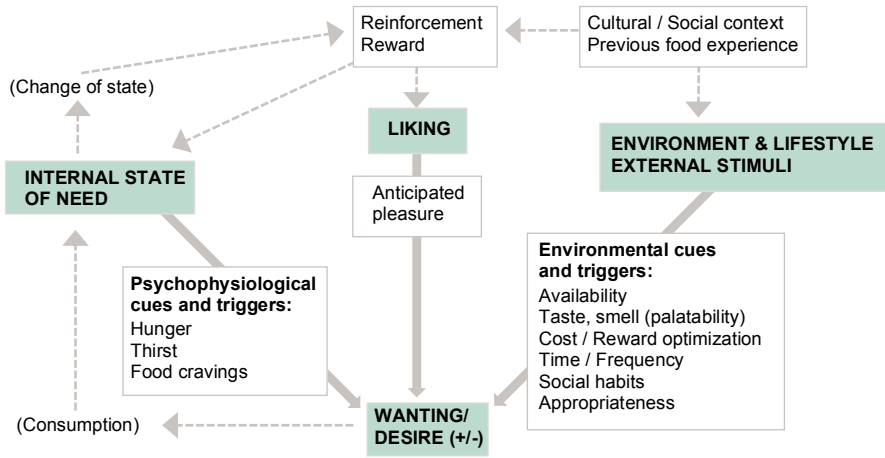

Figure 2. Acquisition and activation of desire for foods.
Wanting/desire is a major determinant of food choice. According to Melas model desire for food is a function of innate and learned liking, the internal need state and environmental cues[65,66,67]. "The metabolic brain" monitors the internal milieu and is under constant influence of the environment and lifestyle – "the cognitive and emotional brain"[68]. In addition, the peripheral and central signaling are subject to individual predisposition: genetic, epigenetic and early life imprinting mechanisms. Reprinted by permission from Elsevier: Appetite, Mela DJ. © 2006, (modified by Stenblom).

Foods that are very tasty and pleasant to eat are called palatable foods[17]. Palatable foods are typically sweet tasting or salty, very energy-dense, rich in refined carbohydrates, sugar and fat, and have a pleasant feel in the mouth. Ingestion of palatable food alters taste sensing compared to regular food[67]. After a regular meal, information about taste and energy content is transmitted to the brain stem and hypothalamus, leading to the release of satiety peptides, with the effect that the rewarding value of food will decrease during consumption, contributing to meal termination[69]. In contrast, highly palatable food also causes the taste information to be sent to the reward system, leading to the release of reward mediators such as dopamine, endocannabinoids and opiates[67]. Then, the taste information is integrated with olfactory, visual and cognitive inputs in secondary taste neurons[69]. The reward circuit interacts with the appetite-controlling neurons in the hypothalamus, which can result in up-regulated expression of hunger signals and decreased or blunted satiety signals[70]. This explains why satiety does not always downregulate a food's palatability or wanting for other foods[67]. Palatable foods have the power to drive hunger, even after a meal, and reinforce overeating and obesity, explaining why there is always room for dessert - even after a big meal[38,55,67].

The motivational value, the wanting to obtain a reward, involves the release of dopamine from neurons in the ventral tegmental area that project to nucleus

accumbens, striatum and other areas in the brain[17]. Dopamine increases wanting for food (or drugs) but is not responsible for the hedonic experience, the liking of a palatable food, which involves μ-opioid receptor signalling in the nucleus accumbens and other areas in the forebrain (activated in part by dopamine). However, wanting and liking usually occur together.

Obesity is associated with altered reward functions in the brain, but for a long time, it has not been clear whether this is the cause or consequence of obesity[17,71]. Neither has it been established whether overeating and reward-related obesity is due to hyper- or hypoactivity of dopamine signalling in the reward system[17,71,72]. Behavioural and neurophysiological data have indicated that obesity may be associated with increased motivation for food consumption, without necessarily any greater pleasure derived from eating[66]. Several studies, using functional magnetic resonance imaging of the brain (fMRI), have shown that obese individuals have increased activation of reward-related brain-areas in response to visual food cues compared to normal weight individuals, especially in response to pictures of energy dense foods[73].

During the last couple of years, several theories have emerged, two of which have gained the most scientific support and will be presented here: The incentive sensitization theory and the reward surfeit theory[74]. The incentive sensitization theory states that repeated intake of high-calorie palatable foods leads to an increased responsivity to cues associated with palatable foods, such as sights and smells[75]. This learned increased responsivity triggers craving and overeating when the cues are encountered. The reward surfeit theory proposes that individuals who are born with greater reward region responsivity to food intake are at elevated risk for overeating and weight gain[74]. Indeed, hyper responsivity to rewards in general also increases the risk for obesity as well as substance use. This has been associated with a genetic propensity for greater dopamine signalling. Since satiety signals such as leptin and insulin are thought to decrease the rewarding value of food, leptin resistance and insulin resistance may in part explain non-homeostatic feeding[76].

Overeating

Unlimited access to palatable food and increased exposure to food cues can induce conditioned hedonic overeating even in the postprandial phase when we are satiated and metabolically replete[17]. Therefore, it has been suggested that there may be nothing wrong with the reward system – it is functioning the way it was supposed to - instead it is the environment that is abnormal, encouraging us to eat all the time.

Excessive intake of palatable foods without need for energy is called overeating[77]. Hedonic hunger without homeostatic hunger favours energy-dense palatable food, rich in sugar, fat and salt such as pastries, sweets, ice cream and fast foods[78,79]. Because palatable foods are very energy-dense and at the same time easy to digest, they make over-eating very easy[55]. Therefore, hedonic hunger plays a part in overeating that over time leads to accumulation of body fat, overweight and obesity[8, 38,66].

A cumulative positive energy balance can come about through adjustments in the various components of energy expenditure or fuel utilization, together with shifts in food selection or eating patterns leading to adjustments in macronutrient intake[80]. For example, regarding food choice, energy density is a key determinant of energy intake[81], and regarding eating patterns one eating pattern associated with overweight and central obesity is increased eating frequency[82,83].

The amount of food consumed is to a large extent determined by the amount of food on the plate or the size of the package[9]. Portion sizes in restaurants and supermarkets have increased during the last 40 years, and so has the amount served at home (Figure 3)[4,5]. Variety and a greater number of foods also lead to increased eating[84]. Finally, if people around you eat more, so will you[9,85].

Environment based risk factors that make you change your eating pattern and choice of foods and the amount of food eaten and ultimately promote a higher energy intake are: Foods eaten outside the home, high-fat foods, large portion sizes and unlimited amounts of food to a set prize[86,87]. Environmental risk factors for overeating also include stress, increased screen time and sleep-deprivation.

Most people increase their food intake in response to stress, specifically the kind of stress that is perceived as threatening, since it causes increased release of cortisol, which in turn stimulates hunger and feeding[76]. Eating highly palatable food relieves the stress[88]. It has also been suggested that stress-induced eating is a result of habits and learned wanting[89]. Due to the hedonic outcome, palatable food is a strong Pavlovian stimulus, therefore making the eating habit more likely to be activated under stress.

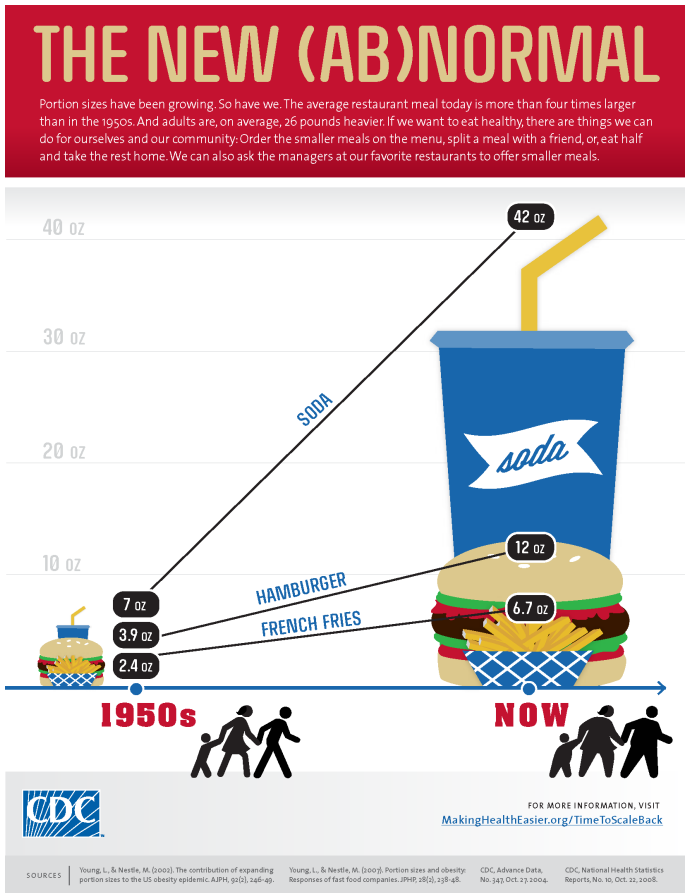

Figure 3. Larger portion sizes promote overeating.
Portion sizes have increased during the last decades, promoting a higher energy intake because the amount of food eaten is to a large extent determined by the amount of food on the plate. Source: Centers for disease control and prevention (CDC).

Another environmental factor is increased screen time. Eating while watching TV is associated with overeating[90], promoting sedentariness and at the same time increasing the exposure to marketing of energy dense foods[91]. A third environmental factor is sleep deprivation. Short sleep-time increases portion sizes and increases snacking[92], alters food choice in favour of calorie-dense foods[93], decreases physical activity[94], decreases energy expenditure[95], increases impulsivity in response to food cues[96], decreases leptin levels[97,98], increases ghrelin levels[92,93,97], reduces and delays the GLP-1 response[98] and increases the risk for obesity[97,98].

Eating addiction

Overeating is not a psychiatric disorder. However, it has been suggested that severe cases of overeating of palatable foods is linked to food addiction, and that food addiction should qualify as a psychiatric disorder according to the diagnostic and statistical manual of mental disorders, fifth edition (DSM-5) [79,99,100]. Indeed, the same neural circuitry is used for motivated intake of both drugs and food[77]. Neuroimaging has shown that obesity, as well as binge eating, is associated with altered dopaminergic signalling and hyperactivation of reward-related brain areas in response to food cues, such as pictures of palatable food[101]. Nevertheless, this is not enough evidence that food addiction is indeed an addiction[102]. As opposed to addictive drugs, we need food for our survival[100]. Furthermore, the ingestion is not of one substance, but mixed nutrients, even if the preferred foods are most often rich in refined carbohydrates, such as white sugar and white flour. Lastly, even if the DSM-5 criteria for addiction are met, the consequences of overeating are, in most cases, not as extensive or severe as those of drug addiction[101]. Therefore, it has been suggested that severe cases of overeating, which results in negative health, social and occupational consequences, instead of food addiction be called food abuse[77] or eating addiction – an expression of strong habits and preferences[102]. Indeed, according to results from studies using the Yale food addiction scale (YFAS) there are many overlaps with binge eating disorder[99].

Eating behaviour

Eating behaviours

There are various types of eating behaviours, including the widely recognized categories "emotional eating", "uncontrolled eating" and "restrictive eating"[103]. Eating behaviours are typically evaluated through self-report questionnaires and may help explain problematic eating, weight-related outcomes such as overweight and obesity, and why diet breakdowns occur. A genetic susceptibility to obesity may also include eating behaviours[104].

Emotional eating

Emotional eaters typically have increased food cravings and overeat when they lose self-control due to dysphoric emotions such as depression, anxiety or stress[76,103,105, 106]. Emotional eaters with high stress-induced cortisol levels typically choose sweet-and-fat foods, such as chocolate and cake, in response to stressors or negative affect[107]. In contrast, underweight individuals and non-emotional eaters

usually eat less when anxious and consequently lose weight during depression[107,108]. Recent studies have shown that eating in response to positive emotions is as common as eating in response to negative emotions[105]. It has also been suggested that emotional eating in respect to negative and positive emotions represent different categories. Emotional eating is more common in women than men[103].

Uncontrolled eating

Individuals with uncontrolled eating overeat because of a high susceptibility to food cues and because they lose control over their food intake[103,105,108]. Uncontrolled eating is sometimes referred to as external eating. Socio-economic status is a strong determinant for eating behaviour such as uncontrolled eating and night eating as well as risk for obesity[109].

Cognitive restraint

Cognitive restraint, also called restricted eating, restrained eating or restrictive eating, is the tendency to consciously restrict food intake in order to control body weight[103,108]. Restricted eaters typically eat more under disinhibiting conditions; such as after an eating an appetizer or after alcohol consumption. Cognitive restraint may be more common in women than men.

Psychiatric eating disorders

Anorexia nervosa, bulimia nervosa and binge-eating disorder are psychiatric eating disorders diagnosed according to DSM-5[110]. The prevalence of eating disorders varies between studies, the overall point prevalence in the general population ranging from 0.2 – 3.7%[111]. Anorexia nervosa is the least common eating disorder and binge eating disorder the most prevalent[112]. Eating disorders are most common in adolescents and young adults, and more common in women than men[113].

Brain imaging has shown alterations in both grey and white matter in several regions of the brain, as well as in connecting fibers and neurotransmitter activity, in anorexia nervosa, bulimia nervosa, and binge eating disorder. These alterations suggest that reward pathways, including the taste-reward system, may have a central role in eating disorders[110]. Individuals with anorexia nervosa have increased dopamine response, whereas individuals with bulimia nervosa have decreased dopamine-related brain activity. These changes are associated with altered dopamine receptor availability. However, in eating disorders, pathological attitudes towards food may outweigh taste responses and physiological regulation of food intake[78]. This may explain why, in both anorexia nervosa and bulimia

nervosa, repeated exposure to sweet taste compared to random exposure gave rise to different responses in reward-related areas of the brain[110]. It clearly made a difference whether the individual was prepared or not for the exposure, to be able to cognitively counteract the spontaneous reaction[78].

Anorexia nervosa

Individuals with anorexia nervosa are typically underweight but feel fat[110]. Anorexia nervosa can be divided in two categories: a restricting type with restricted food intake, sometimes combined with over-exercising, and a binge eating and purging type where individuals eat large amounts of food in a short time and/or use different methods to avoid weight-gain, such as vomiting.

Bulimia nervosa

Individuals with bulimia nervosa engage in binge eating and purging behaviour at least once a week[110]. Due to a combination of undereating and binge eating they are usually normalweight[113]. Binge-type foods are typically fat-and-sweet including doughnuts, bread, cereal, ice cream, sweets and pop corn[78]. The amount consumed in binges is typically between 1000 kcals and 2000 kcals[113].

Binge eating disorder

Most women with binge eating disorder are obese and have a history of weight cycling[78]. However, many individuals with binge eating disorder are not obese and most obese people do not have binge eating disorder[114]. Binge eating disorder involves episodes of excessive eating but without compensatory behaviours like vomiting[110]. The episodes are uncontrolled, often rapid, and occur in isolation, even in the absence of hunger[114]. The eating persists despite physical discomfort and is associated with feelings of guilt and disgust. Binges can be triggered by negative mood states, but these are not necessarily ameliorated by the food ingestion. Binge-type foods are typically fat-and-sweet (see bulimia nervosa)[78].

Body weight regulation

According to the energy balance equation, energy stores reflect the difference between energy intake and energy expenditure[115]. Energy intake consists of food and drink consumption while total energy expenditure is the sum of 1) resting metabolic rate, i.e. the energy expended on basic cellular functions, 2) the energy cost of physical activity and 3) adaptive thermogenesis, including the thermic effect of food[116].

Body weight remains relatively constant over time despite short-term fluctuations in food intake and physical activity[29]. However, since our survival is more acutely threatened by starvation than obesity, the system to regulate the energy-balance is better at ensuring sufficient energy intake than the opposite. Individuals that lose weight and reduce their fat stores secrete less of the adiposity signals, leptin and insulin, leading to a reduced sensitivity to satiation signals like CCK in the brain and hence increased food intake through larger meals[19]. In this way, adiposity and satiation signals interact to defend stable adipose stores. In response to weight loss, both the motivation to find food (wanting) and the size of individual meals tend to increase until energy stores are replenished[69].

Overweight and obesity

Body mass index (BMI) is a measurement widely used to calculate nutritional status[11]. BMI is defined as an individual's weight in kilograms divided by the square of his/her height in metres (kg/m^2). The WHO defines overweight as a body mass index (BMI) ≥ 25 kg/m^2, preobesity as BMI ≥ 25 – 29.9 kg/m^2 and obesity as a BMI ≥ 30 kg/m^2. Overweight and obesity can also be defined as an excessive accumulation of fat that can lead to increased health risks. A limitation with BMI is that it cannot distinguish between fat and muscle mass. In order to better predict increased risk for disease, BMI can be complemented by other measures, such as waist circumference[117]. Because BMI fails to provide information on total body fat, a new type of obesity has been defined; normal weight obesity, which refers to individuals with BMI within the normal range (BMI 18.5 – 24.9 kg/m^2) but with excessive body fat (> 17.6% for men and > 31.6% for women)[118]. Normal weight obesity is associated with higher waist circumference and increased risk for metabolic syndrome.

Excessive accumulation of fat is due to the interaction between genetic factors and environmental conditions such as constant access to energy-dense food and minimal physical demands of daily living[6,115]. Eating energy-dense food, especially high-fat food, is positively associated with obesity for both men and women[24,119, 120]. Fast food intake also predicts weight gain and obesity[79,87]. Eating between meals, eating snack foods and fast foods have also been associated with excess weight[87]. So has skipping breakfast. Ultimately, an increase in body weight is caused by increased food intake, decreased energy expenditure or a combination of the two[9]. WHO Europe estimates that 25% of adults and more than 80% of adolescents are not sufficiently active today, and the trend is towards decreasing levels of physical activity[196]. Unfortunately, sedentariness does not promote a down regulation of food intake[86].

Daily food consumption has increased in the world between the 1960s and today with 21%[197] (Table 1). During the same period, the percent of populations of developing countries with an energy intake of less than 2200 kcal per day decreased from 57% in 1964-66 to 10% in 1997-99[198].

Table 1. Food consumption per capita (kcal/person/day)
Data from national food balance sheets[197].

	1969/1971	2015
World	2373	2860
Developing countries	2055	2740
Developed countries	3138	3390

Pathogenesis

Genetic factors have a large influence on BMI, which explains why not all individuals exposed to an obesogenic environment become obese. Estimates of heritability range from 40 to 90% of the variation in BMI in a population[121,122], indicating that the genetic contribution to body fat content in humans is as strong as that for height[116].

In rare cases of childhood-onset obesity, single gene defects have been identified, such as the Prader-Willi syndrome where high ghrelin levels lead to extreme hyperphagia and juvenile-onset obesity[52]. In the general population of adults with obesity, however, the disorder is polygenic with genetic variations creating susceptibility to environmental factors, i.e. a multifactorial disease[17].

The tendency to gain weight is associated with: a low basal metabolic rate, low energy cost of physical activity, a low capacity for fat oxidation, high insulin sensitivity, low sympathetic nervous system activity and low plasma leptin concentration[80,123]. Individuals predisposed to obesity may also have a gut microbiota that promotes more efficient extraction and/or storage of energy from the diet compared to microbiota of lean individuals[124,125].

Even though total amount fat consumed is a potent food-related risk factor for weight gain, some individuals remain lean even on a high-fat, high-energy diet. Different susceptibility to high-fat diets may be explained by variations in metabolic flexibility, where individuals predisposed to weight gain have a reduced ability to switch from glucose to fat oxidation[24,123]. Impaired hypothalamic nutrient sensing has also been suggested to contribute to obesity[46] and type 2 diabetes in predisposed subjects exposed to a chronic lipid overload[47].

Even though there is a clear heritable component in an individual's susceptibility to become overweight, the short time frame in which the obesity epidemic has developed implies that a change in gene frequencies is unlikely to be the primary causative factor[19]. Instead, the increased incidence of obesity is thought to reflect an interaction between our genotype, evolved in a scarce environment, and an increasingly obesogenic environment and lifestyle - a combination that clearly is a mismatch[17]. Indeed, several of the genes associated with obesity are highly expressed in the brain and hypothalamus, suggesting a role in weight regulation, affecting appetite, energy expenditure and/or behaviour[104,126,127]. Studies in children have shown substantial genetic influence on appetitive traits known to be obesogenic, such as lower sensitivity for satiety and increased responsiveness to food cues. With this in mind, the most important environmental factor is thought to be the ubiquitous access to low-cost energy-dense foods[19]. Another environmental risk factor is stress[76]. Stress that is perceived as uncontrolled and threatening is usually accompanied by high cortisol levels, and, in the presence of insulin, high cortisol levels promote lipid accumulation. Therefore, detrimental stress is associated with visceral adiposity and increased risk for metabolic disease.

Comorbidities

Obesity is associated with metabolic abnormalities including insulin resistance, high triglycerides, low HDL cholesterol, increased small, dense LDL particles and non-alcoholic fatty liver disease[128]. Consequently, an elevated BMI is a major risk factor for cardiovascular diseases, including heart disease and stroke, which were the leading cause of death in 2012[11]. Overweight and obesity also increase the risk for type 2 diabetes, degenerative musculoskeletal disorders (such as osteoarthritis) and various cancers (including endometrial, breast, ovarian, prostate, liver, gallbladder, kidney and colon cancer). Despite the increased risk for a long list of comorbidities, studies in overweight individuals (BMI 25.0 to <30.0 kg/m^2) have shown contradictory results regarding mortality risk - both reduced and increased mortality risk - compared with normal weight individuals[128]. In addition, a meta-analysis of 97 studies in individuals with grade 1 obesity (BMI 30.0 to <35.0 kg/m^2) showed no increased mortality risk. Individuals with grade 2 and 3 obesity, however, had a significantly higher mortality rate compared to the normal weight.

To improve health, a sustained weight loss of at least 5% of a person's body weight is needed[128,129]. For many complications however, the relationship between weight loss and decreased risk for comorbidity is not linear, so the associated health benefits decrease gradually for a weight loss greater than 10%[129].

Metabolically healthy obese

Not all obese individuals have metabolic abnormalities. A subgroup of obese individuals, the so-called metabolically healthy obese, have normal fasting glucose, insulin sensitivity, lipid profile and intrahepatic triglyceride content levels[128] and consequently have a lower risk for cardiovascular disease[130]. The metabolically healthy obese phenotype is more common in younger than in older people and occurs more often in women than men[128]. Due to the lack of uniform definition of metabolically healthy obesity[128,131], the prevalence has been hard to establish, ranging in different studies from 6% to 75%[132]. The question is if, given more time and additional weight gain, the metabolic function will deteriorate, or if the metabolically healthy obese are protected against metabolic abnormalities[128].

Treatment

Lifestyle modification

Standard treatment for overweight and obesity includes lifestyle interventions and behavioural interventions that aim for caloric restriction, reduced sedentary behaviour and increased physical activity level[129]. Current recommendations include limiting the energy intake from total fat and sugars, increasing the consumption of fruit and vegetables, whole grains and nuts, as well as engaging in daily physical activity – 60 minutes per day for children and 150 minutes per week for adults[11].

For most adults, the results of these interventions are poor because the central control of energy balance is fighting against a sustained weight loss to defend the maximum weight once achieved[9,19]. This defense of excess body fat can be illustrated by the phenomenon of yo-yo dieting. The minority who complete weight-loss programmes regain half of the lost weight within a year[9], a contributing cause being that food consumption is affected by cues in the environment. Therefore, weight loss programmes are only successful if individuals learn how to handle these cues. In conclusion, the best diet is the one adhered to by the patient (Table 2) [15,133].

Pharmacotherapy

In Sweden today, there are three pharmacotherapies available, orlistat[134] (Xenical®), liraglutide (Saxenda®)[208] and naltrexone/bupropion (Mysimba®)[209]. Pharma-cotherapy is used in conjunction with reduced caloric intake and increased physical activity in adults with a BMI ≥ 30 kg/m^2 or a BMI ≥ 27 kg/m^2 together with weight-related comorbidity (28 kg/m^2 for orlistat).

- **Orlistat** inhibits pancreatic lipase irreversibly and reduces the digestion and absorption of dietary fat in the small intestine by ~30%[134]. The

inhibited fat hydrolysis causes a reduced secretion of the satiety hormone CCK[135]. Most common side effects are gastrointestinal, such as abdominal pain and fecal urgency[134]. Long-term treatment causes a significant reduction in levels of beta-carotene, vitamin D and vitamin E[136,137]. Placebo-subtracted weight loss achieved after 1 year on the maximum dose is 3-5%[128]. Placebo-adjusted mean weight loss in 15 different studies with up to four years of treatment was 2.9%[134] (Table 2).

- **Liraglutide** was until recently only used for the treatment of type 2 diabetes but since March 2015 liraglutide is also approved for treatment of obesity[208]. Liraglutide is a GLP-1-analogue that increases feelings of satiety and decreases feelings of hunger, which leads to a reduced food intake[138]. Liraglutide increases insulin secretion and reduces glucagon secretion in a glucose dependent way. Most common side effects include nausea and vomiting[139]. Placebo-subtracted weight loss achieved after 1 year on the maximum dose is approximately 6%[128] (Table 2).
- **Naltrexone/bupropion** was also approved for treatment of obesity in March 2015[209]. Naltrexone is an opioid antagonist while bupropion is a weak antagonist of the reuptake of dopamine and noradrelinaline in nervendings. Both substances affect neurons in nucleus arcuatus in the hypothalamus as well as mesolimbic dopaminergic reward pathways. Most common side effects include: nausea, constipation, headache, vomiting, and dry mouth[140]. Placebo-subtracted weight loss achieved after 1 year on the maximum dose is approximately 6%[128] (Table 2).

The effect of medication on weight loss is not sustained when the medication is discontinued[128]. Patients generally regain weight to a level determined by the intensity of the concomitant lifestyle intervention.

Bariatric surgery

Among currently available obesity treatments, bariatric surgery alone routinely achieves substantial, permanent weight loss >15% and a reduction in overall mortality despite the perioperative risks[29,128] (Table 2). For example, bariatric surgery reduced the long-term incidence of type 2 diabetes by 80% in the Swedish Obese Subjects (SOS) study[141]. Micro and macrovascular complications were also reduced compared to in the control group. Surgery is only available for adults with severe obesity (BMI >40.0 kg/m^2 or BMI >35.0 kg/m^2 with obesity-related comorbidity) who have failed to respond to all other possible treatments[128]. The three most common bariatric procedures are: adjustable gastric banding, the Roux-en-Y gastric bypass and the vertical sleeve gastrectomy[142] (Figure 4).

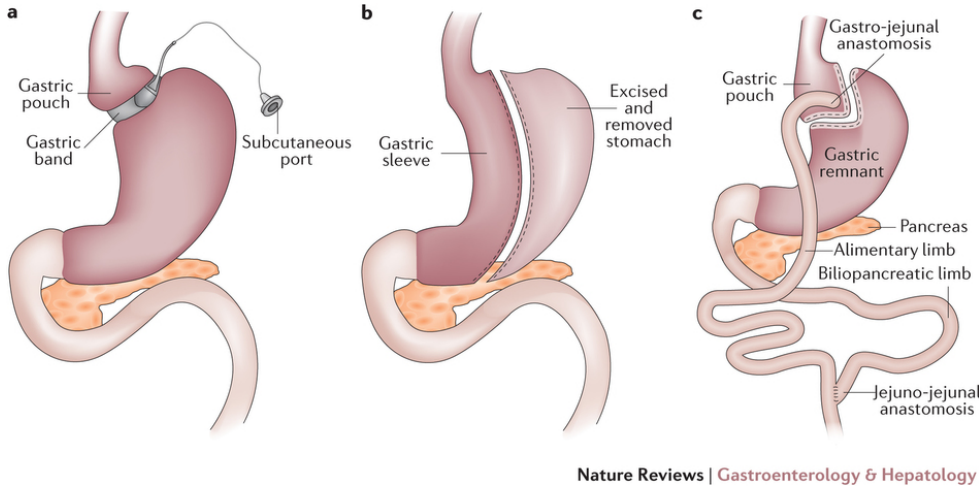

Figure 4. Types of bariatric surgery.
The most frequently performed bariatric operations are either purely restrictive, such as gastric band (a) and sleeve gastrectomy (b) or restricitive and malabsorptive such as the Roux-en-Y gastric bypass (c). Permission obtained from Nature Publishing Group: Nat. Rev. Gastroenterol. Hepatol. Naik RD, Choksi YA, Vaezi MF. © (2016) 10: 575-584.

Gastric bypass has proven to be the most successful and by far the most commonly used method[143], as it is more efficient in reducing appetite, body weight and the occurrence of type 2 diabetes compared to for example gastric banding[29,141,15]. These differences are believed to be due to altered secretion of gut hormones, such as reduced ghrelin[52] and increased GLP-1[27,44], that occurs after gastric bypass but not gastric banding[128]. Elevated levels of GLP-1 are seen also after sleeve gastrectomy[45]. The most serious complications of bariatric surgery include post-operative sepsis, anastomotic leaks, bleeding and venous thromboembolism[144] (Table 2). In addition, studies have shown increased prevalence of alcohol use disorder after bariatric surgery[145].

Table 2. Efficacy of weight loss therapies
Placebo-adjusted weight loss achieved by weight loss therapies currently available in the European Union[15].

Weight loss therapies	Body weight loss	Side effects
Commercial weight loss programmes[15]	1 year: 3%	
Orlistat[15,128,134]	1 year: 3 – 5%	GI symptoms: steathorrea, abdominal pain, fecal urgency, vitamin deficiencies
Liraglutide[15,128,139]	1 year: 4.4 – 6%	Nausea, vomiting
Naltrexone/bupropion[15,128]	1 year: 4.1 – 6%	Nausea, increased heart rate
Bariatric surgery[15]	Mean change in BMI after 3 years (kg/m^2): Gastric bypass: -22.9 Sleeve gastrectomy: -16.8 Gastric banding: -11.4	Sepsis, anastomotic leaks, bleeding, thromboembolism, vitamin and mineral deficiencies, dumping syndrome, gastro-oesophagal reflux, hypoglycaemia, surgical complications, alcohol use disorder

Thylakoids

Thylakoids, the photosynthetic membranes inside the chloroplasts of green leaves, are the most abundant biological membranes on earth[146] (Figure 5).

Thylakoids contain more than a hundred different membrane proteins involved in the photosynthetic electron transport[146]. Together with the main pigments, chlorophyll, carotenoids and xanthophylls, the membrane proteins account for 70% of the thylakoid mass[147,148], and the remaining 30% are membrane lipids, such as galactolipids, phospholipids and sulfolipids. Thylakoids also contain vitamins and antioxidants such as vitamin A, C and E, folate, beta-carotene, lutein, zeaxanthin, plastoquinones and phylloquinones (Table 3).

The thylakoids used for the studies presented in this thesis were extracted from spinach leaves (*Spinachia oleracia L.*) as previously described[149]. Two different thylakoid containing powders have been used: SwePharm AB (Södra Sandby, Sweden) prepared the first powder (used in Papers I and II), and FutureCeuticals (Momence IL, USA) the second powder (Appethyl®) (used in Papers III-V)

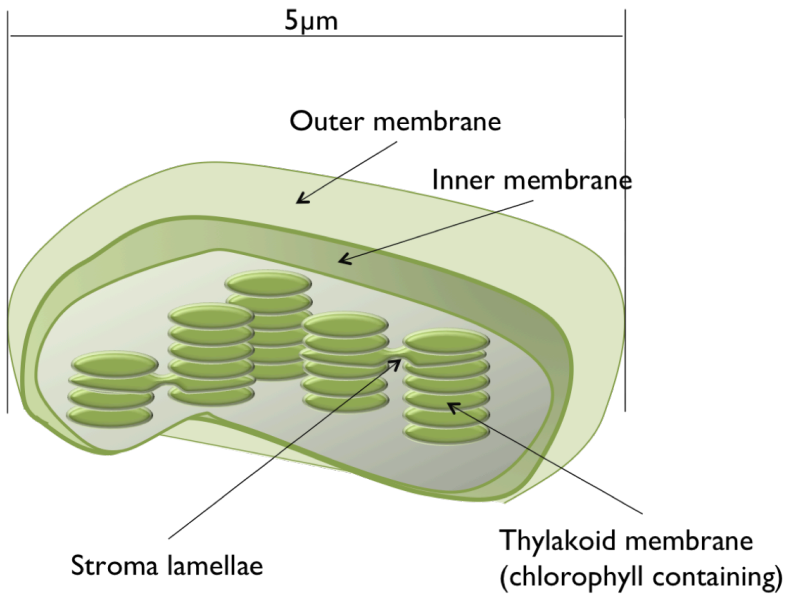

Figure 5. Chloroplast with thylakoids.
Thylakoids are stacked like coins inside the chloroplasts of green leaves (Figure: Magnus Hillman).

Table 3. Table of contents

The contents of fresh spinach compared to thylakoid-containing spinach powder (Appethyl®)[a]

	Spinach (100 g)	Appethyl® (100 g)	Appethyl® (5 g)
Energy (kcal)	24.1	365	18
Carbohydrates (g)	0.8	48.7	2.4
Dietary fiber (g)	2.1	38.7	1.9
Fat (g)	0.4	7.2	0.4
Protein (g)	3.3	26.1	1.3
Vit A (IU)	917.2[a]	5750	287.5
Vit C (mg)	36.7	< 1	n.d.
Vit E (mg)	1.2[a]	6.07[b]	0.3
Vit K (μg)	270[a]	1330[b]	66.5
Vit B9 (Folate) (μg)	202	166[b]	8.3
Beta-Carotene (μg)	7732[a] (= total carotenoids)	3450	172.5
Iron (mg)	2.1	115	5.8
Calcium (mg)	88 [a]	2910	145.5
Sodium (mg)	160[a]	274	13.7
Lutein-Zeaxantin (mg)	(Part of total carotenoids [a])	Lutein: 27.9[b] Zeaxantin: 0.7 [b]	1.4 0.04
Ash (g)	No data	12.5	0.6
Moisture (g)	No data	5.4	0.3

Values for spinach from the National Food Agency Sweden (NFA)[210], except for values marked with [a] that come from the Finnish National Food Database, Fineli[211]. Values for Appethyl come from Van Drunen Farms, Illinois, US 2014-04-11 except for values markes with [b] that come from Eurofins Food & Agro Testing Sweden 2012-12-17.

Previous studies with thylakoids

In vitro studies

When isolated, thylakoids inhibit the pancreatic lipase/co-lipase-mediated hydrolysis of dietary fat[150,205]. This is done by two complementary mechanisms; 1) the membrane proteins bind to the lipase/co-lipase complex and thereby block its active site from getting in contact with the substrate, and 2) thylakoid membranes cover the oil droplet, binding to the oil/water interface, also preventing access to the substrate (Figure 6). Since the thylakoids will eventually be hydrolysed in the gastrointestinal canal, the inhibition is reversible and does not cause steatorrhea[150,151].

Besides the inhibition of the lipase/co-lipase complex, thylakoids also slow down the passage of macronutrients over the intestinal wall, a dose dependent effect, shown *in vitro* using methyl-glucose, dextran and ovalbumin[152].

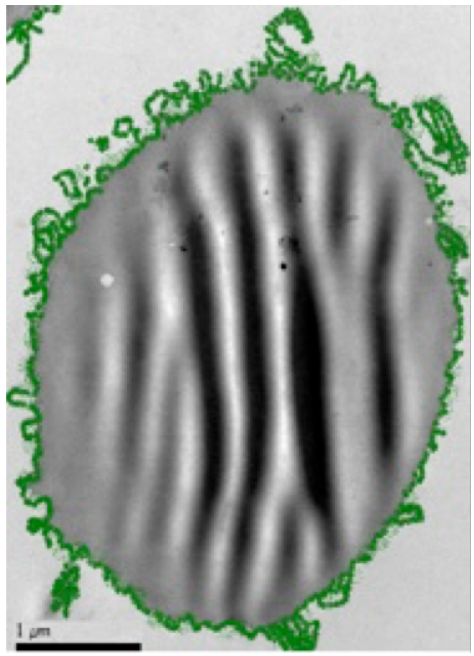

Figure 6. Thylakoid-covered lipid droplet.
The electron microscopy image shows thylakoid membranes covering a lipid droplet[150]. The coloring of the thylakoid membranes was done in Photoshop by Sinan C Emek.

Animal studies

Previous animal studies with thylakoids have shown that supplementation of thylakoids to the diet is associated with reduced food intake[150, 151,153] , reduced body weight gain[149,150,153] , reduced body fat accumulation[149,153], reduced blood levels of leptin[153], reduced free fatty acids[153], and tricylglycerols[150,153], increased postprandial levels of CCK[150,153,154], and enterostatin[150], reduced levels of ghrelin[154,155], altered gut microbiota[151], increased expression of pancreatic lipase[150], and increased secretion of pancreatic lipase/co-lipase[156].

Effects of thylakoid treatment on blood glucose and plasma insulin have shown different patterns; glucose and insulin levels have either been unchanged or reduced in different combinations[151,153, 154,156].

Human studies

In a crossover meal study in human, ingestion of thylakoids caused increased postprandial levels of CCK and leptin, decreased postprandial ghrelin, reduced free fatty acids, and reduced insulin. Blood glucose levels were not affected[155].

Aims and hypotheses

The general aims of this thesis were to study the effects of thylakoids on appetite regulating hormones secreted from the gut, feelings of hunger, satiety and reward, and the implications on body weight regulation and risk factors for comorbidity.

The individual aims and main hypotheses of the papers included in this thesis:

Paper I

The aim was to investigate the effect of thylakoids compared to placebo when served with a high carbohydrate breakfast in a crossover meal study in humans on ratings of hunger, fullness, urge to eat and thoughts of food as well as on blood levels of glucose, insulin, CCK, ghrelin and the inflammation marker tumor necrosis factor-α (TNF-α). The hypothesis was that thylakoid-supplementation would cause a delayed glucose uptake that would affect postprandial levels of appetite regulating hormones, increase ratings of fullness, reduce ratings of hunger, urge to eat and thoughts of food, as well as reduce inflammation and thus TNF-α.

Paper II

The aim was to investigate the effect of daily supplementation with thylakoids for two months in humans, together with an energy-restricted diet, on body weight loss and metabolic parameters associated with obesity as well as ratings of hunger, satiety and cravings for palatable food. The hypothesis was that thylakoids would cause a greater weight reduction in the treated compared to control and to improve the metabolic risk factors for disease as well as reduce hunger, reduce cravings and increase satiety.

Paper III

The aim was to investigate how daily supplementation of thylakoids for three months together with a healthy lifestyle affects body weight and metabolic parameters associated with obesity and to investigate how thylakoid treatment affects appetite-related feelings and appetite-regulating hormones during the first and last days of the study. The hypothesis was that thylakoids, through their effects on appetite-regulating hormones, as well as hunger and wanting for

palatable food, would facilitate weight-loss in the treated group compared to control.

Paper IV

The aim was to investigate how supplementation of thylakoids with a breakfast in the morning affects ratings of hunger, satiety, wanting and liking for palatable food during the day, as well as intake of palatable sweets and snacks in the afternoon, and to see if the treatment effects of thylakoids are associated with eating behaviour scores. The hypothesis was that thylakoids would increase satiety and reduce hunger as well as reduce wanting and liking for palatable food and intake of palatable food and that the treatment effects would be associated with eating behaviour scores.

Paper V

The aim was to investigate the effects of long-term daily supplementation with thylakoids, together with a high-fat diet, in the rat on fatty acid oxidation, body composition, energy expenditure and nutrient-substrate utilisation. The hypothesis was that thylakoids would increase fatty acid oxidation in the intestine and to shift nutrient-substrate utilisation towards fat and that this would result in increased energy expenditure and a reduced body fat accumulation.

Methodology

The purpose of this chapter is to give an overview of the methods used in the thesis as well as a discussion and reflection over the strengths and limitations of the chosen methods. For a detailed description, see the respective papers.

Clinical studies (paper I-IV)

Ethics

The studies were approved by the Ethics Committee for Human Studies at Lund University and conducted in accordance with the Declaration of Helsinki. Participants were recruited through advertising in the local community. All participants gave written informed consent before the studies began. Except for the first meal study (Paper I) the participants did not receive monetary compensation. Individuals with pathological values were followed up and referred to a doctor.

Statistics

Based on data from previous studies, power calculations were performed to calculate sample size with a sensitivity of 0.80 and a significance level of 0.05. Participants in the studies with parallel design (Papers II and III) were randomized into either a thylakoid treated group or a placebo group in order to achieve two similar groups with normal distribution for the parameters studied. In the studies with crossover design the allocation to treatment was randomized and balanced between test days using Latin Square, Williams design.

Data were analysed for statistical significances using GraphPad Prism, versions 4-6 (GraphPad Software, Inc., San Diego, SA, USA). Analyses of blood parameters and anthropometric measurements in Paper II were done using R Development Core Team, version 2.15.3, 2011 (R Foundation for Statistical Computing, Vienna, Austria). Energy and macronutrient composition of test meals were calculated using the Dietist XP software (Kostdata, Bromma, Sweden). Normal distribution of parameters was tested using D'Agostino-Pearson omnibus and Shapiro-Wilk normality tests, verified by boxplot analysis. For normally distributed parameters, unpaired t-test was used for comparisons between groups and paired t-test was

used for comparison within groups. For non-normally distributed parameters, Mann-Whitney test was used for comparisons between the groups and Wilcoxon matched-pairs signed rank test for paired comparisons. The Pearson correlation coefficient was used to study correlations. For comparison between groups of repeated measurements, two-way repeated measures ANOVA was used with treatment and time as fixed factors. Area under the curve (AUC) was used to capture total blood levels or visual analogue scale (VAS) scores over time. In Paper II, blood parameters and anthropometric measurements were analysed together to obtain rates of change over time for all metabolic risk factors using Hotelling's T2-test. In text and figures, data were expressed as mean ± SD/SEM or median ± interquartile range depending on whether parametrical or non-parametrical statistics had been used. p-values < 0.05 were considered statistically significant and p-values < 0.1 a tendency towards significance.

Subjects

For the diet intervention studies (Papers II and III) a screening procedure was performed at a screening visit before the studies began to ensure samples that were reasonably homogenous and to achieve normal distribution for the variables tested as well as to screen for the exclusion factors, which were: diabetes, thyroid disease, inflammatory bowel disease, irritable bowel syndrome, recent use of antibiotics, having followed a diet with the intention of losing weight during the last three months, being vegetarian or vegan. For the meal studies, which were designed as crossover studies, no screening visit was needed, since the participants here served as their own controls. In the second meal study, eligible participants were chosen among volunteers based on their answers to questionnaires. Only female participants were included in the clinical studies, because most volunteers were women. To obtain a homogenous sample, given that the intensity of food cravings may fluctuate across the menstrual cycle[157,158], mainly middle-aged and postmenopausal women were included in the studies. In addition, all women were asked whether they experienced hormonal influences on their appetite.

Study design

The meal studies (Papers I and IV) had crossover design and the diet intervention studies (Papers II and III) parallel design. All clinical studies were single-blinded except the second meal study (Paper IV) that was double-blinded. In the single blinded studies, the investigators performed the randomization and handed out the experimental drinks, containing thylakoids or placebo, to the participants. In the double-blinded study, these tasks were delegated. The participants were not aware of which group they belonged to in any of the studies, neither was the clinic staff.

The first meal study (Paper I) and the two-month diet intervention study (Paper II) were conducted at the Overweight and Diabetes Unit, Skåne University Hospital

(SUS), Lund Sweden. The three-month diet intervention study (Paper III) was conducted at the Overweight/Diabetes Unit and at the Division of Occupational and Environmental Medicine, SUS, Lund Sweden. The second meal study (Paper IV) was conducted at the Biomedical Centre (BMC), Lund University, Sweden.

Standardization, confounding factors, compliance

Working with human participants to study their appetite and eating behaviour there are many potential confounding factors, making standardization of study routines very important[159]. Therefore, in all studies, participants had a standardized meal the night before test days and thereafter fasted over night. They were not allowed to exercise vigorously the day before test days, or drink alcohol. The participants were told not to hurry to the clinic in the morning or run up the stairs; instead they should take the elevator. Fasting blood samples were taken and VAS-questionnaires were filled out before the participants were served a standardized breakfast with or without thylakoid supplementation. Measures were taken to ensure a stable environment without variable external cues that could affect the results. For example, the participants were not allowed to read or talk about food during the test days when they rated their feelings of hunger and cravings. During the diet intervention studies, diaries and interviews were used as tools to assess compliance regarding diet and exercise.

Visual Analogue Scale (VAS) questionnaires

VAS-questionnaires were used in all clinical studies (Papers I-IV) to assess feelings of hunger, fullness and cravings for palatable food, a method that is considered reliable for appetite research[160] (Figure 7). The questionnaires were constructed as booklets with written instructions on the front page. All participants were also instructed how to fill out the questionnaire, one page of questions per time point, or, in the second meal study (Paper IV), two pages. Each VAS-question was 100 mm long with words anchored at each end, expressing the minimum answer to the left and the maximum to the right. Participants were not allowed to discuss the ratings with each other or look at previous answers.

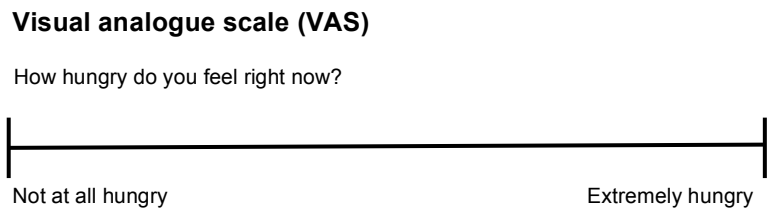

Figure 7. VAS-question.
The participants rated their appetite-related feelings such as hunger, fullness and cravings for palatable food by marking somewhere along the 10 cm long VAS.

First meal study, homeostatic hunger (Paper I)

Twenty healthy overweight women were enrolled in this placebo-controlled crossover study that included three visits to the clinic at least one week apart (Figure 8). The three different conditions were: low (3.7 g) or high dose (7.4 g) thylakoids (SwePharm AB) or placebo, administered in a black currant jam, served together with a typical Swedish high carbohydrate breakfast.

Blood samples were taken before breakfast (time point 0 minutes) and then again 30 minutes after breakfast as well as 60, 90, 120, 180 and 240 minutes after breakfast. At the same time points the participants also rated their subjective feelings of hunger, fullness, urge to eat and thoughts of food in a VAS questionnaire. The blood was tested for glucose and plasma was analysed for insulin, CCK, ghrelin and TNF-α.

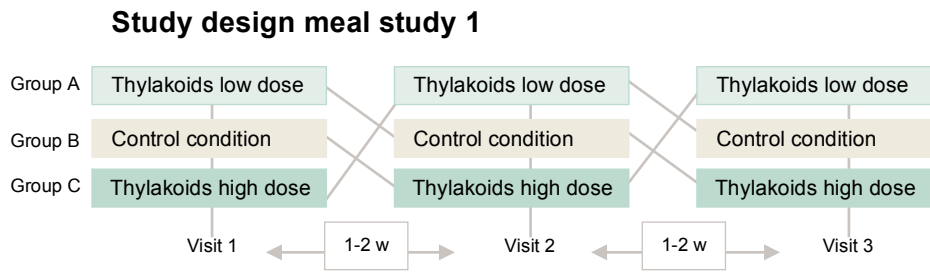

Figure 8. A cross-over study with three different conditions.
The participants visited the clinic on three separate occasions, separated by a minimum of one week. The participants were randomised to receive high dose thylakoids, low dose thylakoids or control on different days in a balanced order.

Two-month diet intervention study (Paper II)

Of the 48 overweight or obese women that were screened for eligibility, 30 women were enrolled in the study and completed it.

During the two-month duration of the study, the participants visited the clinic every second week for measurements of body weight, waist- and hip circumferences and body composition (Figure 9). Fasting blood samples were taken for measurements of glucose, insulin, HbA1c, TAG, cholesterol (LDL, HDL and total cholesterol), ApoB1 and leptin. At each visit the participants received 14 blueberry drinks prepared with 5.6 g thylakoids (SwePharm AB) or placebo to be consumed daily with breakfast during the next two weeks. They also received standardized breakfast at the clinic and a standardized take away lunch. On the first and last days of the study the participants filled out VAS-questionnaires repeatedly throughout the day.

During the two month intervention, the participants were instructed to follow a 15% energy-restricted diet regime consisting of three meals a day, freely chosen from a cookbook compiled exclusively for the study, containing recipes for breakfast, lunch and dinner. The participants were also told to exercise 60 minutes per day at low/medium intensity, such as brisk walking. Every day, the participants recorded their food choices and amount of exercise in a diary. They also wrote down how many steps they had taken during the day according to a pedometer.

Before and after the study, the participants filled out a questionnaire concerning eating behaviours, the three-factor eating questionnaire (TFEQ) which is a self-assessment scale used in studies of eating behaviour[108].

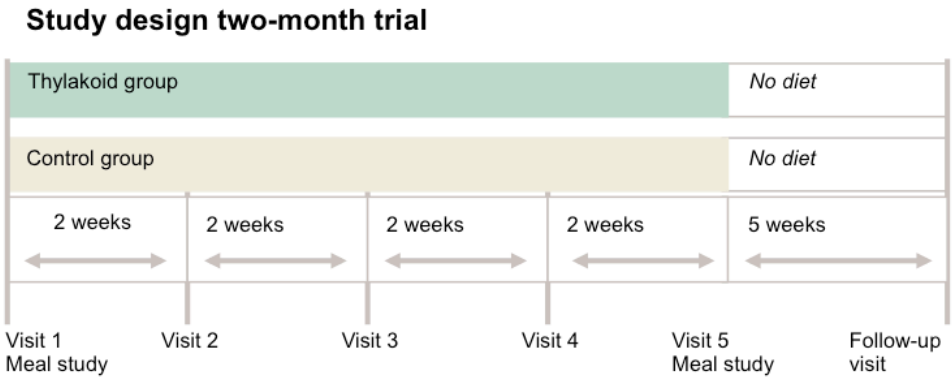

Figure 9. The participants visited the clinic every two weeks during the two-month study.
During all visits fasting blood samples and anthropometric measurements were taken: body weight, body composition and waist- and hip circumferences. At visit 1 and 5 the participants also filled out a VAS-questionnaire before breakfast and repeatedly during the day. Five weeks after the study, there was a follow-up visit.

We also performed a follow-up study[200] (not included in the paper). Five weeks after the end of the study, anthropometric parameters were measured (body weight, waist circumference, hip circumference and body composition) and blood samples analysed (glucose, insulin, HbA1c, TAG, total cholesterol, HDL-cholesterol and LDL-cholesterol). At the follow up, participants also filled out the VAS-questionnaire as well as the eating behaviour questionnaire.

The data from the questionnaires were analysed recently[201] (not included in the paper). In this study, besides looking at answers from the five-week follow up, VAS-ratings for cravings during the two-month intervention were also re-analysed, using a new category of cravings named individual cravings. This category consisted of the highest cravings for each participant – i.e. for every individual the ratings for the kind of food that person craved the most.

Three-month intervention study with meal studies (Paper III)

Fifty-three overweight/obese women volunteered for screening and 38 of them were enrolled in the study.

During the three-month study participants visited the clinic every third week for measurements of body weight, waist- and hip circumferences and body composition (Figure 10). Fasting blood samples were taken for measurements of glucose, insulin, cholesterol (total, HDL and LDL), TAG and leptin.

During the three-month study, the participants had no energy restriction, but they were only allowed three meals per day and no snacking in between, no sweet drinks or alcohol. They were also encouraged to follow the Swedish dietary guidelines according to the Nordic Nutrition Recommendations (NNR)[207], issued by the National Food Agency, Sweden, and to engage in low to medium-intensity physical activity 30 minutes per day (Figure 11). At each visit to the clinic the participants received blueberry drinks prepared with 5 g thylakoids (Appethyl®) or placebo to be consumed daily with breakfast during the next three weeks.

On the first and last days of the study, meal studies were performed. During these two test days additional blood samples were taken at repeated time points before lunch for analysis of blood glucose, p-insulin, p-ghrelin and p-GLP-1. In addition, the participants filled out VAS-questionnaires repeatedly throughout the day. For this reason, the participants received standardized breakfast at the clinic and standardized take away lunch and dinner.

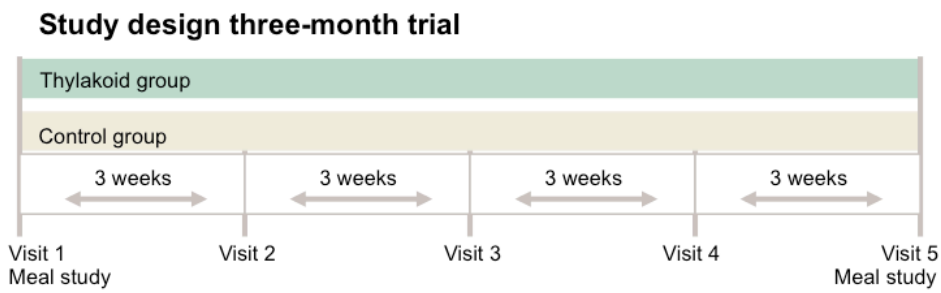

Figure 10. The participants visited the clinic every three weeks.
During all visits, 1-5, fasting blood samples and anthropometric measurements were taken: body weight, body composition and waist- and hip circumferences. At visit 1 and 5 the participants also filled out a VAS-questionnaire before breakfast and repeatedly during the day. Blood samples were taken at fixed intervals during six hours.

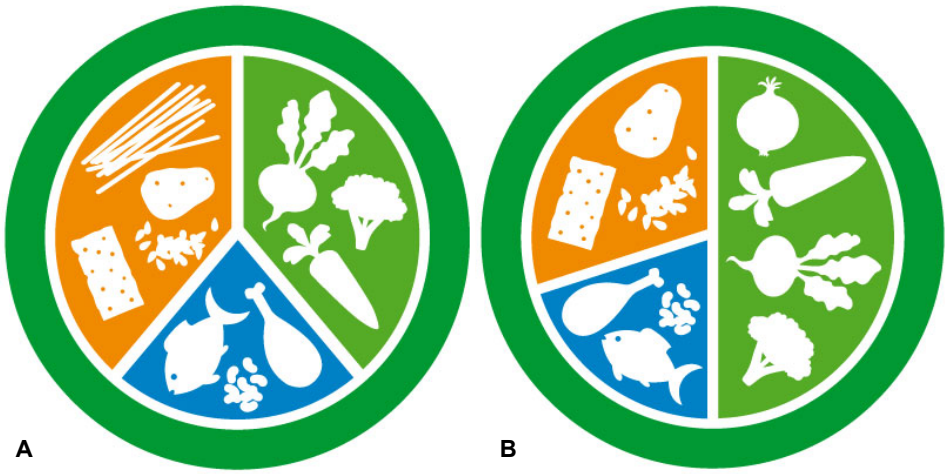

Figure 11. The Swedish dietary guidelines can be illustrated by these two plates.
The green part of the plate should be filled with vegetables and root-crops, the orange with potatoes, pasta, bread and grains, preferably wholemeal products. The blue part of the plate, the smallest part, contains sources of protein such as meat, fish, eggs, beans and lentils. The first plate (A) is recommended for persons who are active in their daily life. The proportions in the second plate (B) are recommended for persons who are sedentary. In the three-month weight loss study, the participants were recommended to use plate B, even though they were not sedentary, in order to create an energy deficit. Source: National Food Agency, Sweden.

Second meal study, hedonic hunger (Paper IV)

Twenty-six healthy overweight women were enrolled in this randomized, double blind, placebo-controlled meal intervention study. The study had a crossover design including two visits for each participant, at least one week apart (Figure 12). The two different conditions were: 5 g thylakoids (Appethyl®) or placebo, administered in a blueberry drink and served together with a typical Swedish high carbohydrate breakfast, consisting of yoghurt with muesli, one slice of white bread with butter, cheese and sweet pepper, coffee or tea.

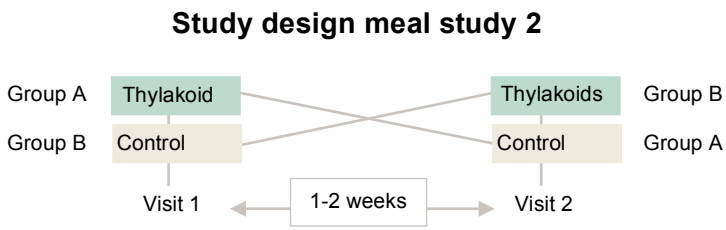

Figure 12. A cross-over study with two different conditions – thylakoid and control.
The participants were randomized to receive either thylakoids or control on the first visit and the other condition on the second visit in a balanced fashion.

Before the study began volunteers for the study answered a questionnaire about general health and another questionnaire about what kinds of sweets and snacks they preferred and how much they liked the different food items. The food questionnaire was later used as the basis for the choice of sweets and snacks for the study. Before the test days, participants also filled out a questionnaire about eating behaviour, a revised version of the Three-Factor Eating Questionnaire (TFEQ) - the TFEQ Revised 18-item Version 2 (TFEQ-R18V2)[103]. In this study, this version was used because it contains fewer questions and has therefore been recommended for research purposes. The TFEQ-R18V2 contains 18 questions to measure three types of eating behaviour: emotional eating, uncontrolled eating and cognitive restraint.

On test days, participants arrived fasted in the morning and filled out the first two pages of the VAS-questionnaire before breakfast. After the standardized breakfast, served with thylakoids or placebo, the participants filled out the questionnaire repeatedly at fixed time points (Table 4). The participants were allowed to leave between breakfast and lunch and were reminded via text messages to fill out the questionnaires. After a standardized lunch participants stayed on the premises until the end of the test day, filling out the questionnaire every hour until a snack buffet was served at 16:00 hours. Finally, the participants filled out the last pages of the questionnaire after the snack buffet - this time the questionnaire included one page of questions about liking.

Table 4. Test day schedule
The same schedule applied for both test days. Time points are calculated as minutes (min) after start of breakfast.

Time	Activity	Time point
07:30	Participants arrived at BMC	
07:45	VAS	Baseline
08:00	Breakfast with blueberry drink with/without 5 g thylakoids	0
08:15	VAS	15 min
09:00	VAS	60 min
10:00	VAS	120 min
11:00	VAS	180 min
12:00	VAS	240 min
12:45	VAS	285 min
13:00	Lunch	300 min
13:20	VAS	320 min
14:00	VAS	360 min
15:00	VAS	420 min
16:00	Snack buffet served. VAS	480 min
16:45	VAS after snack buffet	525 min

Strengths and limitations

Clinical studies

One concern that applies to all the clinical studies (Papers I-IV) was the relatively low number of participants. On the other hand, this was the only possibility, given the limited space and the low number of staff at our facilities. The positive side of performing a small study with a limited number of participants is that the same investigator and staff is taking care of all the participants in exactly the same way in the same facilities, ensuring standardized routines and treatment.

Another limitation was the relatively short duration of the weight loss studies. With duration of one year, it would have been easier to compare the results with results from other weight-loss studies. Again, there are positive sides to shorter study duration, such as better compliance.

In the studies where we analysed insulin repeatedly during the day (Papers I and III), one possible limitation was that we analysed insulin instead of C-peptide. Given that most intervals between blood samples in these studies were 30 minutes or more, C-peptide may have given more reliable results of insulin production compared to insulin itself, since C-peptide is produced in equal amounts but is not cleared by the liver and therefore has a longer half-life[161].

In the diet intervention studies (Papers II and III), the participants were given diaries to keep a daily record of food and activities. These records were helpful tools to judge compliance with the study regulations. This worked well in the two-month study since the participants were obliged to answer questions in the journal on a daily basis, but less so in the three-month study because writing in the diary was voluntary. When questioned about compliance, however, all participants rated their efforts very high. Nevertheless, incompliance with the diet or exercise can never be ruled out. For example, underreporting of food intake is a well-known phenomenon in appetite studies[159,162].

In both weight loss studies the treatment started on day one, which means that baseline measurements were taken fasting, before the first treatment. Therefore, there are no baseline curves for whole-day VAS ratings for comparison (Papers II and III), and no corresponding baseline curves for appetite regulating hormones (Paper III).

In paper III, cumulative body weight changes were used for the statistical calculations of body weight change after 3, 6, 9 and 12 weeks. This question would have been better addressed by ANOVA of the body-weight changes for each interval. This was performed after publication and reported in an erratum to the journal.

Animal study (paper V)

Intestinal fatty acid oxidation study

Ethics

The first part of the study was performed at the Department of Biology, Lund University, Sweden. The study protocol was approved by Lund University Ethical Review Committee for Animal Experiments. The second part of the study was performed by the Centre for Physiology and Bio-Imaging, Core Facilities, Sahlgrenska Academy at the University of Gothenburg, Sweden. This part of the protocol was approved by Gothenburg University Ethical Review Committee for Animal Experiments. The study was conducted according to the European Communities regulations concerning protection of experimental animals.

Statistics

Animals were randomized to achieve a similar distribution of body weight in both groups. Data were analysed for statistical significances as previously described for clinical studies (See Clinical studies, Statistics). In addition, linear regression was used to test for differences in slopes or intercepts.

Diets

The rats were fed either a thylakoid-enriched high-fat diet (1808 kJ/100 g) or a control high-fat diet (1930 kJ/100 g), with the energy distribution of 46 E% fat, 36E% carbohydrates and 18E% protein (Research Diets®, New Brunswick, NJ, USA). The diets were based on the D12451 high fat diet from Research Diets®, and the thylakoid-enriched diet contained 33% w/w spinach powder (Appethyl®, FutureCeuticals (Momence IL, USA). In the first experiment, the rats were given a fixed amount of 15 g thylakoid-enriched or control high-fat diet for consumption during the night and then had *ad libitum* access to control high-fat diet during the day. In the second experiment, the rats had *ad libitum* access to either thylakoid-enriched or control high-fat diet at all times.

Body composition

In the first experiment, adipose tissue was dissected and weighed, and in the second study body fat was assessed using a minispec LF110 whole body composition analyzer (Bruker's, Doubravnik, Czech republic).

Blood sampling

One hour before termination of the experiment, the rats were given 3 g thylakoid high-fat diet or control high-fat diet. The rats were killed by exsanguination one

hour later and in this process blood samples were taken from the heart. The blood was analysed for beta-hydroxybutyrate (BHB), triacylglycerol (TG), and non-esterified fatty acids (NEFA).

Intestinal segments

Three approximately 10 cm long segments were dissected from the intestine representing the duodenum, the proximal jejunum and the distal ileum respectively. The mucosa was immediately scraped off, using glass slides on ice, and frozen for later analysis.

Quantitative real-time polymerase chain reaction (q-RT-PCR)

Expression of genes involved in fatty acid oxidation and ketogenesis were analysed by q-RT-PCR. Each sample was run in triplicate and normalized against *actin, beta*.

Cytochrome P450 (CYP) activity

Livers were analysed for CYP-activity using probe reactions and subsequent analysis of metabolites.

Indirect caloric measurements

Telemetry devices (G2 E-Mitter, MiniMitter, Sunriver, OR, USA) were implanted intraperitoneally for measurements of core body temperature and activity. After 12 days of high-fat diet feeding with or without thylakoids, the rats were put individualy into sealed chambers (SOMEDIC Metabolic System, INCA, Hörby, Sweden) during 48 hours for measurement of oxygen consumption and carbon dioxide production.

Analysis of fecal fat

During the 48 hours of indirect calorimetry, fecal droppings were collected. The fat content was analysed using acid hydrolysis (Eurofins Food & Feed testing laboratory, Linköping, Sweden).

Strengths and limitations

Animal study

Strengths of animal studies are the stable environment with pathogen-free housing, exact times for lights on and lights off, stable temperature and humidity as well as control over external cues and manipulations. With animals it is possible to monitor feeding as well as body temperature and activity level at all times. Another advantage is the small interindividual variation between animals as long

as you use animals of the same strain, sex and age[202]. Finally, in studies with animals you can dissect any tissue for analysis after termination.

There are also limitations to using animals. Even though the rat is a commonly used laboratory animal[163], no matter how controlled, results from animal studies cannot entirely translate to human[164].

Using different sites for experiments was not entirely straightforward. The fixed amount of feed during the night in the first experiment assured that all thylakoid-treated rats ingested the same amount of thylakoids. In the second experiment, it was not possible to implement the same feeding schedule for practical reasons, making it harder to draw conclusions about the thylakoid effects on differences in food intake and body weight gain between the two experiments. Also, using different methods to analyse body composition in the two experiments affects the comparability of the results.

Results and discussion

In this section the results and discussion from the individual papers will be presented.

Paper I

Stenblom E-L, Montelius C, Östbring K, Håkansson M, Nilsson S, Rehfeld JF, Erlanson-Albertsson C. **Supplementation by thylakoids to a high carbohydrate meal decreases feelings of hunger, elevates CCK levels and prevents postprandial hypoglycaemia in overweight women.** *Appetite* (2013) 68:118-123.

Results

In this meal study with crossover design the participants visited the clinic at three different occasions, having a Swedish high carbohydrate breakfast test meal at each occasion. One breakfast was supplemented with a low dose thylakoids, one with a high dose thylakoids and the third was a control breakfast without thylakoids. Since there were no differences between the two doses of thylakoids, the resulting data were analysed as thylakoid and control.

When receiving thylakoids, the participants rated their feelings of hunger, thoughts of food and urge to eat lower compared to control. Since the scores for hunger, thoughts of food and urge to eat were similar, the three questions were analysed together and presented as one category, named hunger motivation. Hunger motivation was suppressed in the thylakoid condition compared to control from time point 180 minutes after breakfast and onwards. There was no significant difference regarding fullness.

Thylakoid supplementation affected levels of blood glucose and plasma insulin between 90 and 240 minutes after breakfast compared to control. In the control condition, after the first peak, the blood glucose declined fast and continued to decline below fasting levels from 60 minutes and onwards. With thylakoids, there was a second peak in blood sugar at 120 minutes, thus preventing the low blood sugar levels in the control condition ($p < 0.1$). Insulin levels followed the same

pattern as glucose levels, decreasing slower after thylakoid treatment, and here the difference between the thylakoid and control conditions was significant ($p < 0.01$).

Thylakoid treatment also increased blood levels of the satiety hormone CCK between three and four hours after breakfast, i.e. until lunchtime.

No differences in ghrelin levels were found between thylakoid and control using AUC and therefore no differences were reported in the publication. However, new calculations using Two-way RM ANOVA with time and treatment as fixed factors followed by a Bonferroni corrected multiple comparison test of all individual timepoints, show there was a significant time and treatment interaction for thylakoids compared to control between breakfast and lunch ($p < 0.05$) as well as significantly reduced ghrelin levels by thylakoids at time point 240 minutes after breakfast ($p < 0.05$), i.e. the last timepoint before lunch.

TNF-α levels decreased to a similar degree in both the thylakoid and the control condition between time point 0 and 240 minutes.

No adverse events were reported.

Discussion

This was the first study with thylakoids using VAS questionnaires to measure feelings of hunger and satiety. In the thylakoid condition, participants rated their hunger motivation slightly lower compared to in the control condition. The two curves started to diverge from 120 minutes and onwards, reaching near significance between 180 minutes and 240 minutes after breakfast, i.e. during the hour before lunch at which time hunger starts to increase, suggesting an effect of thylakoids on the secretion of appetite regulating hormones[58,165, 166].

CCK was indeed increased between 180 and 240 minutes after breakfast – the same time frame as the reduced hunger motivation. Given that CCK suppresses hunger[58], and hunger motivation[167], the reduced hunger motivation seen in this study may be associated with the increased levels of CCK. The release of CCK by thylakoids as shown in this study is in agreement with a previous meal study in humans, where thylakoids significantly increased CCK-levels at 240 and 360 minutes compared to control, the curves starting to diverge already from time point 120 minutes[155]. What is new in this study is that CCK increased after supplementation of thylakoids to a regular high carbohydrate breakfast, whereas the previous study tested a high-fat meal. Similar effects on CCK was also shown in a previous study in pig[154], and two studies in rodents[150,153]. The increased secretion of CCK can be explained by thylakoids causing reversible lipase inhibition, which slows down the digestion of dietary fat in the gastrointestinal canal[135,150].

Reduced ghrelin levels may also participate in mediating the reduced hunger motivation by thylakoids seen in this study. New statistical calculations of ghrelin levels measured in this study show that thylakoid treatment did in fact affect ghrelin secretion over time and that there was a difference between the thylakoid and control conditions specifically regarding ghrelin secretion 240 minutes after breakfast. This finding is supported by previous studies with thylakoids showing similar results on ghrelin secretion[154,155]. However, this is the first time we have demonstrated reduced ghrelin secretion after ingestion of thylakoids together with a regular high carbohydrate breakfast.

Blood glucose was slightly higher in the thylakoid condition from 90 minutes and onwards due to a second peak at 120 minutes. Instead of slowly and continuously decreasing, like it did in the control condition, the blood sugar level stayed above fasting level in the thylakoid condition. Given that low blood sugar levels are known to induce hunger[30,48,168] , thylakoid treatment may have prevented the onset of hunger motivation seen in the control condition from 120 minutes and onwards through counteracting low blood sugar levels. The second peak in blood glucose seen in the thylakoid condition may be due to a slower absorption of glucose compared to control. A previous study has shown reduced passage of macronutrients over the intestinal wall *in vitro* after addition of thylakoids, including methyl-glucose, consequently making the nutrient uptake in the intestine slower[152]. Thylakoids also reduce gastric emptying, which may contribute to the slowed digestion and absorption of nutrients[203].

In response to the increased blood glucose in the thylakoid condition, insulin levels were also increased between 90 and 240 minutes. Thylakoids increase secretion of GLP-1 (paper III), known to increase glucose dependent insulin secretion[29]. Consequently, one may speculate that without the incretin effect, the blood sugar would have been even higher in the thylakoid group at the later time points. GLP-1 has also been shown to suppress food motivation[169], which may have contributed to the reduced ratings for hunger motivation in this study.

In conclusion

The hypothesis was that thylakoid supplementation with a high carbohydrate meal would cause delayed glucose absorption, affect postprandial levels of appetite regulating hormones, and reduce feelings of hunger, which was indeed the case. Thylakoids did not however affect ratings of fullness or blood levels of TNF-α compared to control.

Paper II

Stenblom E-L, Montelius C, Erlandsson D, Skarping L, Fransson M, Egecioglu E, Podgórski K, Erlanson-Albertsson C. **Decreased urge for palatable food after a two-month dietary intervention with green-plant membranes in overweight women.** *J Obes Weight Loss Ther* (2014) 4:4

Results from the two month diet-and-exercise intervention study

All participants lost body weight as well as body fat significantly. There were no differences between the thylakoid (green-plant membrane) and control groups either at the start, the end or during the study.

In the thylakoid-treated group, the participants' feelings of hunger decreased between the first and last days of the study. There was no such change over time in the control group. There were no differences between the thylakoid and the control group on either the first or last days. Fullness was not affected.

Ratings regarding urge for chocolate decreased in the thylakoid group between the first and last days of the study. In contrast, the control group displayed no difference over time. Comparing the urge for chocolate between the thylakoid group and the control group, there was no difference on the first day, but there was a development over time towards a tendency to lower urge for chocolate in the thylakoid group on the last day compared to control.

The urge for a sandwich or a cinnamon bun was not significantly altered between the first and last days of the study in either of the groups but there was a tendency towards reduced urge over time within the thylakoid group.

Regarding metabolic parameters, there were no differences between the thylakoid and the control groups at baseline regarding any of the anthropometric parameters - body weight, waist circumference, hip circumference and body composition – nor any differences regarding the fasting blood samples of glucose, insulin, HbA1c, TAG, total cholesterol, LDL-cholesterol, ApoB1 and leptin. Over time, during the two-month diet intervention study, the overall metabolic profile, based on all anthropometric meaurements and blood values, improved significantly in the thylakoid group compared to control, with significant reductions in the thylakoid group for leptin, hip circumference, LDL-cholesterol, Apo B1 and fasting blood glucose compared to control.

No side effects were reported.

Four participants were excluded from the analyses, due to incompliance with the diet and exercise regime, based on the information in the diaries.

Results from the follow-up study, five weeks later (not published)

The follow-up study showed that the participants in the thylakoid group continued to lose a significant amount of body weight during the five weeks after the diet intervention[200] (Table 5). Body composition analysis showed a reduced body fat mass and reduced body fat percentage. Specifically, the thylakoid group lost visceral fat mass, the visceral fat mass percentage of body weight being significantly reduced. This was reflected in a slightly reduced waist circumference. The hip circumference was also reduced in the thylakoid group. In contrast, in the control group body weight, body fat, waist- and hip circumferences were not altered. Lean mass was not affected in either of the groups.

Total cholesterol, LDL and HDL increased somewhat during the follow-up period in the thylakoid group but the values did not reach above baseline values. Fasting glucose, HbA1c, insulin and TAG were unaltered.

Table 5. Follow-up study

During the five weeks between the intervention and the follow-up, the participants did not follow any diet and received no recommendations regarding activity.

Parameter	Group	Visit 5, end of study	Follow-up, 5 weeks later	Difference	*p*-value within group	*p*-value between groups
Body weight (kg)	Thylakoid	67.8 ± 7.7	66.7 ± 8.3	-1.1 ±1.1	0.01	0.27
	Control	71.7 ±5.3	71.4 ±5.8	-0.3 ±1.8	0.49	
Body fat (kg)	Thylakoid	21.9 ± 4.7	21.1 ± 5.3	-0.7 ± 1.2	0.08	0.21
	Control	23.8 ± 3.	23.7 ± 4.0	-0.1 ± 1.0	0.57	
Body fat (%)	Thylakoid	32.4 ± 3.5	31.6 ± 4.0	-0.8 ± 1.4	0.06	0.15
	Control	33.4 ± 3.0	33.4 ± 3.5	-0.07 ± 1.0	0.9	
Visceral fat (kg)	Thylakoid	10.9 ± 2.8	10.4 ± 3.1	-0.5 ± 0.8	0.06	0.10
	Control	12.0 ± 1.8	12.0 ± 2.2	0.0 ± 0.6	1	
Visceral fat (%)	Thylakoid	29.6 ± 4.2	29.7 ± 6.5	0.1 ± 4.0	0.01	0.27
	Control	30.9 ± 3.2	30.9 ± 3.4	0.0 ± 1.3	0.02	
Waist (cm)	Thylakoid	77.9 ± 8.2	77.1 ± 8.1	-0.8 ± 1.0	0.03	0.90
	Control	82.6 ± 6.4	81.8 ± 6.2	-0.8 ± 2.2	0.22	
Hip (cm)	Thylakoid	98.4 ± 3.8	97.5 ± 3.7	-0.9 ± 1.1	0.03	0.49
	Control	100.5 ± 3.8	99.9 ± 4.7	-0.6 ± 2.2	0.45	
Cholesterol (mmol/L)	Thylakoid	5.2 ± 1.1	5.9 ± 1.3	0.7 ± 0.5	0.01	0.01
	Control	5.1 ± 0.6	5.2 ± 0.7	0.2 ± 0.4	0.07	
LDL (mmol/L)	Thylakoid	3.1 ± 1.0	3.6 ± 1.1	0.4 ± 0.4	0.01	0.02
	Control	3.1 ± 0.5	3.0 ± 0.5	-0.01 ± 0.3	0.8	
HDL (mmol/L)	Thylakoid	1.8 ± 0.4	1.9 ± 0.5	0.2 ± 0.2	0.01	0.15
	Control	1.7 ± 0.3	1.8 ± 0.4	0.1 ± 0.2	0.10	

After the follow-up, VAS-scores from the two-month intervention period as well as from the follow-up were re-analysed[201] (not included in the paper). In addition, scores from the eating behaviour questionnaire were compared between baseline, the end of the study and the follow up. The results show that there were no differences in individual cravings between the thylakoid and control groups on the first day of the study. During the study however, individual cravings decreased in the thylakoid group ($p < 0.05$) but not in the control group, resulting in a difference between the thylakoid group and the control group on the last day of the study ($p < 0.05$), individual cravings being lower in the thylakoid group compared to control after the two-month diet-and-exercise intervention compared to before. At the follow-up five weeks later there was still a tendency towards a lower craving in the thylakoid group compared to control ($p < 0.09$).

The eating behaviour questionnaire data revealed a significant increase in cognitive restraint in the control group during the two-month diet-and-exercise intervention ($p < 0.05$). In contrast, the thylakoid group did not experience increased cognitive restraint. After the study however, at the follow-up, both groups showed significant increase in ratings for cognitive restraint compared to baseline ($p < 0.05$ respectively). Concerning uncontrolled eating, the control group showed a significant decrease in this category both during the two-month intervention ($p < 0.05$) and at the follow-up, five weeks after the intervention ($p < 0.05$). In contrast, there were no differences in the thylakoid group regarding uncontrolled eating. Regarding the emotional eating category, there were no significant differences for either the control group or the thylakoid group during the intervention or the follow-up.

Discussion

Combining energy restriction with an increased activity level will cause accumulating energy deficit and a subsequent weight loss[170]. Consequently, in this two-month diet-and-exercise intervention study, we found that the thylakoid-treated participants and the control group participants all lost body weight and body fat, and they did so to a similar degree. The expected thylakoid effect was not seen in this context. We suggest that this is due to the energy-restricted diet that left no room for possible treatment effects on energy intake and macronutrient choice.

Despite the body weight loss, the body fat loss and decreased leptin levels in this study, participants in the thylakoid group experienced reduced feelings of hunger as well as a reduced urge for chocolate over time, in contrast to the common body reaction to weight loss, which is increased cravings and an increased appetite[86].

Analysis of the eating behaviour questionnaire[201] supports the theory that the thylakoid-treated participants followed the energy-restricted diet with less effort

compared to control. Comparing eating behaviour scores before and after the intervention showed that the control group experienced more restricted behaviour and less uncontrolled behaviour during the restricted diet while there were no differences in the thylakoid group, suggesting that the thylakoid-treated participants found it easier to follow the energy-restricted diet, possibly due to the reduced hunger and cravings discussed above.

With weight loss and body fat loss follows improvement in metabolic parameters[133]. The reason why the metabolic profile, based on all anthropometric meaurements and blood values, was significantly more improved in the thylakoid group compared to control, even though the body weight loss was not, is not known. The possibility cannot be excluded that the participants in the thylakoid group lost more body fat, possibly due to increased fat oxidation by thylakoids, a mechanism induced by thylakoids suggested in Paper V. Supporting this theory are the reduced leptin levels in the thylakoid group that may reflect reduced body fat stores[23]. A reduced body fat mass could explain the improvement of the hip circumference, LDL-cholesterol, Apo B1 and fasting blood glucose in the thylakoid-treated participants.

Results from the follow-up five weeks after the end of the diet intervention show that the thylakoid group continued to lose weight after the intervention and showed a significantly reduced body fat mass compared to control[200]. However, there was no corresponding improvement in metabolic parameters at the follow-up. This may be explained by the change of diet. During the diet intervention, the prescribed diet had a fixed macronutrient content. After the intervention, the participants were free to eat what they wanted, which may have resulted in an alteration of macronutrient content between the groups. Studies have shown that diets with different macronutrient composition have different effects on metabolic risk factors, even if they both cause body weight loss[133].

In conclusion

Both the thylakoid and the control groups reduced their body weight and body fat to a similar degree. The hypothesis was that thylakoid supplementation would cause a greater weight reduction compared to control, which it did not. However, daily ingestion of thylakoids improved metabolic risk factors compared to control. Thylakoids also reduced feelings of hunger and cravings over time compared to control and reduced the experience of cognitive restriction compared to control. Thereby, in an everyday setting, thylakoids may facilitate body weight reduction through increased compliance to a restricted diet.

Paper III

Montelius C, Erlandsson D, Vitija E, Stenblom E-L, Egecioglu E, Erlanson-Albertsson C. **Body weight loss, reduced urge for palatable food and increased release of GLP-1 through daily supplementation with green-plant membranes for three months in overweight women.** *Appetite* (2014) 81:295-304.

The results from this three-month study will be presented as follows: First the results on body weight and anthropometric measurements over time, then the results from the meal study performed on the first day of the study, and finally the results from the last day meal study. This is followed by a discussion of first the long-term results and then the meal study results.

Three-month study, long-term results

The primary outcome was 12-week body-weight loss, which was significantly greater in the thylakoid group compared to control. Comparing body-weight changes for each three-week interval (3, 6, 9 and 12 weeks), the difference between the thylakoid group and the control group was near significant, as reported in an erratum.

Also performed after publication and reported in the mentioned erratum, were analyses of plasma lipids that showed that LDL cholesterol was not significantly reduced in the thylakoid group at any specific time point even though there was a significant effect of treatment for the whole period. Subsequent calculation of tAUC confirmed that LDL-cholesterol was indeed lower in the thylakoid group over the course of the entire study compared to control ($p < 0.05$). Thylakoid-treatment also decreased total cholesterol compared to control.

Fat free mass, body fat mass as well as plasma leptin decreased in both groups during the study and there were no differences between the groups. Fasting glucose, insulin, HDL-cholesterol and triglycerides did not change during the study in either of the groups.

No adverse events were reported.

Two participants were excluded during the study due to incompliance and were therefore not included in the final analyses.

Longterm results not incuded in the paper

After the paper was published, fecal samples were analysed for fat content and changes in microbiota[203]. There were no differences between the groups regarding fat content of the fecal samples. Thylakoids did however affect the amount and composition of the gut microbiota, increasing the total number of 16S rRNA gene copies in the thylakoid group before and after the study, specifically genes

belonging to the *B fragilis* group. No significant differences were shown in the control group.

Results, first day meal study

Thylakoid-treatment reduced the first peak in both blood glucose and plasma insulin compared to control. In addition, glucose peaked later (30 minutes) compared to control (15 minutes). GLP-1 levels were similar in the thylakoid group and control group at baseline. After the first treatment, the secretion of GLP-1 increased significantly more in the thylakoid group compared to control. There were no differences between the groups at any specific time point. Ghrelin levels were not affected.

Food cravings, investigated using VAS-questionnaires, were affected by thylakoid treatment compared to control already on the first day of the study. In the thylakoid group ratings for the urge for sweet and chocolate were significantly lower compared to control in the afternoon. Sensations of hunger and satiety as well as urge for a sandwich or chips were similar in both groups.

Total caloric intake during the day was not affected.

Results, last day meal study

Similar to the first day of the study, the glucose peak appeared later in the thylakoid group (30 minutes) compared to control (15 minutes). Levels of blood glucose and plasma insulin were similar. GLP-1 levels were similar at baseline, but after treatment thylakoids increased GLP-1 secretion during the first hour after breakfast compared to control, the first peak maximum value significantly higher than control. Ghrelin levels were not affected.

Similar to day one, the urge for sweets and chocolate was suppressed by thylakoids compared to control during the afternoon. On the last day however, the effect was evident already at the time points preceeding lunch. Sensations of hunger and satiety as well as urge for a sandwich or chips were similar in the thylakoid and control groups.

Total caloric intake during the day was not affected.

Discussion long term changes

Both groups lost body weight during the study, probably due to the weight loss regime, which was easy to understand and to follow. We propose that eating only three times a day, making healthy food choices and avoiding energy-dense palatable food as well as sweet beverages and alcohol, together with daily activity promotes an energy deficit and subsequent weight loss. Empowering the participants to structure and organize their eating behavior and to take control over the eating pattern and food choices is the key to successful weight loss and

maintenance[86]. In addition, daily ingestion of thylakoids in combination with prevalent recommendations for leading a healthy lifestyle caused a greater body weight loss compared to control. We suggest that this may be due to the increased postprandial secretion of GLP-1, associated with reduced wanting for food as well as reduced food intake[169,171] and proposed to regulate intermeal intervals[38]. Combined, the known effects of GLP-1 may have facilitated the implementation of the dietary recommendations in the present study.

As expected, accompanying the greater body weight loss in the thylakoid group was a reduction in both LDL and total cholesterol compared to control[133]. Fat free mass, body fat mass or leptin levels were reduced to similar extent in both groups. The fact that fasting blood glucose, insulin, HDL-cholesterol and triglycerides did not change in any of the groups is probably due to the values being normal at baseline, leaving little room for significant improvement.

There were no differences between the groups regarding fat content of the fecal samples, suggesting that all dietary fat is indeed absorbed in the intestine. These results are in line with previous studies showing that thylakoids are digested and absorbed in the intestine and the lipase/colipase inhibition therefore reversible[150].

Daily thylakoid ingestion for three months affected the amount and composition of the gut microbiota in a positive direction[203]. This may be an important finding because altered gut microbiota is associated with both obesity and type 2 diabetes[124,125,172] and diversity is important for metabolic health[128]. In addition, gut microbiota can affect secretion of appetite regulating hormones[173,174,61] as well as affect energy homeostasis[124]. Both of these mechanisms may have contributed to the thylakoid effects demonstrated in this study.

Discussion meal studies

Regarding blood glucose, the reduced first peak seen in the thylakoid group on day one may reflect a slower uptake of macronutrients over the intestinal wall, an effect of thylakoids previously described[152]. This is also the probable cause for the glucose peak appearing later compared to control. The reduced insulin peak mirrors the reduced glucose values. The augmented increase in GLP-1 secretion may be a result of the prolonged time for fat digestion[150,60], as well as the slower absorption[152], resulting in nutrients being delivered all the way to the lower small intestine. Nutrients reaching as far as the lower small intestine are known to cause elevated plasma concentrations of GLP-1[60,45]. The fact that GLP-1 peaked later in the thylakoid group (30 minutes) compared to control (15 minutes) on both test days supports this hypothesis.

The augmented first peak GLP-1 secretion compared to control on the last day suggests that there is an accumulative effect of thylakoid treatment over time. This amplification of effects is reflected in the VAS-ratings, seeing that the reduced

cravings in the thylakoid group had an earlier onset on the last day compared to the first day. Both on the first and last days of the study, participants in the thylakoid group rated their urge for sweets and chocolate lower compared to control during the afternoon, but on the last day, this phenomenon started already before lunch. We suggest that these effects o GLP-1 secretion and cravings may be due to the altered gut microbiota (see discussion above).

In conclusion

This study supported the hypothesis that thylakoid supplementation improves body weight and metabolic parameters associated with obesity as well as facilitate weight-loss, possibly due to increased GLP-1 and reduced cravings compared to control.

Paper IV

Stenblom E-L, Egecioglu E, Landin-Olsson M, Erlanson-Albertsson C. **Consumption of thylakoid-rich spinach extract reduces hunger, increases satiety and reduces cravings for palatable food in overweight women.** *Appetite* (2015) 91:209-219.

Results

In this crossover meal study, ingestion of thylakoids with breakfast reduced ratings of hunger and increased ratings of satiety during treatment days compared to the control condition. Feelings of hunger were attenuated in the thylakoid condition at the time points directly before lunch and snack buffet, while satiety-scores were augmented one and two hours after breakfast as well as before the snack buffet.

In the thylakoid-treated condition the participants rated their wanting lower during the whole day for all three categories of palatable foods (salty, sweet, and sweet-and-fat) separately and together (total wanting) compared to the control condition. The treatment effect of thylakoids on wanting sweet-and-fat snacks was positively correlated to the treatment effect on intake of sweet-and-fat snacks. A similar correlation was shown for wanting all snacks and intake of all snacks. The treatment effects of thylakoids on wanting sweet-and-fat snacks as well as total wanting for snacks were also positively correlated with emotional eating scores.

The difference in food intake from the snack buffet between the thylakoid and control days was not statistically significant. There was however a tendency towards reduced intake of salty snacks in the thylakoid-treated condition.

Thylakoids reduced liking for sweet foods after consumption compared to control. Liking for all snacks together was also reduced, but not significantly. The treatment effect of thylakoids on liking for sweet foods was positively correlated to the treatment effect on intake of sweet snacks. Similar correlations were shown for sweet-and-fat snacks and all snacks.

Of the 32 women who volunteered, 26 were enrolled and 22 completed the study. No side effects were reported.

Discussion

This crossover meal study showed for the first time immediate effects of thylakoid treatment on satiety as well as hunger and cravings for different categories of palatable food compared to a control condition. These findings are supported by previously demonstrated results included in this thesis: reduced hunger motivation (Paper I), reduced hunger and urge for chocolate after two months daily ingestion of thylakoids (Paper II) and reduced urge for sweet and chocolate both on the first treatment day and after three months daily ingestion of thylakoids (Paper III). In addition, the effects of thylakoids increasing fullness and reducing hunger as well as the desire for something salty and savoury have been repeated[175].

In this study, feelings of hunger were attenuated in the thylakoid condition at the timepoints preceeding lunch and the afternoon snack buffet. Satiety-scores were augmented during two hours after breakfast as well as before the snack buffet. It is the first time a promotion of satiety is observed during the whole day. Taken together, these data suggest that thylakoid-ingestion promotes a prolonged intermeal interval. This is important because eating frequency and snacking frequency are associated with overeating and obesity[82,83]. Food-cravings (wanting) were also affected during the whole day in the thylakoid condition, thylakoids acting to reduce wanting for salty, sweet, sweet-and-fat snacks and all snacks together compared to the control condition. This is an important finding because wanting is also associated with overeating and obesity[17,72,73], as well as binge eating[176].

Liking, which was measured for the first time in a study with thylakoids, was reduced for sweet after consumption. This reduction was positively correlated with a reduction in intake of sweet snacks. Liking is involved in establishing the reinforcing value of foods, in the learning process to want something[67]. However, it is possible for wanting to become more important for ingestive behaviour than liking, suggesting that reducing liking for palatable foods is not as important as reducing wanting for palatable foods if the desired outcome is reduced food intake. The effect of thylakoids on liking needs to be examined further.

The correlations between the treatment effects on wanting and liking and a reduced snack intake are also novel findings. Furthermore, the correlation between

the treatment effect on wanting and higher emotional eating scores is another novel finding, suggesting that participants with higher perceived emotional eating behavior have greater treatment effect of thylakoids on wanting, sweet-and-fat snacks in particular. Since emotional eaters typically choose sweet-and-fat foods, such as chocolate and cake, in response to negative affect[107], the current findings are very interesting. Studies have also shown that especially women and children choose fat and sweet foods, such as chocolate or ice cream, as comfort foods[177,78]. However, the findings need to be substantiated in studies including a larger group of emotional and non-emotional eaters.

In order to study food cravings in a normal environment and avoid external interference with the VAS-ratings[159], this study was performed outside of the laboratory setting in an attempt to mimick everyday life. Consequently no blood samples were taken, even though analysis of appetite regulating hormones would have substantiated the discussion. Nevertheless, the reduced wanting, hunger and liking as well as the increased feelings of satiety are probably due to altered secretion of appetite regulating hormones by thylakoids, as shown in previous studies, affecting areas in the brain that are associated with hunger and reward. Reducing wanting and hedonic hunger is crucial to reduce overeating, which is a major contributor to overweight and obesity.

Between breakfast and lunch, the participants were allowed to choose location as long as they could keep a low activity level and refrain from eating. Consequently, the participants were in their normal environment and not in a sterile and uninspiring laboratory with a cannula in their arm, hurrying back and forth between the nurses' station (for blood sampling) and their seat (to rate their appetite-related feelings). The change of environment in this study probably affected the VAS-ratings, seeing that the greatest differences between thylakoid and control occurred between breakfast and lunch, when they were subjected to environmental cues and temptations that occur in our everyday environment.

Despite the effects of thylakoids on wanting there were no significant differences in food intake between the groups. This may be explained by the unnatural situation that is bound to affect the eating behaviour - the results regarding food intake from this study may therefore not be representative for the free-living situation[86].

In conclusion

The hypothesis was confirmed that thylakoid supplementation would increase satiety and reduce hunger as well as wanting and liking for palatable food. The treatment effects were also associated with eating behaviour scores.

Paper V

Results

Stenblom E-L, Egecioglu E, Montelius C, Ramachandran D, Bonn B, Weström B, Mansouri A, Langhans W, Erlanson-Albertsson **C. Dietary thylakoids reduce visceral fat mass and increase expression of genes involved in intestinal fatty acid oxidation in high-fat fed rats.** *Am J Physiol Regul Integr Comp Physiol* (2016) 311: R618-R627.

This study included two sets of experiments. In the first experiment, the rats had limited access to either thylakoid or control food during the night and *ad libitum* access to control food during the day. In this setting, the total energy intake during 24 hours was similar between the thylakoid rats and the control rats. In the second experiment, all rats had *ad libitum* access to either thylakoid diet or control diet at all times. In this setting, thylakoid-enriched high-fat diet reduced food intake compared to control high-fat diet.

In both experiments, the body weight of the thylakoid-fed and the control rats were similar. However, in the second experiment there was a reduced body weight gain in the thylakoid treated rats compared to control, as well as a significant time and treatment interaction for the body weight development over time. On day 12, the thylakoid treated rats had gained 17.5% less weight compared to control. There were no differences in body weight gained per ingested calorie.

In the first experiment, fat pads were dissected and weighed, showing that thylakoid-feeding reduced visceral fat pad mass by 25% compared to control. In the second experiment, body fat mass was estimated using body composition analysis (BCA), revealing a reduced total body fat in the thylakoid-fed rats compared to control, however not statistically significant.

Expression of intestinal fat oxidative genes, examined in the first experiment, was enhanced in the jejunum of the thylakoid-fed rats compared to control: *Hmgcs2, Cpt1a* and *Fat/Cd36*. There was also a tendency to increased *Lcad*, whereas no effect was observed on *Fabp2* and *Ppara*. No differences were seen in the duodenum or ileum. In the liver, a reduced expression of *Ppara* was noted.

During the indirect calorimetry, performed in the second experiment, thylakoids reduced the respiratory quotient, but did not affect whole body oxygen consumption, core body temperature, activity levels or fecal fat content.

There were no differences regarding plasma levels of BHB, TG or NEFA and no differences in liver weights or CYP-activity.

Discussion

In this study, including two experiments of two weeks each, rats were either fed a thylakoid-enriched high-fat diet or a control high-fat diet to study the effects on fat metabolism in the intestine as well as whole body energy expenditure.

We showed, for the first time, that ingestion of thylakoids induced enhanced expression of intestinal fat oxidative genes in the jejunum: *Hmgcs2*, *Cpt1a*, *Fat/Cd36* and *Lcad*, key enzymes of fatty acid transport, fatty acid oxidation and ketogenesis. Another novel finding was a reduced respiratory quotient, indicative of increased fatty acid oxidation. Increased fatty acid oxidation caused by thylakoids is an important finding since decreased ability for fatty acid oxidation may contribute to overweight and obesity[24,123].

Thylakoid-feeding also reduced body fat mass and visceral fat depots; a desirable capacity considering accumulation of abdominal fat is associated with adipose tissue inflammation[178], increased cardiovascular risk[14,179] and hepatic steatosis[180,181]. The reduced body weight gain in the thylakoid group compared to control suggests that had the experiment been longer than two weeks, a difference in body weight would have developed over time.

Thylakoid-enrichment of a high-fat diet reduced caloric intake compared to a control high-fat diet in rats with *ad libitum* access to food. Food intake was consistently lower in the thylakoid group compared to control, thus ruling out palatability as a factor. Palatability of thylakoids has been tested in conjunction with earlier studies and shown to have no effect on appetite suppression[153]. The reduced food intake may either be caused by decreased hunger and increased satiety due to altered appetite-regulating hormones by thylakoids as shown previously[150,153], and/or increased fatty acid oxidation, found to be associated with reduced food intake[182,183,184].

Thylakoids did not cause steatorrhea. Hence, the body weight and body fat loss was not an effect of energy lost in faeces.

Taken together, the findings from this study suggest that daily intake of thylakoids together with a high-fat diet increases fatty acid oxidation in the intestine, reduces food intake and visceral adipose tissue mass. The mechanism may be increased activation of PPAR-α-dependent signalling, known to increase fat oxidation. Previous studies have shown that when when animals are fed a high-fat diet fatty acid oxidation and ketogenesis is induced through increased activation of PPAR-α-dependent signaling in the intestine and liver, increasing the expression of enzymes that promote fatty acid oxidation, such as FAT/CD36 and CPT1[184,185,186]. It also stimulates ketogenesis through HMG-CoAS2[187]. Our findings suggest that thylakoids enhance this effect even though we failed to demonstrate an increased expression of PPAR-α in this study. It remains to be elucidated whether there is

instead an increased protein expression corresponding to the genes enhanced in this study and thereby establish if the thylakoid-effects are mediated through PPAR-α.

In conclusion

This study demonstrated that thylakoid supplementation increases intestinal fatty acid oxidation and shift nutrient-substrate utilisation towards fat, and that this is associated with reduced body fat accumulation. Energy expenditure and fecal fat content were not affected, suggesting that the reduced food intake contributed to the body fat reduction, possibly caused by increased satiety.

General discussion

In this work we have shown that ingestion of thylakoids brings several beneficial effects, originating in the intestine, that may help prevent the development of overweight and obesity as well as associated comorbidities. The included studies demonstrate that thylakoids increase secretion of the satiety hormones cholecystokinin and GLP-1, reduce secretion of the hunger hormone ghrelin, reduce feelings of hunger, increase satiety and reduce food-cravings (Figure 13). Through these effects, thylakoid treatment targets food choice and overeating of palatable foods, with the potential to decrease energy intake through reduced eating frequency, snack frequency in particular, increasing the intermeal interval, thus promoting body weight loss and body fat loss. Thylakoid supplementation also reduces blood lipid levels and increases fatty acid oxidation, adding to improved metabolic health.

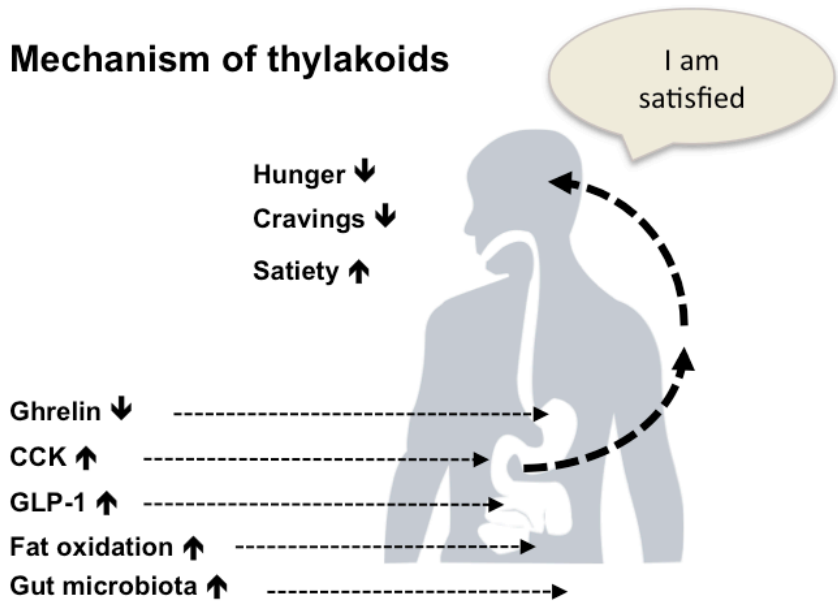

Figure 13. Effects of thylakoid supplementation with the diet.
Thylakoids reduce ghrelin and increase CCK and GLP-1, leading to reduced hunger, increased satiety and reduced cravings for palatable food. Increased fat oxidation may also lead to increased satiety, and increased gut microbiota may further increase the secretion of GLP-1.

The studies included in this thesis exclusively involved female participants. Thylakoid effects have also been studied in mixed groups and males, showing effects on hunger, fullness, desire for food, food intake, anthropometric and metabolic parameters[155,175,204].

Hunger, satiety, cravings, appetite regulating hormones

Four studies included in this thesis (Papers I, II, III, IV) investigated the effects of ingestion of thylakoids on subjective feelings of hunger, satiety as well as cravings for palatable foods. Three of the studies (Papers I, II, III) also examined the effect of thylakoids on appetite regulating hormones.

Paper I

In the first meal intervention study, a crossover study with three conditions (high dose thylakoids, low dose thylakoids and control) we showed that ingestion of thylakoids with a high-carbohydrate breakfast reduced hunger motivation before lunch. The reduced hunger motivation may be caused by altered levels of appetite regulating hormones originating from the gastrointestinal tract[51,38], given that at the same time blood samples revealed increased levels of the satiety hormone CCK and reduced levels of the hunger hormone ghrelin in the thylakoid-treated condition compared to control. Ingestion of thylakoids also caused increased levels of glucose and insulin between 90-240 minutes after breakfast probably due to altered intestinal absorption[152], thus preventing postprandial hypoglycaemia, which is also known to stimulate hunger[42].

Paper II

In the second study, the two-month diet-and-exercise intervention with meal studies on the first and last days, thylakoids reduced feelings of hunger between the first and last days of the study while no effect was seen in the control group. In this study, the VAS-questionnaire also included questions about cravings for palatable foods. The results showed that the participants in the thylakoid-treated group experienced reduced cravings for chocolate and carbohydrate snacks on the last day compared to the first day. These are important findings since the normal response to weight loss is increased hunger and cravings due to decreased leptin and a compensatory increase in ghrelin[86]. Counteracting the compensatory increase in hunger and cravings, thylakoids may be useful to prevent snacking in between meals and the associated weight gain. Thylakoids may also help people stick to an energy-restricted diet and thereby facilitate weight loss.

The suggested mechanism for the reduced hunger and cravings in this study is altered secretion of appetite regulating hormones from the gastrointestinal tract, as

demonstrated in other studies (Papers I and III). However, since these were not analysed in this study, no clear conclusions can be drawn on the mechanism.

Paper III

In our next study, the three-month diet-and-exercise intervention study, immediate effects of thylakoid ingestion included reduced cravings for sweets and chocolate. The reduced cravings may be caused by the increased secretion of intestinal GLP-1 seen in the thylakoid group already on the first day, given that GLP-1 is known to reduce wanting for palatable food[169]. This pattern of results was repeated on the last day of the study with even greater differences between the thylakoid and control groups.

We suggest that the increased secretion of GLP-1 induced by thylakoids is caused by a delayed digestion of dietary fat in the intestine[150], which prolongs the time and distance for absorption of fat, thereby stimulating enteroendocrine cells further down the intestine to secrete satiety hormones[60,45]. The additionally increased secretion of GLP-1 seen after three months daily ingestion of thylakoids may be caused by altered gut microbiota, given that the gut microbiota has previously been shown to affect secretion of appetite regulating hormones[173,174,61]. Indeed, fecal samples from the participants, analysed at a later date, showed increased amount of and diversity in the microbial flora[203], the opposite effect of obesity och metabolic disease[124,173,174,128,61].

Paper IV

The previously shown effects of thylakoids on GLP-1 and ghrelin made us want to focus on feelings of hunger and wanting in the next study. Therefore, in the second crossover meal study, we focused on hunger, satiety, wanting and liking for palatable foods and their association with eating behaviour. For the participants to be able to fully concentrate on these questions, no blood samples were taken in this study. We suggested that by avoiding the laboratory setting and the stressful blood-sampling situation, the participants could relate more easily to their normal everyday feelings about food and snacks. In addition to the altered study design, the food products tested were carefully chosen and pictures and questionnaires changed accordingly which may be important for the results.

For the first time, we could show that thylakoids increased ratings for satiety, reduced liking for sweet after consumption and reduced intake of salty snacks in the thylakoid-treated condition. We also replicated the immediate effects of thylakoids on cravings for palatable foods from the previous three-month study (Paper III), but in this study thylakoids reduced cravings for all the tested categories throughout the day: salty, sweet, sweet-and-fat and all snacks together. We also replicated the immediate effect on hunger from the first meal study (Paper I) but in this study, the effect was evident not only during one hour, but throughout

the day. Finally, we could show that the treatment effect of thylakoids on wanting and liking for sweet-and-fat snacks and all snacks was positively correlated with the treatment effect on intake of the same snacks as well as scores for emotional eating behaviour.

Because appetite-regulating hormones were not analysed in this study, no clear conclusions can be drawn as to the mechanism for the thylakoid-induced effects in the study. However, the suggested mechanism for the increased satiety, reduced hunger, reduced cravings and liking in this study is altered secretion of appetite regulating hormones, as demonstrated in Papers I and III.

In conclusion

We suggest that the demonstrated effects on hunger, satiety and cravings for palatable food in Papers I-IV are caused by altered secretion of appetite regulating hormones from the gastrointestinal canal, CCK, GLP-1 and ghrelin, acting on hunger- and reward related areas of the brain to reduce hunger, increase satiety and reduce cravings for palatable food and reward[52,56,58,169,171]. We suggest that the altered secretion of appetite regulating hormones seen in this work is caused by a reduced rate of digestion and absorption of macronutrients in the intestine. Previous studies have shown that thylakoids reduce lipase/co-lipase activity reversibly, making lipolysis slower[150], but still complete[148]. In addition, thylakoids cover the inside of the intestinal wall and slow down the absorption of ovalbumin and methyl-glucose[152]. The reduced absorption rate of macronutrients causes them to stay longer in the intestine and consequently reach further down the gastrointestinal canal, where they may stimulate additional populations of enteroendocrine cells to secrete satiety hormones such as CCK and GLP-1[60,45]. A reduced gastric emptying by thylakoids, an effect seen in a recent study in the rat[203], may also contribute to the slowed down food processing. Reduced gastric emptying is a known effect of CCK[135,23] and GLP-1[29] as well as of reduced ghrelin levels[52].

Body weight and metabolic parameters

Two studies included in this thesis investigated the effect of daily ingestion of thylakoids on body weight in human participants. In the first study (Paper II) all participants followed an energy-restricted diet and exercised 60 minutes per day for two months. In the second study (Paper III) the participants did not follow an energy-restricted diet but were advised to consume only three meals per day, including a lot of fruit and vegetables, and to avoid sweet drinks, alcohol and snacking in between meals for three months. They were also recommended to exercise 30 minutes per day.

Two-month study (Paper II)

Due to the effective diet-and-exercise intervention with fixed energy intake, participants in both the thylakoid group and the control group lost body weight and body fat to a similar degree. The average body weight loss was 5.3 kg (7.3%) in the thylakoid group and 4.9 kg (6.4%) in the control group. In addition, the follow-up five weeks after the two-month intervention period showed that the thylakoid group continued to lose weight significantly after the intervention (Table 5)[200].

Both the thylakoid group and the control group lost body fat during the two-month study; 4.6 kg (4.5%) for the thylakoid group and 4.0 kg (3.5%) for the control group. The five-week follow up showed that the thylakoid group continued to lose body fat after the intervention[200]. The thylakoid group lost on average 2.6 kg abdominal fat and the control group 2.2 kg during the intervention. The follow-up five weeks after the two-month intervention period showed that the thylakoid group continued to lose abdominal fat after the intervention, while the control group gained abdominal fat[200].

Even though there were no significant differences between the groups during the two-month study regarding reduction of body weight and body fat, the overall improved metabolic profile in the thylakoid group including a reduced hip circumference and reduced fasting levels of leptin and blood glucose suggested that thylakoid treatment may still be more efficient than control for body weight and body fat loss over time. In addition, even though the thylakoid and control groups lost body weight and body fat to a similar degree during the two-month intervention, data from the follow up showing that the thylakoid group continued to lose body weight, body fat and abdominal fat during the five weeks following the intervention suggests that thylakoid-treatment has a lasting effect compared to control[200]. Also, thylakoids seem to favour a reduction of abdominal fat, which is important considering the increased risk for cardiovascular disease associated with visceral fat.

The lasting effect of thylakoids after treatment may be caused by an altered microbiota, as shown in fecal samples from the three-month study[203], or an activation of fat oxidative genes as seen in paper V in rat. In addition, the reduced hunger and cravings induced by thylakoid treatment during the study may have made it easier for the previously thylakoid-treated participants to maintain a healthy lifestyle after the intervention[201].

Three-month study (Paper III)

After performing the two-month study with no significant differences seen in body weight or body fat between the thylakoid and control groups we performed a three-month study without energy restriction. With this study design, the thylakoid group lost significantly more body weight compared to control, 5.0 kg (6.3%)

versus 3.5 kg (4.4%). Total cholesterol was also reduced significantly in the thylakoid group compared to control. Body fat, fat free mass, waist circumference and fasting leptin levels decreased in both the thylakoid group and the control group during the study while fasting glucose and insulin as well as HDL-cholesterol, LDL-cholesterol and triglycerides were unaffected.

In conclusion

Data from the two-month and three-month weight-loss studies (Papers II and III) show that daily ingestion of thylakoids promoted an average body weight loss of approximately 7%, both with a restricted diet and a three-meal diet regime without restriction. In addition, thylakoid treatment together with diet restriction and 60 minutes of daily exercise improved metabolic parameters and therefore has the potential to reduce the risk for cardiovascular disease.

Compared to other weight-loss studies, two and three months are relatively short study durations. Achieving a 7% weight loss in this short time is very successful, compared to weight loss achieved by weight loss pharmacotherapies available today (Table 2). Furthermore, the initial rate of weight loss during the first two months can predict weight loss at 4 and 8 years, suggesting that the early rate of weight loss is important for outcome in the future[15].

In our studies, the placebo-groups were also successful in losing weight, on average 5.4%. However, the follow-up after the two-month study showed that the control group did not continue to lose body weight, body fat and abdominal fat during the five weeks after the intervention while the thylakoid-treated group did, indicating that there is a lasting effect of thylakoid-treatment compared to control[200]. The results from the VAS- and eating behaviour questionnaires during and after the two-month study showed that the thylakoid-treated participants felt less restrained during the energy-restricted diet and experienced less cravings for palatable food[201]. These data suggest that the thylakoid group found it easier to follow the energy-restricted diet during the study and also to continue leading a healthy lifestyle afterwards.

Fatty acid oxidation

Study V

The background to this study was that several previous animal studies with thylakoids have shown reduced body weight gain and reduced body fat accumulation in the thylakoid-treated animals compared to control. After seeing body fat reduction also in thylakoid-treated humans compared to control despite a similar energy intake (Paper II) and no steatorrea[203], we wanted to examine

whether ingesting thylakoids together with a high-fat diet increases energy expenditure and fatty acid oxidation.

In the first experiment, we found that the thylakoid-treated rats accumulated 25% less visceral fat compared to controls despite a similar food intake. In addition, several genes associated with fatty acid transport, fatty acid oxidation and ketogenesis were upregulated in the jejunum of these rats. In the second experiment, the thylakoid-treated rats showed decreased food intake, gained less weight and their respiratory quotient was lower. There was no steatorrhea. Taken together, these results suggest that thylakoid-treatment shifted nutrient utilization towards fat, stimulated fatty acid oxidation and ketogenesis in the intestine and that this, together with a reduced food intake, may have affected body fat accumulation in thylakoid-treated rats. Indeed, more recent experiments have shown effects of thylakoids on visceral adipose tissue; increasing lipid turnover and decreasing mean adipocyte cell size[206].

Since thylakoids cause fat to stay longer in the intestine through reduced lipase/colipase activity, slowed down fat digestion and absorption, the fat is registered for longer time over a larger area. Therefore, one hypothesis is that the intestine reacts as if there are more fatty acids present, i.e. as if the diet had a higher fat content. High fat diets are known to upregulate fatty acid oxidation[185,186,184]. They also stimulate ketogenesis through HMG-CoAS2[187].

PPAR-α is a transcription factor that regulates the expression of numerous genes, among them the genes upregulated in this study[188]. Therefore, we suggest that thylakoids cause activation of PPAR-α in the intestine, either through dietary fatty acids or through direct activation by the thylakoids or a component of thylakoids.

Among the most abundant natural PPAR-agonists are dietary fatty acids and fatty-acid derived compounds[189]. PPARs have general preference for polyunsaturated fats. In addition, the large hydrophobic binding cavity allows PPARs to interact with structurally diverse compounds[190]. Numerous dietary plant bioactive compounds have been suggested to serve as natural ligands for PPARs, although the in vivo relevance of PPAR activation by these compounds remains uncertain[188]. To understand which component or components of thylakoids that may act as a ligand to PPAR-α, besides polyunsaturated fatty acids, the exact content of the thylakoid powder needs to be further explored. One hypothesis is that the thylakoid effects may be caused by antioxidants, because pigments and antioxidants may stay in the intestine and have local effects.

Several studies on the effects of green tea rich in catechins have shown similar results to the ones demonstrated in studies on thylakoids, even though the catechins' effects have not been examined in the intestine specifically. Catechins have been shown to decrease body weight, improve weight loss maintenance and

reduce body fat and waist circumference as well as RQ[191,192]. The reduction in the RQ results from a shift in substrate utilization towards fat oxidation[193]. Also, green tea, black tea and a component from green tea leaves, epigallocatechin gallate, have been shown to activate PPAR-α in vitro[194]. Green tea also has the ability to increase energy expenditure and fat oxidation depending on genotype[195]. Taken together, the effects of thylakoid ingestion on fat oxidation may be mediated by similar mechanisms as the effects shown for components in tea, which are possibly caused by antioxidants. Because the exact nature of antioxidants in thylakoids is not yet established, this issue needs to be further investigated.

Conclusions

Thylakoid effects on hunger and reward

- Thylakoids alter levels of appetite regulating signals from the gut, increasing CCK and GLP-1 and reducing ghrelin, affecting feelings of hunger, satiety and reward.
- Thylakoids affect blood levels of glucose and insulin, preventing postprandial hypoglycaemia.

Implications and significance

Altering the secretion of appetite regulating hormones may promote reduced food intake, increased intermeal interval and reduce snacking in between meals. It may also have an effect on food choice, reducing the intake of palatable energy-dense foods. Preventing postprandial hypoglycaemia may also reduce wanting for palatable food and and snacking between meals. These results are important because total energy intake, snacking and eating frequency as well as intake of palatable foods and fast foods are associated with overeating and obesity.

Thylakoid effects on body weight and body fat loss

- Thylakoids facilitate body weight loss during weight loss programmes in human participants, and promote body weight loss and body fat loss after the treatment period.
- Thylakoids reduce body weight gain and body fat accumulation in rodents on a high-fat diet, affect fatty acid oxidation in enterocytes, alter substrate utilization towards fat, and reduce visceral body fat accumulation.

Implications and significance

Body weight loss and body fat loss reduce the increased risk for comorbidity associated with accumulation of body fat, overweight and obesity. Given that visceral fat accumulation is associated with inflammation and increased risk for cardiovascular disease, thylakoid ingestion may help to improve metabolic health.

Future perspectives

To further investigate by what mechanism thylakoids affect feelings of hunger, satiety and reward, we would like to perform both animal studies and human studies:

- In rats, we would like to look at the development of anticipation for palatable food, with or without thylakoid feeding, during an entrainment schedule during which the rats are trained to expect a piece of chocolate every day at the same time. In this study, we will also look at appetite regulating hormones in the blood (ghrelin and GLP-1) as well as expression of neurotransmitters in areas of the brain associated with hunger and reward (such as dopamine).
- In humans, we would like to use fMRI in a crossover study to investigate possible differences in brain activation in areas associated with hunger and reward after thylakoid ingestion compared to control. The task will involve looking at pictures of palatable food and possibly food tasting.
- In humans, we would like to investigate in a crossover study whether thylakoids affect wanting and liking for palatable food in individuals with self-perceived eating addiction.
- Finally, we would like to identify biologically active components in thylakoids and study their effects.

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Research report

Supplementation by thylakoids to a high carbohydrate meal decreases feelings of hunger, elevates CCK levels and prevents postprandial hypoglycaemia in overweight women ☆

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ABSTRACT

Thylakoids are chlorophyll-containing membranes in chloroplasts that have been isolated from green leaves. It has been previously shown that thylakoids supplemented with a high-fat meal can affect cholecystokinin (CCK), ghrelin, insulin and blood lipids in humans, and can act to suppress food intake and prevent body weight gain in rodents. This study investigates the addition of thylakoids to a high carbohydrate meal and its effects upon hunger motivation and fullness, and the levels of glucose, insulin, CCK, ghrelin and tumour necrosis factor (TNF)-alpha in overweight women. Twenty moderately overweight female subjects received test meals on three different occasions; two thylakoid enriched and one control, separated by 1 week. The test meals consisted of a high carbohydrate Swedish breakfast, with or without addition of thylakoids. Blood samples and VAS-questionnaires were evaluated over a 4-h period. Addition of thylakoids suppressed hunger motivation and increased secretion of CCK from 180 min, and prevented postprandial hypoglycaemia from 90 min following food intake. These effects indicate that thylakoids may intensify signals of satiety. This study therefore suggests that the dietary addition of thylakoids could aid efforts to reduce food intake and prevent compensational eating later in the day, which may help to reduce body weight over time.

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Introduction

Obesity is a multifactorial disease developing from an interaction of the genotype and environment, which involves social, behavioural, cultural, physiological and metabolic factors. Under contemporary circumstances of modern society, our inherent appetite regulation may often be considered too weak to control energy homeostasis in that satiety signals may not always be strong enough to inhibit the effect of hunger signals (Erlanson-Albertsson, 2005). New ways to strengthen the appetite signalling system may be achievable by the enhancement of satiety hormones and the decreasing of hunger hormones. Even though by the most energy dense nutrient of fat also generates potent satiety signals via cholecystokinin (CCK), it is not able to inhibit energy intake fast enough to prevent overconsumption (Holt, Miller, Petocz, & Farmakalidis, 1995). In Sweden, intake of carbohydrates and proteins increased during the last decades, while the intake of fat has remained stable (Swedish Board of Agriculture, 2009). The increased intake of refined carbohydrates, such as corn syrup, and a decreased consumption of fibres have appeared to evolve in parallel with the increased prevalence of type 2 diabetes during the 20th century (Gross, Li, Ford, & Liu, 2004; Holt et al., 1995). This increased ingestion of refined carbohydrates has also been shown to correlate with elevated levels of triacylglycerides and low density lipoprotein-cholesterol, and decreased high density lipoprotein-cholesterol, all of which are risk factors for cardiovascular disease (Siri-Tarino, Sun, Hu, & Krauss, 2010).

Today, popular diets rich in fat or in wholegrain carbohydrates are advertised as good ways to lose weight, by resulting in a lower over-all consumption of energy, and in many cases, high-fat diets

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result in short term weight reduction and improved glycemic control. However, in the long-term this type of diet may have detrimental affects on cardiovascular health, and cognitive function, and could be associated with inflammation (Frigolet, Ramos Barragán, & Tamez González, 2011). On the other hand, diets rich in wholegrain carbohydrates are generally less tolerated by patients with various bowel diseases and syndromes (Smith, Humes, & Spiller, 2010; Unlü, Daniels, Vrouenraets, & Boermeester, 2012). There is therefore a need to find alternative and healthier satiety-promoting food supplements and additives, which are not associated with drastic changes in the macromolecular energy distribution of the meal.

Thylakoid membranes, part of the chloroplast extracted from green leaves, have been shown to prolong *in vitro* lipolysis by binding to both dietary fat, lipase and colipase (Albertsson et al., 2007). Thylakoids have also been shown *in vitro* to prolong the passage of glucose through the intestinal wall due to steric hindrance, and by the binding of macromolecules present on the mucosal side (Montelius et al., 2011). In both short-term and long-term studies with rats, thylakoids were shown to be associated with decreased food intake and body weight (Emek et al., 2010; Köhnke et al., 2009). In humans, thylakoids have been suggested to increase satiety signals such as CCK, and decrease hunger signals such as ghrelin, in a single meal study consisting of 45% fat (Köhnke, Lindbo, et al., 2009). A reduction of the insulin secretion by thylakoids has also been found in porcine and human studies (Köhnke, Lindbo, et al., 2009; Köhnke et al., 2010).

The aim of the present study is to investigate the effects of thylakoid supplementation in a high carbohydrate breakfast on ratings of hunger, fullness, urge to eat and thoughts of food, as well as serum levels of glucose, insulin, CCK, ghrelin and tumour necrosis factor (TNF)-alpha in overweight women.

Materials and methods

Subjects

The study was conducted on 20 healthy women aged 39–69 years with BMI 24–30 (non-vegetarian and non-smoking). The baseline characteristics of the participants are listed in Table 1. Participants were recruited through advertisement in the local community. Exclusion criteria included diabetes, inflammatory bowel disease, thyroid disease, food allergies, food intolerance and recent use of antibiotics. Procedures, objectives and requirements of the study were explained in detail to the participants, and a written consent was signed before the study started. All participants received a compensation of 1100 SEK (taxable) after completing the study. The Ethical Committee of Lund University approved the study protocol, and the research adheres to the tenants of the Declaration of Helsinki.

Experimental study design

The study was conducted at the Overweight and Diabetes Unit, Skåne University Hospital, Lund, Sweden, in 2011, as a single-blinded, randomized, single centre meal supplementation study. Participants were given a high carbohydrate breakfast on three occasions, with one acting as a control and two being thylakoid-enriched. The washout period was of at least 1 week in duration.

Table 1
Baseline characteristics of the 20 subjects of the study.

Variable	Range	Mean	SD
Age (years)	39–69	53.3	7.49
Body weight (kg)	64.6–85.7	74.7	6.42
BMI (kg/m^2)	24.6–31.8	27.0	1.65
Waist circumference (cm)	78–102	88.9	6.25
Waist/hip ratio	0.73–0.98	0.86	0.06

The subjects were instructed not to consume high-fibre foods and alcohol, and avoid excessive physical activity on the day prior the experiment. After 8.00 pm the evening before each test day, no further intake of food or liquid was allowed, and subjects arrived fasting the next morning. Subjects were weighed and measured the first day. The first blood sample was taken before breakfast was served. The addition of thylakoids to the thylakoid breakfasts was 3.7 g and 7.4 g respectively. All subjects were told to finish their breakfasts in under 15 min, after which no food or fluid was allowed for the next 4 h. The subjects stayed on the premises during experimental days, and were asked to keep relatively still in a quiet, non-stressful environment, though sleeping was not allowed. Blood samples were taken and questionnaires filled out at 0, 15, 30, 45, 60, 90, 120, 180 and 240 min after the start of the meal. Subjects received the diet of low dose thylakoids, high dose thylakoids and control in a randomised order. All reported adverse events during and after trial days were registered.

Biochemical analyses

Blood samples were taken through a venous catheter in the arm continuously during trial days. Plasma concentrations of glucose, insulin and CCK were measured at all time points, ghrelin was measured at 0, 30, 60, 90, 120, 180 and 240 min, and TNF-alpha was measured at 0 and 240 min after the start of the meal.

Plasma glucose was measured with an direct apparatus, HemoCue Glucose 201 (HemoCue AB, Ängelholm, Sweden). Plasma insulin and TNF-alpha were analysed by standard methods in the Department of Clinical Chemistry at Skåne University Hospital, Lund, Sweden. Plasma CCK was measured with a radio immunoassay using a highly specific antiserum (No. 92128) (Rehfeld, 1998). Plasma immunoreactive ghrelin was measured with a RIA human kit that recognises the acylated and desacyl forms of the hormone (Phoenix Pharmaceuticals, Belmont, CA, USA).

Test breakfasts

A common Swedish breakfast with high carbohydrate content (Table 2) was served at three separate occasions. The energy content of the breakfast was calculated upon the needs of a moderately active woman aged 31–60 years. The thylakoid breakfasts were adjusted (Table 2) for caloric and nutritional values, as the energy content of 100 g of thylakoid powder is 1470 kJ (351 kcal) (20.6 g carbohydrates, 45.8 g protein and 9.2 g fat). Total energy content and distribution of the breakfasts are presented in Table 3. The thylakoid powder was mixed with blackcurrant jam. Before serving, the blackcurrant jam was blended with yoghurt and muesli was placed on top. The jam contained no thylakoids in the control breakfast. Thylakoids have a taste of green tea and a dark green colour. However, subjects were unable to distinguish any difference between the thylakoid and control breakfasts, since taste, colour and texture were concealed in the blackcurrant jam.

The thylakoids used in the present study were prepared by SwePharm AB (Södra Sandby, Sweden), using previously described methods (Albertsson et al., 2007). The particle size of the thylakoids was <315 μm. The thylakoid powder contained 36.4 mg chlorophyll per gram.

Questionnaire

At regular intervals throughout the experiment, participants answered questions about their state of appetite (Table 4). The questionnaires were designed as Visual Analogue Scales (VASs) (Flint,

Table 2
The composition of the control and thylakoid supplemented breakfasts.

Ingredients	Control meal (g)	Low-dose thylakoid meal (g)	High-dose thylakoid meal (g)
Muesli – fruits and nuts, homemade	55	60	60
White bread (Skogaholmslimpan, Pågen AB, Malmö, Sweden)	40	40	40
Vanilla yogurt 2.5% fat (Skånemejerier, Malmö, Sweden)	180	175	175
Cheese 17% fat (Herrgårdsost, Skånemejerier, Malmö, Sweden)	20	20	18
Ham 6% fat	31	20	18
Butter 60% fat	2.0	2.8	3.5
Black currant jam	50	50	50
Orange juice	230	220	220
Red pepper	25	20	20
Banana	10	25	20
Orange	50	0	0

Table 3
Nutritional composition of the control and thylakoid supplemented breakfasts.

Nutritional value	Control meal		Low-dose thylakoid meal		High-dose thylakoid meal	
Energy content	554 kcal	2319 kJ	546 kcal	2282 kJ	537 kcal	2245 kJ
Carbohydrates	112.6 g	71 E%[a]	113.1 g	71 E%[a]	113.0 g	71 E%[a]
Sucrose	11.0 g		11.0		11.0 g	
Fibres	8.5 g		8.0		8.0 g	
Fat	16.9 g	11 E%[a]	17.5 g	11 E%[a]	17.9 g	11 E%[a]
Protein	28.6 g	18 E%[a]	28.4 g	18 E%[a]	29.6 g	18 E%[a]

[a] E% = Energy%.

Table 4
Questions and anchors for line ratings of strength of appetite.

Questions	Anchor phrases	
	Score 0	Score 100
How hungry are you right now?	Not at all hungry	Hungrier than I ever felt before
How full are you right now?	Not at all full	More full than I ever felt before
How strong is your urge to eat right now?	I have no urge to eat at all	Very strong urge to eat right now
How preoccupied are you with thoughts of food right now?	Not thinking of food at all	I can hardly think of anything but food

Raben, Blundell, & Astrup, 2000). Written instructions were given on the front page of the questionnaire. In addition, each subject was individually instructed on how to complete the questionnaire so as to avoid misinterpretation. Each question was followed by a 100 mm line anchored at each end by a descriptor (Table 4). Subjects were instructed to place a vertical line across the scale, thus rating objective sensations at every specific timepoint. Ratings were scored as mm between "not at all" and the rater's mark. Three of the parameters (hunger, urge to eat and thoughts of food) were analysed together, with the mean of all three parameters presented as one single graph termed "hunger motivation".

Statistics

Energy and macronutrient composition of the test meals were measured using the programme Dietist XP (Kostdata, Bromma, Sweden). All data were normally distributed, and were analysed for statistical significances using GraphPad Prism, version 4 (GraphPad Software, Inc., San Diego, CA, USA). Numerical calculations of area under the curve (AUC), mean score across time, were used to compare the outcome of control versus thylakoid diets. Wilcoxons signed rank test was used for all comparisons between thylakoid and control. Data are expressed as mean +/− standard error of the mean (SEM). Baseline characteristics (Table 1) are expressed as standard deviation (SD). *P*-values $\leqslant 0.05$ were considered to be statistically significant.

Results

There were no statistically significant differences between the two concentrations of thylakoids (3.7 g and 7.4 g), using either objective or subjective measurements ($p > 0.5$ for all parameters). The material was therefore analysed and presented as thylakoid versus control.

The supplementation of a high carbohydrate breakfast by thylakoids resulted in decreased ratings of hunger, thoughts of food and the urge to eat. These graphs had identical shape and the same score, and were therefore analysed and presented as a single graph, termed hunger motivation (Fig. 1). As seen in this graph, hunger motivation was suppressed following feeding in a similar way between thylakoids and control up to 120 min. From 180 min there was a significant difference in hunger motivation between thylakoid and control ($p = 0.05$), with the thylakoid diet being associated with the suppression of hunger motivation (Fig. 1). Numerical differences in rated fullness were not statistically reliable.

The secretion of CCK was increased from 180 min ($p = 0.05$) following the thylakoid breakfast, compared to control (Fig. 2). The concentrations of TNF-alpha decreased significantly from 0 to 240 min after both the thylakoid ($p < 0.001$) and the control breakfasts ($p < 0.05$), but no difference between the two meals was observed (Fig. 3).

The supplementation of thylakoids resulted in a tendency towards higher plasma glucose levels from 90 min ($p = 0.09$), compared to control (Fig. 4). However, calculation of total AUC for

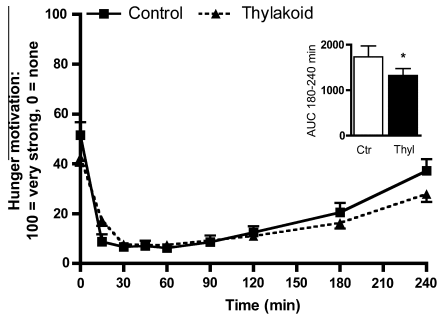

Fig. 1. Hunger motivation (mean of feelings of hunger, the urge to eat and thoughts of food), rated with questionnaires over 4 h, after intake of either a thylakoid (▲) or control meal (■). Rating of 100 at *Y*-axis means very hungry, very strong urge to eat and I cannot think of anything but food (Table 4). Rating of 0 means not hungry, no urge to eat and not thinkning of food. From 180 min after the start of the thylakoid-supplemented meal (Thyl), compared to the control (Ctr) meal, subjects rated their hunger motivation lower (AUC, $p = 0.05$).

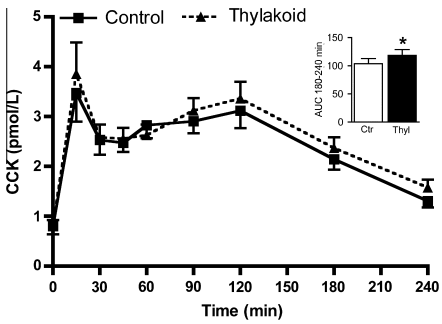

Fig. 2. The secretion of cholecystokinin (CCK) measured over 4 h, after intake of either a thylakoid (▲) or a control meal (■). Thylakoid supplementation (Thyl) resulted in increased secretion of CCK from 180 min (AUC, $p = 0.050$) compared to control (Ctr).

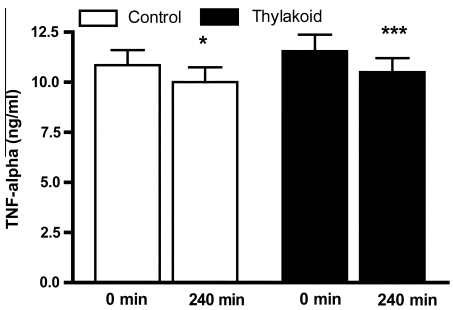

Fig. 3. Levels of TNF-alpha measured in plasma before the start of the meal (0 min) and 240 min after intake of either a thylakoid or a control meal. Both meals resulted in significantly decreased levels; $p < 0.05$ for the control meal and $p < 0.001$ for the thylakoid meal (AUC). No difference between thylakoid and control meals was observed.

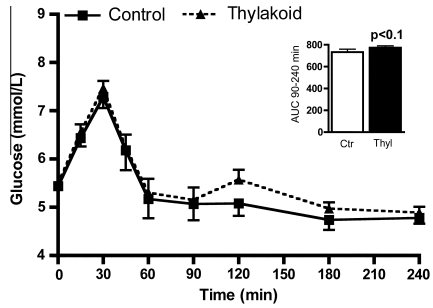

Fig. 4. Plasma glucose levels measured over 4 h after food intake. The supplementation of thylakoids (▲) resulted in a tendency of higher glucose from 90 min after the start of the meal (AUC, $p < 0.1$) compared to control (■), thus preventing hypoglycaemia. There were no significant differences in AUC between thylakoid (Thyl) and control (Ctr) breakfasts for the entire 4-h study ($p > 0.05$).

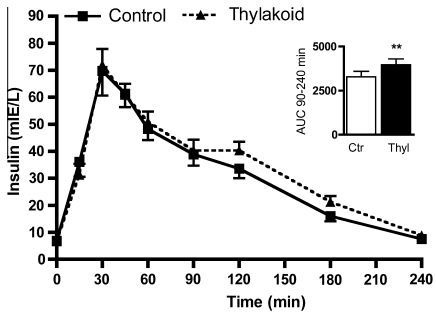

Fig. 5. Plasma insulin measured over 4 h after the start of the meal. The supplementation of thylakoids (Thyl, ▲) resulted in stabilised insulin levels from 90 min (AUC, $p < 0.01$) compared to control (Ctr, ■).

control versus thylakoid breakfasts was statistically not significant ($p > 0.05$). The concentration of insulin was also significantly higher than control from 90 min after supplementation with thylakoids ($p < 0.05$) (Fig. 5).

All subjects completed the entire study. No adverse events were reported during or after the trial.

Discussion

Supplementation by thylakoids resulted in a greater increase in CCK levels from 180 min than control. In an earlier single meal study with humans, a high fat diet and higher concentrations of thylakoids (10, 25 and 50 g respectively) were used, resulting in increased CCK levels with thylakoid supplementation (Köhnke, Lindbo, et al., 2009). In the present study, we demonstrate that supplementation of a high carbohydrate/low fat breakfast by thylakoids at lower doses results in increased CCK levels 3 h postprandially. The release of CCK is known to be dependent on the hydrolysis of triglycerides into fatty acids in humans (Beglinger et al., 2010) and an irreversible lipase inhibitor such as orlistat attenuates the release of CCK (Ellrichmann et al., 2008). The observed augmented release of CCK by thylakoids in our experiments

suggests that fat hydrolysis does occur, though a longer time is needed since the augmented CCK response is observed only at late time points, i.e. after 180 min, which is in agreement with previous observations (Köhnke, Lindbo, et al., 2009). Elevated levels of CCK are consistent with the observed reduced sensation of appetite. Furthermore, thylakoids, unlike orlistat (Baretić, 2012), do not cause steatorrhea.

Plasma glucose levels increased rapidly after intake of both control and thylakoid breakfasts, which was to be expected following a high carbohydrate breakfast. The first peaks in glucose levels are identical after the control and thylakoid breakfasts, suggesting that easily digested carbohydrates, with high concentration of sugars, were not affected by the supplementation of thylakoids. However, supplementation resulted in higher glucose levels from 90 min after the start of the breakfast, and a second peak in blood glucose was observed at 120 min. This did not occur after the control breakfast, where glucose levels dropped below fasting level at 60 min and continued to decrease. An explanation of the second blood glucose peak at 120 min may be a prolongation of carbohydrate digestion and absorption, caused by thylakoids. Such a hypothesis is supported by calculations of the total AUC of glucose in the present study, where there was no statistically significant difference between thylakoid and control breakfast. Furthermore, findings in a previous *in vitro* study support the result of the present study, in that thylakoid membranes appear to prolong the uptake of free glucose through the intestinal wall of the small intestine (Montelius et al., 2011). The mechanism of action for thylakoids is not clear, but may involve binding to disaccharidases in the brush border of the mucosa, which are responsible for the hydrolysis of oligosaccharides to glucose. We propose that preventing postprandial hypoglycaemia is important for weight control in the long run. Such a hypothesis is supported by a recent report showing that a reactive hypoglycaemia was predictive of a more pronounced weight gain in humans (Tremblay & Chaput, 2012). Increased hypothalamic glucose sensing has also been shown to be important for the suppression of hunger hormones, such as Agouti related peptide and neuropeptide Y (Jordan, Könner, & Brüning, 2010).

The secretion of insulin was identical after the thylakoid and control breakfasts during the first 90 min following the start of the meal. Thereafter, the supplementation of thylakoids resulted in stabilised levels of insulin compared to control. The slightly elevated insulin concentrations at 120 and 180 min may indicate that thylakoids have an incretin effect (Hardikar, 2004; Holst & Gromada, 2004). Also, these increased insulin levels postprandially may be an effect of CCK and glucagon-like peptide-1 (GLP-1), since both peptides act to promote satiety.

Different foods and meal compositions have various effects on inflammatory markers (Egger & Dixon, 2010), such as TNF-alpha. Meals high in refined carbohydrates with a high GI-value have been regarded as pro-inflammatory, while low-GI foods have been regarded as anti-inflammatory. The present test-meals, although carbohydrate-rich with high GI-values, resulted in significantly decreased TNF-alpha concentrations between 0 and 240 min. We speculate that this could arise from the high content of dietary fibre and antioxidants in the blackcurrant jam, orange and red pepper. Indeed, no difference in TNF-alpha concentration between the thylakoid and control breakfasts was found.

The effects found after supplementation of thylakoid in the present study indicate a prolongation of the phase satiation seen after the control meal. The increased levels of CCK at later timepoints might explain, at least in part, the decreased ratings of hunger motivation described. Subjects experienced less hunger motivation from 180 min after the thylakoid breakfast compared to control. The higher blood glucose levels from 90 min, preventing postprandial hypoglycaemia, may also explain this observation. These findings agree with Jean Mayers' proposal 50 years ago that blood glucose concentrations act to regulate energy intake and that a trend towards hypoglycaemia and/or glucose instability might induce excess energy intake and overweight in humans (Chaput & Tremblay, 2009).

A possible limitation of this study was the limited number of time points that the subjects were followed after intake of the test meals. In future studies, subjects may be best followed during the entire day, with all meals eaten under observation to monitor second meal effects.

In summary, we have found that supplementation of a single, carbohydrate rich breakfast with thylakoids suppress sensations of hunger. We also show that thylakoid supplementation increases secretion of CCK 3 h postprandially, and prevents postprandial hypoglycaemia from 90 min after the commencement of the meal. If the supplementation of thylakoids can result in a consistent reduction in food intake and prevent compensatory eating later in the day, we suggest that thylakoids may help in a program of weight reduction over time.

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Paper II

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Research Article Open Access

Decreased Urge for Palatable Food after a Two-month Dietary Intervention with Green-plant Membranes in Overweight Women

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Abstract

Background/Aim: The present study investigates the effect of daily green-plant membrane (thylakoid) supplementation for two months on body weight, body composition, metabolic profile and rating of appetite sensations in overweight women on a restricted diet.

Methods: 26 women, Body Mass Index (BMI) 27.5 ± 1.9, randomized into a thylakoid (n=12) and control group (n=14), followed a 7500 kJ/day diet with or without 5.6 g of thylakoids supplemented in a blueberry drink, and exercised 60 minutes per day. Fasting blood samples were taken with two weeks interval. On the first and last day of the study subjects answered Visual Analogue Scale (VAS) questions regarding hunger and cravings.

Results: Both control and thylakoid-treated groups lost body weight and body fat over the course of the study, but no differences were found between the groups. Thylakoid supplementation resulted in decreased hunger (p=0.016) and decreased urge for chocolate (p=0.052) in contrast to the control group. Leptin levels were significantly reduced at the end of the study in the thylakoid-treated group (p=0.012) compared to control, suggesting a decreased fat mass. The overall metabolic profile was also improved in the treated group compared to controls, based on body weight, waist and hip-circumference, trunk and total body fat, p-leptin, p-LDL, p-ApoB1, p-total cholesterol, p-TAG, blood glucose, p-HbA1C and p-insulin (p=0.024).

Conclusions: Thylakoids added to food in adjunct to lifestyle intervention may be helpful in enabling overweight subjects to lose weight by suppression of hedonic hunger.

Keywords: Obesity; Hedonic; VAS; Hunger; Leptin; Palatable food; Thylakoids

Abbreviations:

ApoB1: Apolipoprotein B1; BMI: Body Mass Index; CCK: Cholecystokinin; E%: Energy Percentage; GLP-1: Glucagon-like Peptide 1; LDL: Low Density Lipoprotein; tAUC: Total Area under the Curve; TAG: Triacylglycerol; VAS: Visual Analogue Scale

Introduction

An increasing proportion of food consumption in affluent societies is driven by pleasure. This type of hunger has been described as hedonic hunger as opposed to homeostatic hunger caused by energy deficiency [1]. The increased incidence of overweight and obesity since 1970 [2] is suggested to be caused by hedonic eating, i.e. the consumption of food items such as sweets, sweet drinks, chocolate, chips and pizza [3].

One reason for overeating is the inability of palatable food to promote appetite control [4]. Appetite control occurs through the release of various gut hormones [5] including the hunger hormone ghrelin, and the satiety promoting hormones Cholecystokinin (CCK) and Glucagon-like Peptide 1 (GLP-1) [6].

Previously, we have shown that green-plant membranes, thylakoids, have a hunger suppressing, as well as a satiety promoting, effect. In one single-meal study, general hunger was decreased three hours following intake of thylakoids with breakfast [7]. A sustained suppression of hedonic hunger for the whole day has also been shown following intake of thylakoids with breakfast [8]. Suppression of general and hedonic hunger was related to the release of the satiety hormones CCK [7] and GLP-1 [8]. Furthermore, suppression of ghrelin levels in humans has previously also been demonstrated following intake of thylakoids [9]. Thus thylakoids affect hunger and the release of three hormones important for appetite control; ghrelin, CCK and GLP-1.

In a previous study, thylakoids were shown to decrease body weight in human following daily treatment for three months [8]. These effects were achieved without any caloric restriction. However, most weight loss programs are based on a caloric restriction. Hence, we were interested to find out whether treatment with thylakoids would augment body weight loss during a caloric restriction commonly used in weight loss studies [10].

In this study, a daily supplementation of thylakoids for two months in overweight women was used, together with a caloric restriction of 15 Energy % (E%). In addition to following body weight and metabolic

parameters we also studied subjective ratings of hunger, satiety and cravings for palatable food.

Methods and Procedures

Subjects

Forty-eight middle-aged (40-65 years) non-smoking overweight women who were not, currently or recently, on a diet were recruited for screening through advertisement in the local newspaper. Exclusion criteria were diabetes, inflammatory bowel disease, thyroid disease, food allergies and a Body Mass Index (BMI) over 33. Thirty individuals were enrolled as participants in the study. All of the 30 enrolled participants finished the study. Compliance was measured through diary entries, interviews and the daily use of pedometers. Four participants (one from the control group and three from the thylakoid group) were excluded from the data analysis due to incompliance with the diet and exercise regime. Baseline characteristics of the 26 included subjects are listed in Table 1. One participant in the control group was not included in the analysis of subjective ratings of hunger, satiety and urges for specific food items due to non-attendance.

	Control group (n=14)	Thylakoid group (n=12)	p-values for differences in baseline characteristics
Age (years)	53.0 ± 5.9	51.6 ± 5.2	p=0.54
Weight (kg)	76.6 ± 5.3	73.1 ± 7.4	p=0.24
BMI (kg/m^2)	27.7 ± 2.2	27.4 ± 1.7	p=0.65
Waist circumference (cm)	88.9 ± 6.6	85.7 ± 8.3	p=0.28
Hip circumference (cm)	103.9 ± 3.9	103.3 ± 4.5	p=0.70
Waist/Hip ratio	0.86 ± 0.07	0.82 ± 0.06	p=0.16

Table 1: Baseline characteristics of the 26 women included in the study (average ± SD). There were no differences between the groups (t-test).

Procedures, objectives and requirements of the study were explained in detail to the participants, and written consents were signed both before screening and before the study started. The study was approved by the Ethical Committee of Lund University, and conducted in accordance with the declaration of Helsinki. After completing the study, all participants received a compensation of 2000 Swedish Crowns (SEK) (taxable).

Experimental study design

The study was conducted at the Overweight and Diabetes Unit, Skånes University Hospital (SUS), Lund, Sweden, and designed as a single-blinded, randomised, diet and exercise intervention study with duration of two months. The participants were randomised into two groups to ensure normal distribution within and between the groups based on body weight, BMI, blood glucose, insulin, Triacylglycerides (TAG) and cholesterol (total and LDL). One of the groups received supplementation by thylakoids in a blueberry drink every day, while the other group served as control, receiving a daily blueberry drink without thylakoids.

Every second week, at the same time in the morning, the participants visited the clinic for measurements of body weight, body composition, waist- and hip circumferences and for blood sampling. To optimise conditions for all measurements, the participants were instructed to have a standardised dinner in the evening before each test day and to abstain from further intake of foods or liquid after 8.00 pm.

The participants had a total of five individually scheduled appointments during the study. On the first and the last day (day 1 and 56), following the anthropometric measurements and blood sampling, an isocaloric breakfast (2114 kJ/505 kcal) consisting of the blueberry drink with or without thylakoids, yoghurt with apple, breakfast cereal, nuts and coffee or tea was served (Table 2). After 240 minutes an isocaloric take-away lunch (2593 kJ/630 kcal) consisting of a frozen thai-curry meal, bean salad and a banana was administered. VAS questionnaires measuring subjective parameters of hunger, satiety and urge for specific foods were filled out at given time points throughout the day on both day 1 and 56.

Thylakoids

The thylakoids used in the present study were prepared from baby spinach leaves using the pH-method, as described [11]. The thylakoid-slurry was dried to obtain a thylakoid powder, prepared by Swepharm AB (Södra Sandby, Sweden). 100 g thylakoids consists of 41.1 g protein, 14.5 g fat, 36.8 g carbohydrate, 3.5 g salt as well as pigments such as 3640 mg chlorophyll, 28 mg lutein, 730 ug zeaxantin, 4760 mg betakaroten, 21 ug vitamin A, 1330 ug vitamin K, 6.07 mg vitamin E and 166 ug folic acid.

The thylakoid group received 5.6 g of thylakoid powder mixed with 2.8 g rapeseed oil (Zeta, Di Luca & Di Luca AB, Stockholm, Sweden) and 50 g of blueberry soup (Ekströms original, Procordia Food AB, Eslöv, Sweden). The control group received 2.8 g rapeseed oil mixed with 50 g blueberry soup. The blueberry drinks with and without thylakoids contained 209 kJ/50 kcal versus 188 kJ/45 kcal respectively. The drinks were taken before breakfast every day.

Caloric restriction and diet recommendations

Before the study started, average daily energy requirement for the participants was calculated to 8800 kJ (~2100 kcal), with respect to age, weight, height and presumed daily energy-consumption, according to Harris Benedict equation using Dietist XP (Kostdata, Bromma, Sweden). During the study the calculated energy intake was reduced by 15 E% to ~7500 kJ/day (~1800 kcal/day). The participants were provided with a collection of selected recipes (3 breakfasts, 29 lunches/dinners and 4 desserts) to choose from. The recommended energy intake per day during the study was divided into three meals/day: 2100 kJ (~500 kcal) for breakfast; and 2500 kJ (~600 kcal) for lunch and dinner respectively. An additional 400 kJ (~100 kcal) was allowed for milk in coffee/tea and individual adjustments. Water, coffee and tea were allowed between meals, but no additional foods or snacks. The diet did not allow any sweetened drinks.

Subjects were also instructed to accomplish 60 minutes of low/medium intensity exercise each day, such as power walking, swimming, basic aerobics etc.

Each day, the participants answered questions regarding their choice of meals, health-status and exercise in a diary. These data were used to analyse the compliance with the study guidelines.

Meal components	Amount (g)	Calories (kJ/kcal)	Protein (g)	Fat (g)	Carbohydrate (g)	Fiber (g)
Breakfast						
Natural yoghurt	300	753/180	10.2	9.0	14.4	0.0
Muesli wt. fruits and nuts	45	775/185	2.9	4.9	30.4	3.4
Apple (Granny Smith)	60	139/34	0.2	0.1	7.4	1.1
Almonds	10	255/61	2.0	5.2	1.3	0.7
Blueberry shot, wt or wth thylakoids	50	192/45	2.7	3.4	5.5	0.5
Total intake	465	2114/505	18.0	22.6	59.1	5.7
Lunch						
Red curry chicken (frozen product)	380	1558/380	15.2	13.3	45.6	1.9
Bean salad (ready product)	100	591/144	6.0	5.0	18.0	Not stated on product
Banana	105	444/106	1.0	0.5	23.1	1.8

Table 2: Contents of breakfast and lunch served on first and last days of the study.

Somatic analyses

Body weight was measured with a digital scale (TANITA WB-100A, class III, Amsterdam, The Netherlands). The composition of body fat mass (kg) and trunk fat (kg) were measured with a Bioelectric Impedance Analyser (TANITA-BC 418 MA). Waist circumference, midway between the lower rib margin and the iliac crest, and hip circumference were measured to the nearest 0.5 cm by using a non-stretchable tape measure.

Biochemical analyses

Fasting blood samples were taken through a venous catheter in the arm. Blood glucose was measured directly using HemoCue Glucose 201 (HemoCue AB, Ängelholm, Sweden). Plasma (p) insulin, p-HbA1c, p-TAG, p-cholesterol (total and LDL) and p-Apo B1 were analysed by standard methods at the Department of Clinical Chemistry at Skåne University Hospital (Lund, Sweden). P-Leptin was measured with a RIA human/multi species kit using the double antibody/PEG technique (XL-85K, Millipore Corporation, Billerica, MA, USA).

Questionnaires

Questionnaires constructed as VAS [12] were used to measure sensations of hunger, fullness and urge for specific food items. Pictures assisted the evaluation of the urge for specific food items. For high carbohydrate snack pictures of a sandwich and a sweet cinnamon bun were presented, for salt and fat; pictures of potato chips and salted peanuts, for sweet snack; pictures of candy and a popsicle; and for fat and sweet; pictures of cake and chocolate were presented. First (day 1) and last day (day 56) of the study, subjects answered questions before breakfast (0 min) and at time points 15, 60, 120, 180, 240 (before lunch was served), 270 (after lunch was served), 330, 390, 450 and 630 minutes. Written instructions were given on the front page of the questionnaire, and each subject was individually instructed in how to fill out the questionnaire to avoid misinterpretation. Questions were followed by a 100 mm line anchored by descriptors on each side of the line (Table 3). Subjects were instructed to place a vertical line across the scale, thus rating how strong their sensations were at every time point. Ratings were scored as mm between "not at all" and the individual subjects mark.

Questions	Anchor: 0 mm	Anchor: 100 mm
How hungry are you right now?	Not hungry at all	Extremely hungry
How full are you right now?	Extremely full	Not at all full
How much would you like to have a sandwich or a cinnamon bun right now?	Not at all	Extremely much
How much would you like to have salted peanuts or chips right now?	Not at all	Extremely much
How much would you like to have sweets/candy right now?	Not at all	Extremely much
How much would you like to have cake or chocolate right now?	Not at all	Extremely much

Table 3: Questions in the VAS-questionnaires asked during the first and last days of the study.

Statistics

Power calculations were based on previous pilot-studies examining thylakoid supplementation in humans with respect to changes in blood-glucose. With a sensitivity of 0.80 and a significance level of 0.05, the power calculations indicated a sample size of 14 in each group, when the clinical difference was set to 0.6 and the within-subject standard deviation of 0.56. Statistical data analyses of all blood samples and body measurements were done using R Development Core Team, version 2.15.3, 2011 (R Foundation for Statistical Computing, Vienna, Austria). The analysis was performed in two steps. First, the simple regression model was fitted for each subject and for each of the 13 measured variables by taking time as explanatory variable (5 time points) and the measured variable as the response

variable. The obtained slope values represented fitted rate of change for a particular individual and variable. Second, multivariate analysis was performed on the so obtained rates of change, using two-sample Hotelling´s T2-test with all 13 variables treated together. Deviations from the normality assumption were examined and were determined not to be severe. Additionally, for interpretation purposes, the mean difference in slope variables between the two groups were analysed with the t-test for a univariate two-sample problem for each of the 13 measured variables. The obtained p-values from these individual variable comparisons should be treated with caution due to the effect of multiple testing and thus are only used to discuss and interpret which of the variables contribute most to the significant difference between the groups.

Statistical analyses of the VAS questionnaires were done using Prism, version 6 (GraphPad Software, Inc, San Diego, CA, USA). On the first and last days respectively, the variations in ratings during the day were analysed with a two-way repeated measures ANOVA with treatment and time as fixed factors. Individual time points were further analysed with a multiple comparison test followed by Fisher´s LSD test. Numerical calculations of Total Area under the Curve (tAUC) were analysed with Wilcoxon matched-pairs signed ranks test to compare the difference between first and last days of the study within the groups. The Mann-Whitney t-test was also used to compare differences between the groups.

Objective data exhibited normal distribution for most variables and some deviations from normal assumption for certain variables (HbA1c, Apo B and body weight in the thylakoid group), but not critical for the result of the analysis. The latter was assessed by a resampling study. In the figures, data are expressed as mean ± SEM, while in the tables data are given as mean ± SD. P-values <0.05 were considered to be statistically significant, and p-values <0.1 to be of interest.

Results

After two months of restricted diet all participants lost body weight and decreased their total body fat with no significant difference between the control and thylakoid treated groups (Table 4).

Analysis of subjective ratings of hunger, using VAS questionnaires, revealed a decreased sensation of hunger within the thylakoid group at the end of the study compared to the first day (p=0.016, Figure 1A), whereas no change of hunger sensation was found in the control group (Figure 1A). No differences in hunger sensations were found between the thylakoid group and control on the first day (F(10,240)=1.6, ns) or on the last day of the study (F(10,210)=0.83, ns) (Figure 1B and 1C).

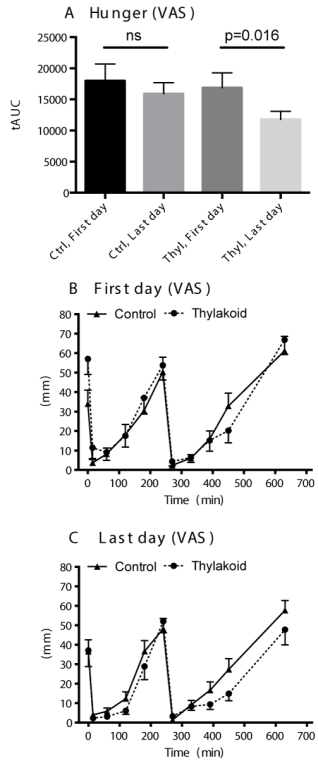

Figure 1: Visual Analogue Scale (VAS) ratings of hunger presented as **A)** Total Area under the Curve (tAUC) for the first and the last day, **B)** the first day and **C)** the last day of the study. Daily thylakoid supplementation for two months resulted in suppressed ratings of hunger the last day compared to the first day. No differences (ns) in hunger ratings were observed in the control group over the course of the study or between the treatment groups on the first or last day. **A)** Wilcoxon matched-pairs signed ranks test was used for within-group analysis and the Mann-Whitney t-test for between-group analysis, **B)** and **C)** was analysed by a two way ANOVA.

A strong tendency for a reduction in the urge for chocolate within the thylakoid group was observed at the end of the study compared to the first day (p=0.052) but not in the control group (p=0.62, Figure 2A). The ANOVA analysis of the urge for chocolate between treated and control on the first day and the last day respectively revealed a significant interaction between time and treatment first day; (F(10,240)=1.9, p<0.05), last day (F(10,230)=1.9, p<0.05). Analysis of individual time points showed a decreased urge for chocolate in the treatment group prior to lunch on the first day and in the afternoon on the last day (Figure 2B and 2C).

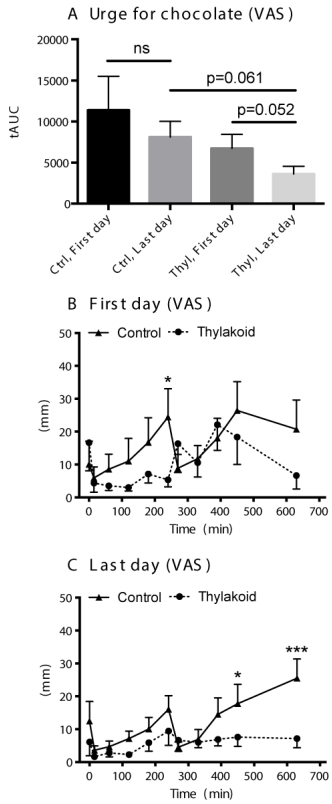

Figure 2: Visual Analogue Scale (VAS) ratings of the urge for chocolate presented as **A)** Total Area under the Curve (tAUC) for the first and the last day, **B)** the first day and **C)** the last day of the study. Thylakoid supplementation tended to suppress ratings of the urge for chocolate on the last day compared to the first day. No differences (ns) were observed within the control group between the first and the last day. Thylakoid treatment decreased urge for chocolate compared to the controls on both the first and the last day. **A)** Wilcoxon matched-pairs signed ranks test was used for within-group analysis and the Mann-Whitney t-test for between-group analysis, **B)** and **C)** was analysed by a two way ANOVA followed by Fischer's LSD test.

The urge for a carbohydrate snack was not significantly altered over the course of the study in any of the groups (Figure 3A). There was however an interaction between time and treatment in the urge for a carbohydrate snack on the first day of treatment ($F(10,240)=2.1$, $p<0.05$), but not on the last day ($F10,230)=1.3$, ns, Figure 3C). The urge for a carbohydrate snack was decreased prior to lunch and in the afternoon on the first day (Figure 3B).

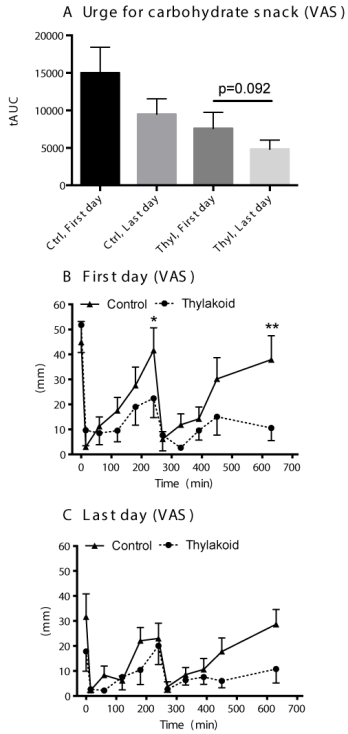

Figure 3: Visual Analogue Scale (VAS) ratings of the urge for a carbohydrate snack presented as **A)** Total Area under the Curve (tAUC) for the first and the last day, **B)** the first day and **C)** the last day of the study. Thylakoid supplementation tended to suppress ratings of the urge for a high carbohydrate snack ($p=0.092$) on the last day compared to the first day of the study. No differences (ns) were observed in the control group. A decreased urge for a carbohydrate snack was found in the thylakoid-treated group compared to controls on the first day of the study. **A)** Wilcoxon matched-pairs signed ranks test was used for within-group analysis and the Mann-Whitney t-test for between-group analysis, **B)** and **C)** was analysed by a two way ANOVA followed by Fischer's LSD test.

Regarding metabolic parameters, there was a significant difference between the control and treatment groups, when comparing the whole series of weekly changes, using Hotelling´s two-sample T2-test (Table 4, $p=0.024$, $T2=3.28$). When analysed individually, a significant difference between control and treatment groups was found in p-

leptin (p=0.012) (Figure 4B) and hip circumference (p=0.046) (Figure 4A).

There were no differences in baseline values between thylakoid and control groups. No side effects of the thylakoid supplementation were reported.

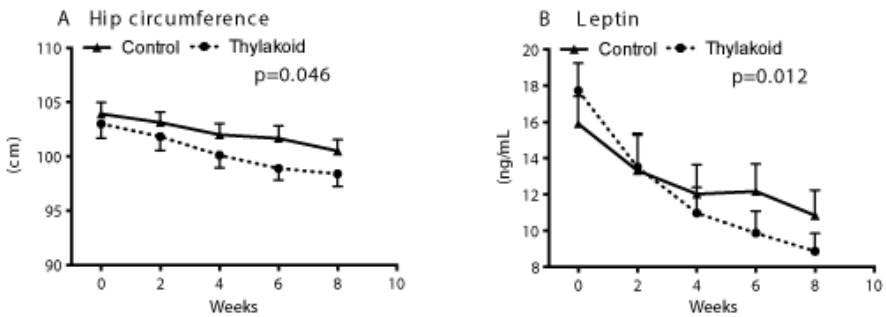

Figure 4: Hip circumference and plasma concentrations of leptin decreased following treatment with thylakoids compared to controls. Weekly change in hip circumference and leptin were analysed using a Univariate t-test.

	Control group (n=14)			Thylakoid group (n=12)			
	First day	Last day	Change per week	First day	Last day	Change per week	Difference, measured in change per week, between groups
Bodyweight (kg)	76.59 ± 5.27	71.72 ± 5.50	-0.59 ± 0.13	73.07 ± 7.49	67.79 ± 8.06	-0.65 ± 0.27	p=0.448
Total body fat (kg)	27.83 ± 3.37	23.80 ± 3.50	-0.39 ± 0.22	26.43 ± 5.18	21.85 ± 4.90	-0.50 ± 0.17	p=0.197
Trunk fat (kg)	14.20 ± 2.06	11.99 ± 1.89	-0.26 ± 0.13	13.48 ± 3.13	10.89 ± 2.90	-0.32 ± 0.09	p=0.250
Waist circumference (cm)	88.89 ± 6.58	82.57 ± 6.67	-0.79 ± 0.28	84.50 ± 7.31	77,93 ± 8.61	-0.84 ± 0.47	p=0.726
Hip circumference (cm)	103.9 ± 3.89	100.5 ± 3.96	-0.42 ± 0.22	103.0 ± 4.55	98.40 ± 3.94	-0.61 ± 0.24	*p=0.046*
p-Leptin (ng/mL)	15.9 ± 5.67	10.85 ± 5.23	-0.56 ± 0.43	17.74 ± 5.25	8.88 ± 3.39	-1.07 ± 0.52	*p=0.012*
p-LDL-cholesterol (mmol/L)	3.18 ± 0.50	3.05 ± 0.56	-0.02 ± 0.04	3.73 ± 1.06	3.14 ± 1.06	-0.07 ± 0.08	*p=0.049*
p-Apo B1 (mmol/L)	0.94 ± 0.14	0.94 ± 0.17	0.00 ± 0.01	1.01 ± 0.31	0.95 ± 0.28	-0.01 ± 0.02	*p=0.036*
p-tot-Cholesterol (mmol/L)	5.23 ± 0.61	5.06 ± 0.67	-0.03 ± 0.04	5.83 ± 1.23	5.16 ± 1.19	-0.08 ± 0.10	p=0.106
p-TAG (mmol/L)	1.06 ± 0.37	0.88 ± 0.24	-0.02 ± 0.03	0.99 ± 0.45	0.71 ± 0.23	-0.03 ± 0.03	p=0.613
p-Insulin (mIE/L)	7.43 ± 3.57	6.29 ± 2.43	-0.17 ± 0.27	8.42 ± 2.47	7.01 ± 2.07	-0.18 ± 0.35	p=0.925
Blood glucose (mmol/L)	5.33 ± 0.47	5.74 ± 0.55	0.04 ± 0.06	5.50 ± 0.66	5.31 ± 0.41	-0.02 ± 0.06	*p=0.023*
p-HbA1c (mmol/L)	36.79 ± 2.91	37.29 ± 3.84	0.06 ± 0.23	35.83 ± 3.56	35.67 ± 3.31	-0.2 ± 0.33	p=0.462
Hotelling´s multivariate T2-test, for all variables: thylakoid vs control groups							*p=0.024*

Table 4: First and last day values (average ± SD), and changes per week (± SD) calculated by regression analysis, for the control and thylakoid groups. Statistical differences between thylakoid and control groups are done for changes per week with the univariate t-test, and for the total multivariate analysis for all parameters examined by Hotellings T2-test.

Discussion

Daily supplementation of thylakoids for two months in combination with a restricted diet resulted in a body weight loss of similar magnitude in the thylakoid-treated and the control groups. The overall decrease in body weight was 0.65 kg/week in the thylakoid group and 0.59 kg/week in the control group. Feelings of hunger and urge for chocolate were reduced by thylakoid treatment over the

course of the study. In contrast, they were not reduced in the control group. Furthermore, the urge for chocolate and a carbohydrate snack was decreased in the thylakoid treated group compared to controls on the first day of the study and for chocolate also on the last day.

Even though the body weight reduction was similar between the two groups, it would appear that this level of weight loss was reached with less effort in the thylakoid-treated group compared to the control group, based on the reduced subjective ratings of hunger and urge for palatable food in the thylakoid group. This suggests that thylakoids exert their appetite controlling effect even during caloric restriction and that this effect is sustained following weight loss. This is an important property of thylakoid treatment, since hunger is common upon weight loss and often leads to overeating and body weight regain [13].

In the present study, the overweight women treated with thylakoids had a larger decline of p-leptin compared to control, indicating that loss of fat tissue was increased by thylakoid treatment. Low leptin values following body weight and body fat reduction is a natural consequence, which often leads to hunger and overeating, since leptin is a postprandial satiety signal. Low leptin levels are said to constitute an important drive for eating; thus explaining the rapid weight gain that often follows a weight loss program [13]. However, thylakoids appear to have the ability to counteract the drive for hunger associated with low leptin levels.

In addition to the effects of thylakoids on single parameters (Table 4), there was an overall improved metabolic profile in the thylakoid-treated group, based on measures of body weight, trunk and total body fat, waist and hip circumference, p-leptin, p-LDL-cholesterol, p-Apo B1, p-total cholesterol, p-TAG, b-glucose, p-insulin and p-HbA1c, compared to control. Further studies are needed to substantiate these effects, in specific patient cohorts with diabetes and/or dyslipidaemia.

A reduction in the urge for palatable food following thylakoid treatment has been observed earlier, in a study where overweight women were treated for three months with a daily supplement of thylakoids [8]. In contrast to the present study, there was no caloric restriction in the weight loss programme during the 12 weeks of intervention, only a recommendation to eat no more than three meals per day. With such a regimen, the thylakoid-treated women lost significantly more weight than controls (0.41 kg/week in the thylakoid group and 0.29 kg/week in the control group). Hence, thylakoids are more efficient for weight loss in the absence of any caloric restriction. In the 12-week study, there was also a reduced urge for palatable food in the thylakoid treated group that was sustained throughout the treatment period [8]. Thus thylakoids appear to be able to suppress hunger, in particular hedonic hunger, irrespective of caloric restriction.

Previous studies have demonstrated that thylakoids inhibit fat digestion transiently due to a reversible inhibition of lipase/colipase in the intestine [11,14,15]. Through this effect the whole gastrointestinal processing of food is extended. This is likely the explanation for the suppression of ghrelin levels following thylakoid consumption [9,16]. The prolonged gastro-intestinal food digestion also explains the increased release of CCK [7,9] and GLP-1 by thylakoids [8]. Other effects are a prolonged uptake of glucose [16-18] and an improved intestinal microflora [18]. The differences between the thylakoid and the control groups found in the present study may be related to one or several of these effects of thylakoids in the intestine.

The reduced feelings of hunger could thus be due to the suppression of ghrelin and/or an increased release of CCK or GLP-1 by thylakoids. Likewise, the observed suppression of urge for chocolate could be an effect of the above gut hormones. Most importantly, the suppressed hunger and urge for palatable food remained throughout the diet intervention, even following body weight loss.

In conclusion, the present study, even though limited by a short time-period of intervention and a relatively small number of participants, demonstrates that a caloric restriction conceals the effect of thylakoids on body weight loss. However, the effects on homeostatic and hedonic hunger remain. We suggest that thylakoid treatment may alleviate some of the strains coupled to caloric restriction during body weight loss programs.

Conflict of Interest Statement

CEA is a scientific advisor for Greenleaf Medical AB and a founder of Thylabisco AB.

Acknowledgements

The study was funded by grants from FORMAS, VINNOVA, the Carl Trygger Foundation, the Royal Physiographic Society of Lund and the Swedish Medical Research Council. ELS and CM planned and performed all parts of the study as well as wrote the manuscript. DE assisted in analysing blood hormones and in writing, LS and MF assisted in performing the study, EE assisted in the statistical analysis and the writing the manuscript and KP performed statistical calculations. CEA assisted in planning the study and writing the manuscript. All authors have been involved in finalising the paper, and have given final approval of the submitted and published version.

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Paper III

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Research report

Body weight loss, reduced urge for palatable food and increased release of GLP-1 through daily supplementation with green-plant membranes for three months in overweight women ☆

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ABSTRACT

The frequency of obesity has risen dramatically in recent years but only few effective and safe drugs are available. We investigated if green-plant membranes, previously shown to reduce subjective hunger and promote satiety signals, could affect body weight when given long-term. 38 women (40–65 years of age, body mass index 25–33 kg/m^2) were randomized to dietary supplementation with either green-plant membranes (5 g) or placebo, consumed once daily before breakfast for 12 weeks. All individuals were instructed to follow a three-meal paradigm without any snacking between the meals and to increase their physical activity. Body weight change was analysed every third week as was blood glucose and various lipid parameters. On days 1 and 90, following intake of a standardized breakfast, glucose, insulin and glucagon-like peptide 1 (GLP-1) in plasma were measured, as well as subjective ratings of hunger, satiety and urge for different palatable foods, using visual analogue scales. Subjects receiving green-plant membranes lost significantly more body weight than did those on placebo ($p < 0.01$). Mean weight loss with green-plant extract was 5.0 ± 2.3 kg compared to 3.5 ± 2.3 kg in the control group. Consumption of green-plant membranes also reduced total and LDL-cholesterol ($p < 0.01$ and $p < 0.05$ respectively) compared to control. Single-meal tests performed on day 1 and day 90 demonstrated an increased postprandial release of GLP-1 and decreased urge for sweet and chocolate on both occasions in individuals supplemented with green-plant membranes compared to control. Waist circumference, body fat and leptin decreased in both groups over the course of the study, however there were no differences between the groups. In conclusion, addition of green-plant membranes as a dietary supplement once daily induces weight loss, improves obesity-related risk-factors, and reduces the urge for palatable food. The mechanism may reside in the observed increased release of GLP-1.

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Introduction

Overweight subjects have an increased liking for palatable food (Blundell & MacDiarmid, 1997; Ettinger, Duizer, & Caldwell, 2012), inducing hyperphagia and the obese state. Whether this is a cause or a consequence of obesity is not known (Berthoud & Zheng, 2012). Gut hormones have been demonstrated to regulate the liking and wanting of sweet and fatty foods, ghrelin acting to stimulate wanting (Egecioglu et al., 2010) and GLP-1 to suppress liking (Shin et al., 2008). A common adaptive response upon weight loss by dieting in the obese is an increased hunger and liking for palatable food, in part due to reduced secretion of satiety hormones, like leptin and GLP-1 (Adam, Jocken, & Westerterp-Plantenga, 2005; Blundell & Gillett, 2001). However, bariatric surgery does not trigger the same response. Instead a diminished preference for sweet and fatty foods is observed along with a reduction in ghrelin secretion and an increase in the secretion of GLP-1 and PYY (Miras et al., 2012). The increase in satiety hormone secretion from the distal small intestine following bariatric surgery is partly explained by food digestive products directly reaching the distal part of the small intestine.

We have found that retardation of fat digestion through reversible pancreatic lipase/colipase inhibition by chlorophyll-containing membranes found in green plants, leads to increased satiety and

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Table 1
Anthropometric, body composition and fasting serum parameters at baseline and at the end of the study. A decrease in body weight, BMI, FFM (fat free mass), body fat, % body fat, waist and leptin concentrations was found over the course of the study. Alterations in body weight were dependent on treatment. Fasting glucose and insulin did not change during the study.

	Control group			Thylakoid group		
	Baseline (n = 19)	End of study (n = 17)	Change (n = 17) start → end	Baseline (n = 19)	End of study (n = 19)	Change (n = 19) start → end
Age (yrs)	54.6 ± 7.5			50.7 ± 7.0		
Body weight (kg)	80.2 ± 8.2	76.3 ± 8.9[a,b]	−3.5 ± 2.3	79.9 ± 10.8	75.0 ± 10.5[a,b]	−5.0 ± 2.3
BMI (kg/m^2)	28.6 ± 2.3	27.6 ± 2.7[a]	−0.7 ± 0.6	28.9 ± 2.2	27.1 ± 2.2[a]	−1.0 ± 0.5
FFM (kg)	47.6 ± 3.2	46.8 ± 3.3[a]	−0.6 ± 1.1	47.6 ± 4.9	46.3 ± 4.7[a]	−1.3 ± 1.4
Body fat (kg)	32.6 ± 6.2	29.5 ± 6.8[a]	−2.9 ± 1.9	32,3 ± 6,8	28.7 ± 7.0[a]	−3.7 ± 2.5
Body fat (%)	40.4 ± 4.1	38.2 ± 4.9[a]	−2.4 ± 2.1	40.1 ± 4.0	37.8 ± 4.8[a]	−2.3 ± 2.3
Waist (cm)	97.6 ± 9.6	93.3 ± 8.6[a]	−4.4 ± 3.5	93.9 ± 6.7	88.0 ± 7.2[a]	−5.9 ± 3.5
Insulin (mIE/L)	10.6 ± 5.0	9.1 ± 4.5	−0.2 ± 3.4	8.4 ± 4.0	8.3 ± 3.5	−0.1 ± 3.4
Glucose (mmol/L)	5.30 ± 0.50	5.24 ± 0.47	−0.08 ± 0.26	5.34 ± 0.69	5.1 ± 0.56	−0.24 ± 0.46
Leptin (ng/mL)	44.0 ± 22.6	32.3 ± 22.1[a]	−10.5 ± 12.2	42.9 ± 17.7	29.2 ± 17.0[a]	−13.7 ± 17.2

[a] Effect of time, $p < 0.001$, two-way RM ANOVA.
[b] Time and treatment interaction, $p < 0.05$, two-way RM ANOVA.

an elevated release of the gut satiety hormone CCK in response to a high fat diet (Albertsson et al., 2007; Kohnke et al., 2009). In man, the uptake of fatty acids into the circulation is retarded (Kohnke et al., 2009), supporting a delay of fatty acid absorption. In addition to increased satiety following intake of a meal high in fat (Kohnke et al., 2009), green-plant membranes reduce hunger after a carbohydrate-rich meal, with a mechanism suggesting a release of incretin hormones (Stenblom et al., 2013). Long-term studies in rat and mouse have demonstrated that daily consumption of green-plant membranes reduced body weight gain, body fat mass and blood lipid levels (Kohnke et al., 2009) (Montelius et al., 2013). The effects of long-term treatment with green-plant membranes in humans are unknown.

The chlorophyll-containing parts of the green plant cell, called thylakoids, contain a hundred different membrane proteins, galactolipids and sulpholipids as well as various vitamins (A, E and K) and antioxidants like carotenoids, lutein, zeaxantin and chlorophyll. They are thus a mixture of bioactive compounds that could be responsible for the retardation of fat digestion previously described (Albertsson et al., 2007). Most importantly, following ingestion of the green-plant membranes, fat digestion is prolonged, but nevertheless complete, and in the end the thylakoids themselves are also digested. Therefore, no steatorrea or rapid excretion of fat is seen following intake of green-plant membranes, in contrast to the effects of irreversible lipase inhibitors (Goedecke, Barsdorf, Beglinger, Levitt, & Lambert, 2003).

In this study we were interested to find out if long-term treatment with green-plant membranes through its satiating effects could affect body weight and metabolic parameters related to obesity. We were also interested in measuring the release of GLP-1, a gut hormone promoting satiety (Flint, Raben, Astrup, & Holst, 1998; Holst, 2007). Based on the fact that GLP-1 regulates reward-induced behaviour for food (Dickson et al., 2012; Egecioglu, Engel, & Jerlhag, 2013a, 2013b; Egecioglu et al., 2013), the urge for sweet, salt and fat was evaluated during one day meal tests in the beginning and the end of the study.

Materials and methods

Subjects

Fifty-three healthy non-smoking women, aged 40–65 years, with a BMI between 25 and 33 were recruited through public advertisement and volunteered for screening, after which 38 women were enrolled in the study (detailed flow chart, Supplementary Fig. S1). The exclusion criteria were diabetes, food allergies, irritable bowel syndrome, food intolerance and recent use of antibiotics. The subjects were not vegetarian and had not followed any diet for the last three months. Baseline characteristics of the 38 women are shown in Table 1.

Experimental study design

The study was conducted at the Overweight and Diabetes Unit and at the Division of Occupational and Environmental Medicine, Skåne University Hospital (SUS), Lund, Sweden, and designed as a single-blinded, single-centered, randomized and placebo-controlled, 12-week diet intervention study. The participants were divided into two groups (n = 19 per group) by a non-algorithmic randomization method (ballot, performed by CM). Normal distribution within and between the groups based on body weight, BMI, blood glucose, insulin, triacylglycerol (TG) and cholesterol (total and LDL) was confirmed after randomization.

Every third week the participants arrived to the laboratory in the morning for anthropometric measurements and blood sampling in the fasted state. Waist circumference was measured with a non-stretchable tape, and body weight, fat-mass and fat free mass (FFM) were measured with a body composition analyser (TANITA-BC 418 MA, Amsterdam, The Netherlands). Fasting blood samples were taken through a venous catheter in the arm.

For meal studies the subjects arrived to the laboratory in the morning in the fasted state on day 1 and day 90 of the supplementation period. After filling in questionnaires, a venous catheter was inserted in the arm for blood sampling. Thereafter, the subjects received a 50 g blueberry drink with or without 5 g of green-plant membranes. Five minutes later, a standardized breakfast was served consisting of vanilla yoghurt, muesli, bread, butter, cheese, juice and coffee or tea (Table 2). The macronutrient composition of the breakfast was 60 E% carbohydrate, 28 E% fat and 12 E% protein. The subjects were instructed to eat all the food within 15 minutes.

Venous blood samples for glucose, insulin, ghrelin and GLP-1 analyses were taken at time point zero, ie prior to breakfast, and thereafter at 15, 30, 45, 60, 90, 120, 180, 240, 300 and 360 minutes. Blood samples were collected in chilled 6 mL Vacutainer EDTA-plasma tubes for all analyses, with the addition of 100 µL DPP-IV inhibitor (Cat # DPP4-010; Millipore Corp. Billerica, MA, USA) for GLP-1 analysis. The tubes were centrifuged at 4 °C, and plasma was immediately stored at −80 °C until analysis.

At time point 360 minutes lunch was served consisting of a pizza (Grandiosa Extra Godfather, Procordia AB, Eslöv, Sweden), water and coffee/tea. The subjects were told to eat and drink until satisfied. Thereafter the subjects left the laboratory. At time point 660 minutes

Table 2
Composition of the breakfast.

Ingredients	Amount	Caloric content
Vanilla yoghurt, 2.5% fat	150 g	121,5 kcal
Muesli with tropical fruits	1 dl = 45 g	185 kcal
White bread	1 piece = 40 g	98 kcal
Butter	1 tsp	33 kcal
Cheese, 28% fat	2 pieces = 20 g	77 kcal
Red bell pepper	2 pieces = 20 g	6 kcal
Orange juice	2 dl	86 kcal
Coffee/tea (with 1 tbsp milk 0.5% fat if preferred)	1 cup	~10 kcal
Blueberry drink (± green-plant membrane supplement)	50 g	45 kcal
Total		660 kcal

the participants consumed a third meal, consisting of salmon and potatoes (Laxpytt, Findus AB, Eslöv, Sweden).

Green-plant membranes

The green-plant membranes (thylakoids) used in the present study was provided by Greenleaf Medical AB, Stockholm, Sweden, prepared from baby spinach leaves using the pH-method, as described (Emek et al., 2010), followed by drum drying. 100 g of green-plant membranes contain 23.5 g protein, 11.9 g fat, 41.7 g carbohydrate, 3.5 g salt, 3000 mg chlorophyll, 27.9 mg lutein, 730 ug zeaxantin, 4 760 ug betakaroten, 21 ug vitamin A, 1313 ug vitamin K, 6.07 mg vitamin E and 166 ug folic acid.

The green-plant membranes were mixed with 2.8 g rapeseed oil (Zeta, Di Luca & Di Luca AB, Stockholm, Sweden) and 50 g of blueberry soup (Ekströms original, Procordia Food AB, Eslöv, Sweden) and given to the participants from day 1. The control group received 2.8 g rapeseed oil mixed with 50 g blueberry soup. The blueberry drinks with and without the green-plant membranes contained 209 kJ/50 kcal versus 188 kJ/45 kcal respectively. The blueberry drinks were taken before breakfast every day.

Diet regimen

In the period between the test-days (days 1 and 90) the participants were told, besides taking the daily blueberry drink with or without green-plant membranes, to consume three meals a day containing a large quantity of vegetables and fruit, and to avoid sweet drinks and snacks. They were also told to exercise at low intensity 30 minutes each day.

Questionnaires

Questionnaires constructed as Visual Analogue Scale (VAS) (Flint, Raben, Blundell, & Astrup, 2000) were used to measure sensations of hunger, fullness and urge for specific food items during the whole day on the first and last days of the study. The questionnaires were filled in at time points 0, 15, 60, 120, 180, 240, 300 and 360 minutes, when lunch was served, and thereafter at time points 420, 480, 540, 600 and 660 min. The VAS-questionnaires included pictures, to facilitate the evaluation of the urge for specific food items. For high carbohydrate snack a picture of a sandwich was presented; for salt a picture of potato chips; for sweet a picture of candy and for fat and sweet; a picture of chocolate was presented. Written instructions were given on the front page of the questionnaire, and each subject was individually instructed in how to fill out the questionnaire to avoid misinterpretation. Questions were followed by a 100 mm line anchored by descriptors on each side of the line. Subjects were instructed to place a vertical line across the scale, thus rating how strong their sensations were at every time point. Ratings were scored as mm between "not at all" and the individual subjects mark.

Biochemical analyses

Glucose, insulin, TG, cholesterol (total, LDL and HDL) in blood were analysed by standard methods at the Department of Clinical Chemistry at Skåne University Hospital (Lund, Sweden). Leptin was measured with a RIA human/multi species kit using the double antibody/PEG technique (XL-85K, Millipore Corporation, Billerica, MA, USA). Active GLP-1 was measured using EGLP-35K (Millipore, Molsheim, France) and for measures of total ghrelin, EZGRT-89K (Millipore, Molsheim, France) was used.

Ethics

The Ethics Committee in Lund, Sweden approved the study (2006/361). The trial was conducted in accordance with the Declaration of Helsinki. All subjects gave written and oral consent before the study began.

Statistics

Participants were included in each analysis by original assigned groups. All statistical analyses were done using the Prism version 6, statistical software (GraphPad Software, Inc, San Diego, CA, USA). Anthropometric measures and fasting leptin, insulin, glucose and cholesterol concentrations over the course of the study were analysed by two-way repeated measures (RM) ANOVA. Differences at individual time points and changes from baseline at the end of the study were analysed with unpaired t-test. Area under the curve (AUC), time to peak value, increase peak value versus baseline (%) and baseline values for glucose, insulin, ghrelin and GLP-1 during days 1 and 90 were analysed by unpaired t-test or Mann–Whitney U test.

The variations in VAS ratings over time were analysed with a two-way RM ANOVA in order to test time, treatment and time × treatment interaction for the first and the last day respectively. Differences in VAS at individual time points were analysed with unpaired t-tests.

In figures and text data are expressed as mean ± SE or for peak times as median followed by interquartile range. Statistics are based on Mann–Whitney U test if not otherwise stated. p-values <0.05 were considered statistically significant.

Results

All 38 participants are included in the statistical analysis of subjective appetite and hormonal release on day 1. One participant did not follow the dietary and exercise recommendations given and one individual moved abroad during the study and were therefore not included in the statistical analysis at day 90 or in the analysis of longitudinal changes.

Body weight, anthropometric and plasma parameters

Supplementation with green-plant membranes for three months produced an increased weight loss compared to control (Fig. 1). For absolute body weight, an interaction between time and treatment was found over the course of the study ($F(4, 136) = 2.94$, $p < 0.05$, two-way RM ANOVA, Table 1). The ANOVA analysis of weight loss revealed an effect of treatment ($F(1, 34) = 4.18$, $p < 0.05$, two-way RM ANOVA) and an interaction between time and treatment ($F(4, 136) = 2.94$, $p < 0.05$, two-way RM ANOVA, Fig. 1). The weight loss in the treated group was increased at weeks 6, 9 and 12 (Fig. 1).

Anthropometric measures, FFM, body fat and serum concentration of fasting leptin decreased in both groups over the course of

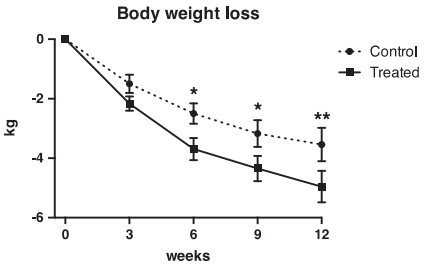

Fig. 1. Body weight loss with and without green-plant membrane supplementation. Dietary green-plant membranes produced a continuous body weight loss that was more marked compared to control at the end of the study. Values are Means ± SE. * $p < 0.05$, ** $p < 0.01$, two-way RM ANOVA followed by unpaired t-test.

the study while fasting glucose and insulin were unaltered (Table 1). No differences between the groups were found (Table 1).

Green-plant membrane supplementation decreased total and LDL-cholesterol compared to control (Fig. 2). An effect of treatment and an interaction between time and treatment were found in total cholesterol ($F(1, 34) = 5.07$, $p < 0.05$ and $F(4, 136) = 3.83$, $p < 0.01$, respectively). Analysis of individual time points revealed that total cholesterol was decreased at 3, 6, 9 and 12 weeks in the thylakoid group compared to control (Fig. 2A). For LDL-cholesterol an effect of treatment was found in the ANOVA analysis ($F(1, 34) = 4.62$, $p < 0.05$) and the levels were decreased at time points 3, 6, 9 and 12 weeks in the treated group (Fig. 2B). HDL-cholesterol and triglycerides were not affected over time or by treatment (Fig. 2C and D).

Glucose, insulin, ghrelin and GLP-1 on day one

In both experimental groups blood glucose increased after breakfast consumption and reached maximal concentration at median time point 15 (15–30) min for control and 30 (15–30) min for the treated group ($p = 0.30$). The glucose concentration was lower at time point 15 min in the treated group compared to control (Fig. 3A).

Plasma insulin increased following breakfast and reached maximum concentrations at median time point 45 (30–45) min for control and 45 (30–45) min for the treated group ($p = 0.33$). Insulin concentrations were lower at time point 15 min in the treated group compared to controls (Fig. 3B).

GLP-1 concentrations increased following breakfast for both experimental groups, reaching a first peak at median time 15 (15.0–33.8) min in the control group and 30 (15.0–30.0) min for the treated group ($p = 0.082$) (Fig. 4A). A second GLP-1 peak occurred at 90 (90.0–120.0) min for both groups respectively ($p = 0.80$). The % maximum increase versus baseline was 2.6 fold higher for the first peak and 1.8 fold higher for the second peak in the treated group compared to control (Fig. 4D). No differences in GLP-1 concentrations were found at baseline, in absolute peak value or at any specific time point between the treated and the control groups (Fig. 4A and C).

Ghrelin concentrations decreased following breakfast in both experimental groups (Fig. 4B). Thereafter both curves reverted back to fasting values at time point 360 min. There was no difference in ghrelin concentrations at any time point (Fig. 4B).

Glucose, insulin, ghrelin and GLP-1 on day 90

Glucose concentrations following breakfast on day 90 were similar in the control group and in the treated group (Fig. 5A). Maximum

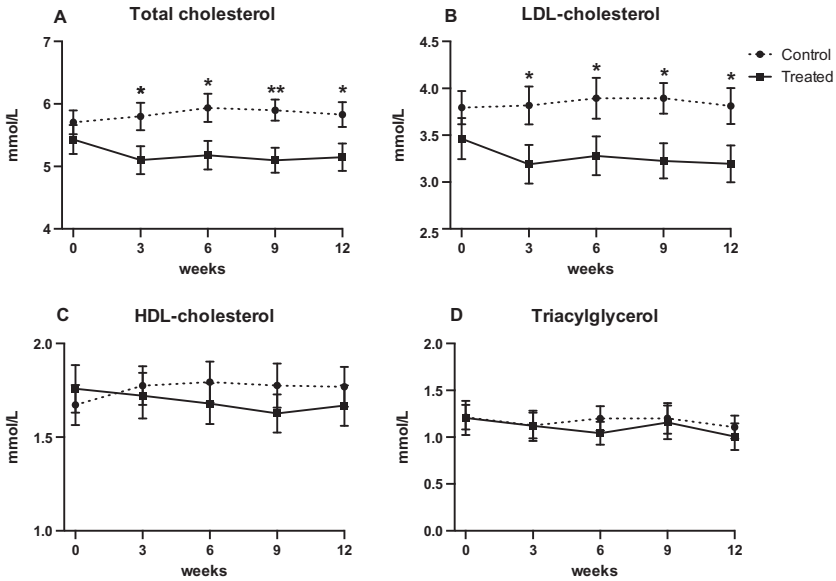

Fig. 2. Blood lipid parameters with and without supplementation with green-plant membranes. Green-plant membranes decreased total and LDL-cholesterol while HDL-cholesterol and TG were unaltered. Values are Means ± SE. * $p < 0.05$, ** $p < 0.01$, two-way RM ANOVA followed by unpaired t-tests.

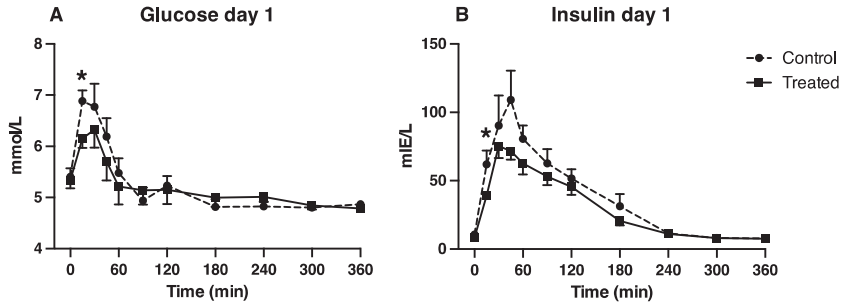

Fig. 3. Postprandial glucose and insulin day 1. Blood glucose (A) and plasma insulin (B) in response to breakfast served with or without green-plant membranes on the first day of the intervention study. Values are Means ± SE. * $p < 0.05$, unpaired t-tests.

concentrations were reached at median time point 15 (15–30) min in the control group and at 30 (15–30) min in the treated group ($p = 0.16$).

Insulin concentrations were increased in a similar way in the two experimental groups after breakfast on the last day (Fig. 5B). No difference in the time to reach maximal concentrations was found between the groups (control: 30, (30–45) min, treated: 30 (30–45) min, $p = 0.69$).

GLP-1 was released after breakfast on day 90 in both experimental groups (Fig. 6A). The concentrations of GLP-1 were similar at baseline and increased at time points 30, 45 and 60 min in the treated group compared to control as was the AUC_{0-60} (control: 393.1; 290.1–595.1, treated: 565.2; 448.37–747.61, $p = 0.039$). Furthermore, the maximum peak value for the initial peak was elevated in the treated group compared to control while no difference in the absolute values for the second peak was seen ($p = 0.14$, Fig. 6C). The initial GLP-1 peak was reached at median time point 15 (15–30) min in the control group and at 30 (15–33.75) min in the treated group ($p = 0.10$). The second GLP-1 peak occurred at median time point 120 (90–120) min for the control group and 90 (60–120) min

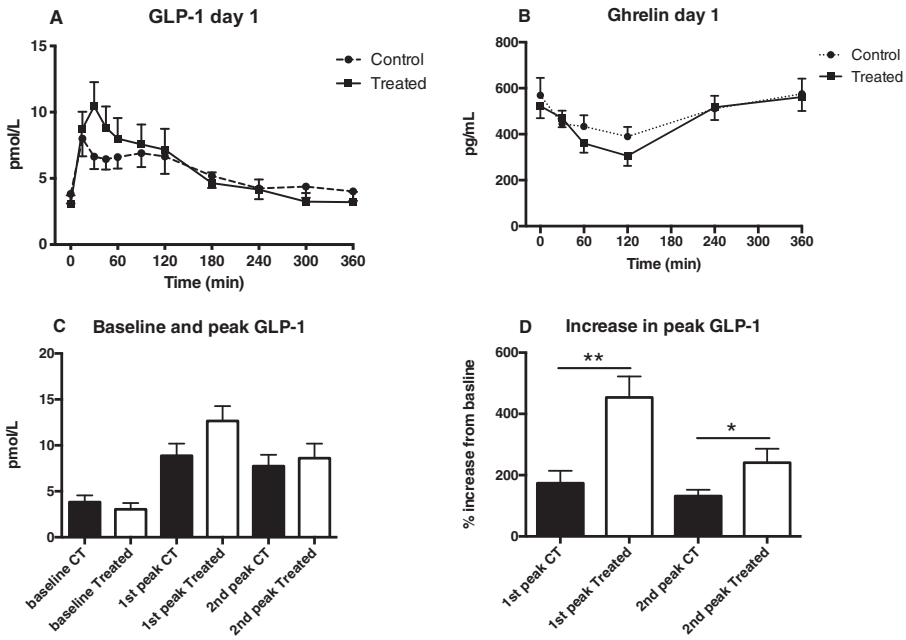

Fig. 4. Postprandial GLP-1 and ghrelin day 1. Plasma concentrations of GLP-1 (A), ghrelin (B), baseline and peak values of GLP-1 (C), percentage increase in peak values of GLP-1 (D) on the first day of the study. Values are Means ± SE. * $p < 0.05$, ** $p < 0.01$, Mann–Whitney U test.

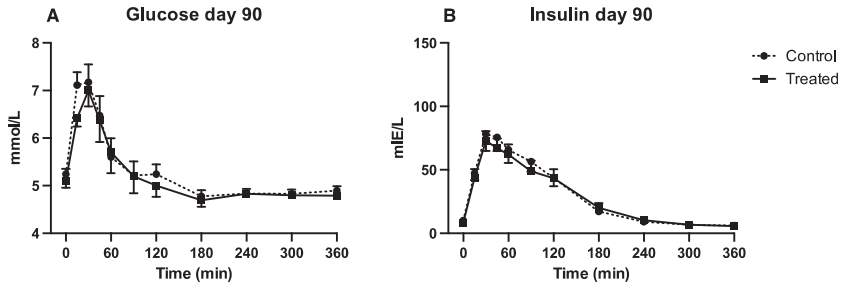

Fig. 5. Postprandial glucose and insulin day 90. Blood glucose (A) and plasma insulin (B) concentrations following breakfast on day 90. Values are Means ± SE.

in the treated group ($p = 0.28$). The % maximum difference versus baseline was not different in the treated group on day 90 compared to control (Fig. 6D).

Ghrelin concentrations were suppressed following breakfast in both the treated group and the control group (Fig. 6B). Thereafter the ghrelin concentrations rose to original values at time point 240 min. There was no difference in response between the two groups (Fig. 6B).

Sensation of hunger, satiety, urge for specific foods and food intake on day 1

Subjective ratings for hunger, satiety and urge for specific food items on day one are presented in Fig. 7. All VAS-ratings were influenced by time ($p < 0.001$ respectively, two-way RM ANOVA) such that hunger and urge for specific food items were increased before mealtime and to a varied extent decreased following meals

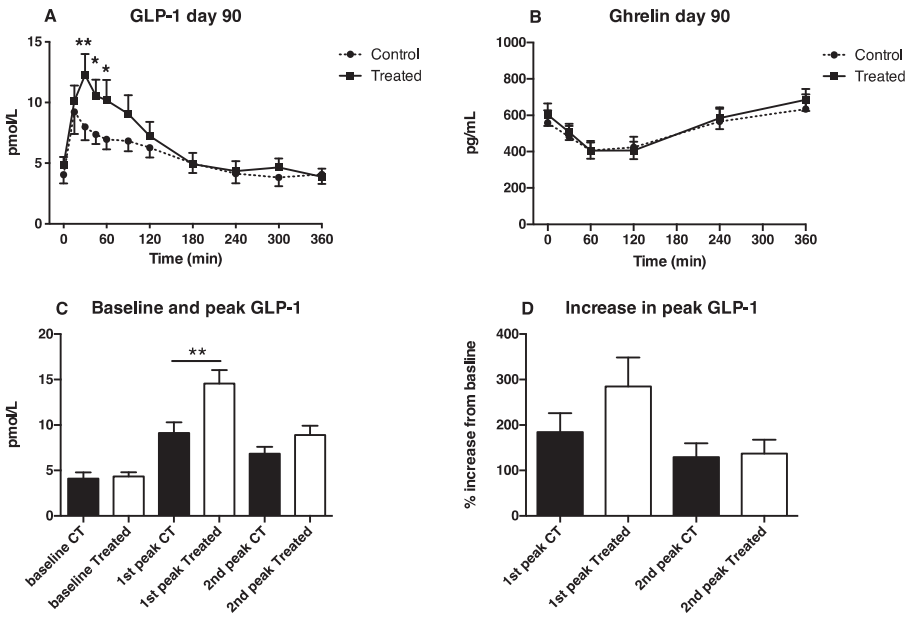

Fig. 6. Postprandial GLP-1 and ghrelin day 90. Plasma concentrations of GLP-1 (A), ghrelin (B), baseline and peak values of GLP-1 (C) and percentage increase in peak values of GLP-1 (D) in response to a breakfast on the last day of the intervention study in overweight women. Values are Mean ± SE. * $p < 0.05$, ** $p < 0.01$, Mann–Whitney U test.

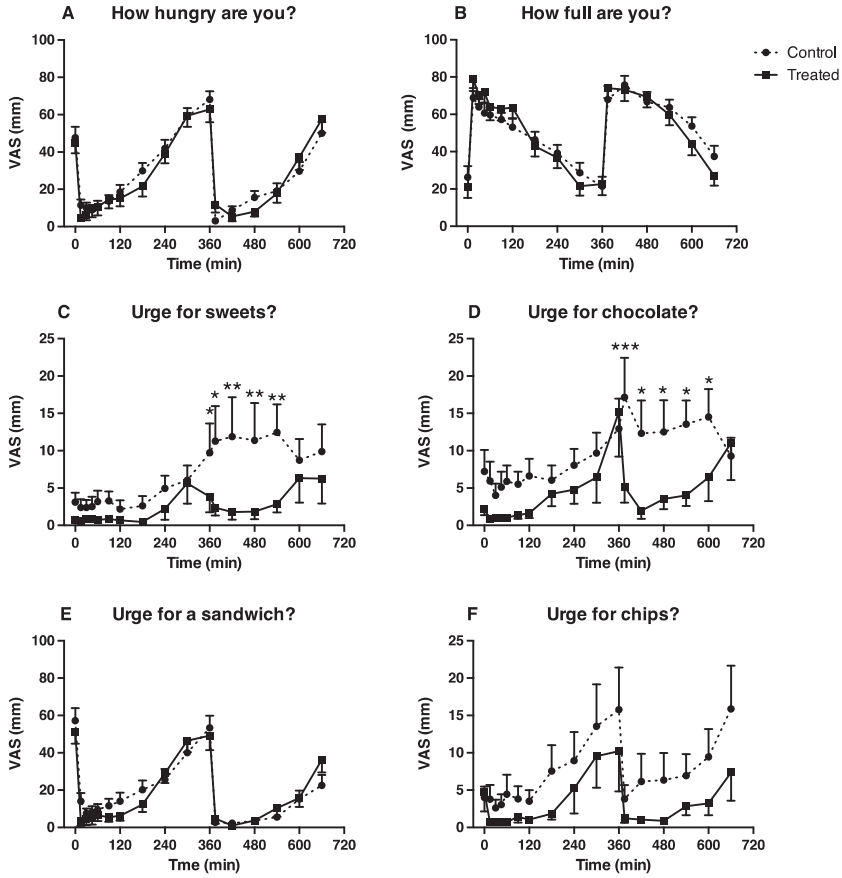

Fig. 7. Hunger, satiety and urge for palatable food day 1. VAS ratings of hunger (A), satiety (B) and urge for sweets (C), chocolate (D), sandwich (E) and chips (F) during day 1. Values are given as Mean ± SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-way RM ANOVA followed by unpaired t-tests.

while ratings of satiety demonstrated the opposite curve (Fig. 7A and B). There was also an effect of treatment on the ratings for the urge for sweet and chocolate (sweet; $F(1, 36) = 5.79$, $p < 0.05$ and chocolate; $F(1, 36) = 5{,}892$, $p < 0.05$, two-way RM ANOVA, Fig. 7C and D). The urge for sweet was lower in the treated group at the time point prior to lunch and remained lower for the following 3 hours compared to control. Ratings for chocolate were lower immediately following lunch in the treated group and remained lower in the following 3 hours compared to the controls. In contrast, the ratings of hunger, satiety, urge for a high carbohydrate snack or salt were not different between the treated and the control groups (Fig. 7A, B, E and F). No time by treatment interaction was found in any of the VAS ratings on day 1. Total caloric intake during day 1, excluding the standardized breakfast, was similar between the groups (control: 1366 ± 48 kcal, green-plant membranes: 1270 ± 65 kcal, $p = 0.24$, unpaired t-test).

Sensation of hunger, satiety and urge for specific foods on day 90

Subjective ratings for hunger, satiety and urge for specific food items on day 90 are presented in Fig. 8. An effect in ratings over time was found for all VAS measures ($p < 0.001$ respectively, two-way RM ANOVA). Similar to day one there was an effect of treatment on the ratings for the urge for chocolate ($F(1, 34) = 6.2$, $p < 0.01$, two-way RM ANOVA, Fig. 8D). Furthermore, a time by treatment interaction was found for both the urge for sweet ($F(16, 544) = 1.88$, $p = 0.05$, two-way RM ANOVA) and for the urge for chocolate ($F(16, 544) = 1.91$, $p < 0.05$, two-way RM ANOVA). The urge for sweet and fat/sweet (chocolate) was lower in the treated group prior to lunch on day 90 and remained low in the treated group for the rest of the duration of the session compared to control. The ratings of the urge for a high carbohydrate snack or salt were not different between the treated and the control groups (Fig. 8E and F). Caloric intake

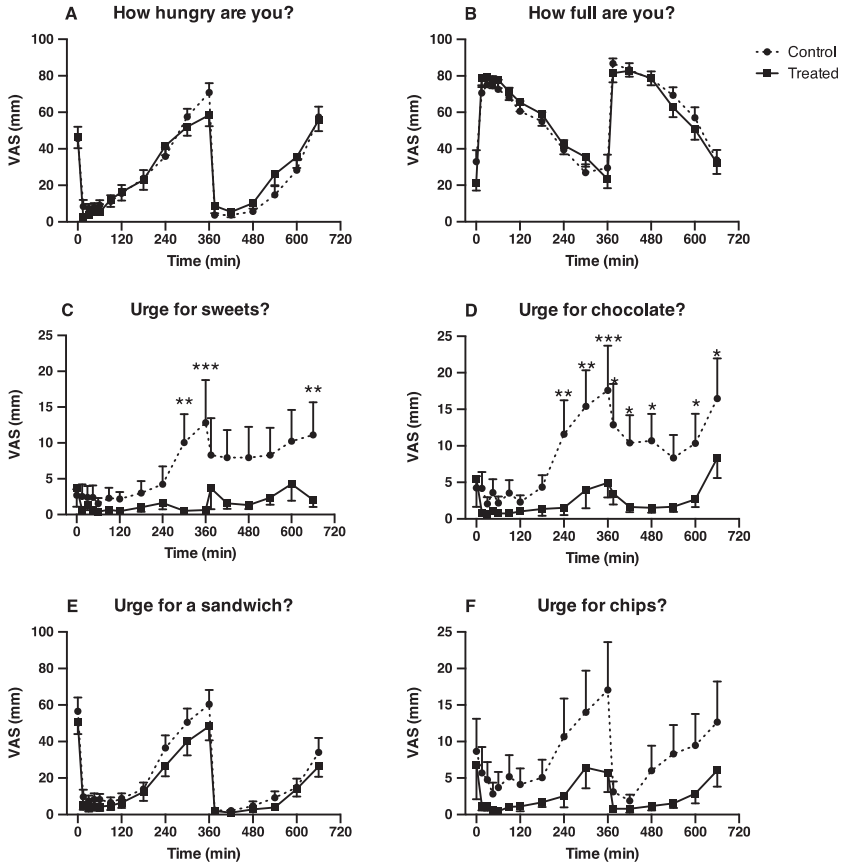

Fig. 8. Hunger, satiety and urge for palatable food day 90. VAS ratings of hunger (A), satiety (B) and urge for sweets (C), chocolate (D), sandwich (E) and chips (F) during the last day of the intervention study. Values are given as Mean ± SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-way RM ANOVA followed by unpaired t-tests.

during day 90, excluding the standardized breakfast, was similar between the groups (control: 1241 ± 55 kcal, green-plant membrane: 1297 ± 104 kcal, $p = 0.65$, unpaired t-test).

No adverse events or effects were reported in any of the groups.

Discussion

In this study we demonstrated for the first time that green-plant membranes, when added to the diet daily for three months, markedly reduced body weight in overweight women. In addition, total and LDL-cholesterol levels were reduced compared to control. These findings are supported by animal studies, where body weight loss and blood lipid lowering effects have been observed (Kohnke et al., 2009; Montelius et al., 2013).

The mechanism underlying the effect of green-plant membranes to reduce body weight was investigated by two test-meals, one at the start and one at the end of the study. These demonstrated a reduced urge for sweet and fatty foods. The decreased urge following treatment was apparent already on day 1, as well as on day 90, indicating both an immediate and a sustained effect of green-plant membranes. We also observed an increased release in GLP-1 postprandially both on day 1 and day 90, suggesting that green-plant membranes influence pathways that are important for satiety and for the determination of the reward value of food. Furthermore, given that diet-induced weight loss has been shown to reduce postprandial GLP-1 levels (Jarry et al., 2006), it is remarkable that the increased secretion of GLP-1 in the treated group was sustained during weight loss in the present study.

The effect of green-plant membranes on body weight loss (6.3%) after 12 weeks was comparable to the effect of treatment with the GLP-1 analogue liraglutide (Astrup et al., 2009). Thus increasing the endogenous release of GLP-1 may be as efficient for obesity treatment as pharmacological agents. It is very likely however that treatment with green-plant membranes involves other additional mechanisms for body weight reduction.

Palatability and pleasantness are important determinants of what and how much we eat (Erlanson-Albertsson, 2005). Increased intake of energy-dense food high in fat and sugar adds energy but also disrupts functions in the brain controlling appetite and motivation (Berthoud, 2012). In the control group the urge for palatable food increased prior to lunch, was reduced following lunch and thereafter reverted. With consumption of green-plant membranes a decreased urge for palatable food was noted prior to lunch, which persisted following lunch despite no further consumption of green-plant membranes. The mechanism may be related to the increased release of GLP-1.

Release of GLP-1 from enteroendocrine L-cells in the distal intestine occurs through nutrients present in the lumen of the intestine and is dependent on the nutrient composition. The most potent secretagogues for GLP-1 are carbohydrate and fat. The release of GLP-1 following administration of pure glucose results in one peak, occurring at around 15 min after oral glucose load (Steinert et al., 2011). Following consumption of a mixed diet with carbohydrate, fat and protein the release of GLP-1 may be twofold, one early peak at time point 15 minutes and one later peak, at 90 minutes. Rapid release of GLP-1 occurs through neuronal activation and/or endocrine factors of the proximal-distal loop of the intestine and the second peak through direct interaction of nutrients with the enteroendocrine L-cells in the distal ileum.

In the present study the peak concentrations of initial GLP-1 release occurred at time point 30 min in the treated group and at 15 min in the control group, suggesting that the green-plant membranes may interfere with factors that stimulate rapid GLP-1 release. The second peak of GLP-1 was also increased on day 1 in the treated group. A possible mechanism for these effects of the green-plant membranes to increase GLP-1 secretion could be related to CCK. Ingestion of green-plant membranes has been found to release CCK, demonstrated both in rodents and in man (Kohnke et al., 2009) and the importance of CCK for mediating GLP-1 release is supported by studies where blockade of CCK-receptors markedly reduced fatty acid-stimulated GLP-1 secretion (Beglinger et al., 2010). Furthermore, the green-plant membranes retard fat digestion without causing an irreversible lipase inhibition (Albertsson et al., 2007). This is important, since hydrolysis of fat is required for the GLP-1 releasing effects of fat and free fatty acid infusion directly into the intestinal lumen cause a substantial release of GLP-1 (Beglinger et al., 2010). In contrast, orlistat, that irreversibly inhibits fat digestion, leads to a lower release of GLP-1 and CCK (Beglinger & Degen, 2004; Ellrichmann et al., 2008). The mechanism for the green-plant membrane-induced GLP-1 release could thus be a direct luminal effect of fatty acids on enteroendocrine cells via prolonged digestion and absorption of dietary fat and/or an indirect effect mediated through neurohumoral signals such as CCK. In rodents green-plant membranes have been demonstrated to act in a pre-biotic way, changing the intestinal microflora. The release of GLP-1 by these bacterial products via short chain fatty acids (Yadav, Lee, Lloyd, Walter, & Rane, 2013) may be an additional explanation for the sustained effect of green-plant membranes over time.

Various mechanisms influence reward in relation to food, among these are gut appetite hormones, such as ghrelin (Abizaid et al., 2006; Egecioglu et al., 2010; Jerlhag et al., 2006; Merkestein et al., 2012; Skibicka, Hansson, Egecioglu, & Dickson, 2012) and GLP-1 (Alhadeff, Rupprecht, & Hayes, 2012; Dickson et al., 2012). Analogues of GLP-1 have been shown to decrease preference and consumption for palatable foods in both humans and rodents (Inoue et al., 2011; Raun et al., 2007; Zhang et al., 2013). These studies have been extended to demonstrate that GLP-1 analogues suppress sucrose-induced reward and motivation in rats (Dickson et al., 2012). Furthermore, blockade of GLP-1 receptor signalling diminishes food reward-related behaviour in rats indicating that endogenous GLP-1 signalling is involved in the regulation of food reward (Alhadeff et al., 2012). The effect of green-plant membranes to reduce the urge for sweet and fat may thus be linked to the elevation of GLP-1 concentrations and/or alterations of peak GLP-1 levels. Ghrelin is known to stimulate reward-related behaviour and food preference (Egecioglu et al., 2010; Merkestein et al., 2012). Green-plant membranes have previously been demonstrated to decrease ghrelin concentrations in humans and pigs (Kohnke et al., 2009; Montelius et al., 2013) indicating that ghrelin signalling may also be involved in the regulation of urge for palatable food by green-plant membranes. However, in this study we did not find any effects on ghrelin secretion by the green-plant membranes.

Another general mechanism important for reward seeking, particularly related to urge for sweet, is glucose and insulin homeostasis, a lowering of blood glucose that leads to search for sweet food (Figlewicz & Benoit, 2009). In this study we found no pronounced effects on glucose or insulin by the green-plant membranes suggesting that insulin and glucose are not central for the treatment-induced effects on the urge for palatable foods.

The urge for palatable food tends to increase over the course of the day and hence any influence on these urges would be more pronounced in the afternoon. Accordingly, inhibition of the urge for palatable food by green-plant membranes was more evident following lunch in the present study. This long lasting reduction could be due to the relative resistance to hydrolysis of green-plant membranes in the intestine (Emek et al., 2011), thereby influencing hormonal release also following lunch. The possible prolonged effect of green-plant membranes to alter gut hormone secretion after a second meal needs to be further elucidated.

The mechanism for the reduction in blood cholesterol is not known, but may involve an increased production of bile salt, needed for the prolonged intestinal fat digestion and fat absorption (Borgstrom & Erlanson, 1978). The LDL-cholesterol lowering effect of green-plant membranes is in the magnitude of the bile acid sequestering therapeutics (Rosenson & Underberg, 2013).

In conclusion, we demonstrate that consumption of chlorophyll-containing parts of green plants, in overweight patients results in significant weight reduction, and reduction in blood cholesterol together with a decreased urge for palatable food. The mechanism suggests an increased meal-related GLP-1 release that sustained during the intervention period. Green-plant membranes may thus be a new agent for control of appetite and body weight.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.endend.2013.05.004.

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Corrigendum

Corrigendum to "Body weight loss, reduced urge for palatable food and increased release of GLP-1 through daily supplementation with green-plant membranes for three months in overweight women" [Appetite 81 (2014), 295–304]

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We wish to correct some aspects the data presentation and analysis in our paper.

First, we failed to identify the measure of variability for the data in Table 1. The data were mean ± SD.

Second, in order to characterize the time course of the effect of green-plant membrane consumption of body weight, we performed ANOVA on the cumulative body-weight changes in the control and green-plant-membrane groups after 3, 6, 9 and 12 weeks. Our question would have been better addressed by ANOVA of the interval body-weight changes (0–3 wk, 3–6 wk, 6–9 wk, 9–12 wk). We have now done this, but the outcome was not significant, $F(1,34) = 3.35$, $p = 0.076$.

Third, the primary body-weight outcome of the study was total 12 week body-weight change. Twelve-week body-weight changes were 3.5 ± 0.6 kg (mean ± SEM) in the in 17 control participants who completed the study and 5.0 ± 0.5 kg in the group ingesting green-plant membranes, a significant difference, $t(34) = 1.83$, $p = 0.038$. A revised Table 1 now displays the baseline measures for the 17 control participants who completed the study.

We also re-analysed the plasma lipid data using a Bonferroni correction for the post-hoc t-tests. This resulted in no differences from the reported values regarding HDL and TAG. For LDL cholesterol, post-hoc analysis of the effect of treatment showed no significant effect of treatment at any of the time points. For total cholesterol there was a significant effect of treatment to lower total cholesterol at the 9 week time-point.

The authors would like to apologise for any inconvenience caused.

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Paper IV

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Research report

Consumption of thylakoid-rich spinach extract reduces hunger, increases satiety and reduces cravings for palatable food in overweight women ☆

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ABSTRACT

Green-plant membranes, thylakoids, have previously been found to increase postprandial release of the satiety hormone GLP-1, implicated in reward signaling. The purpose of this study was to investigate how treatment with a single dose of thylakoids before breakfast affects homeostatic as well as hedonic hunger, measured as wanting and liking for palatable food (VAS). We also examined whether treatment effects were correlated to scores for eating behavior. Compared to placebo, intake of thylakoids significantly reduced hunger (21% reduction, $p < 0.05$), increased satiety (14% increase, $p < 0.01$), reduced cravings for all snacks and sweets during the day (36% reduction, $p < 0.05$), as well as cravings for salty (30%, $p < 0.01$); sweet (38%, $p < 0.001$); and sweet-and-fat (36%, $p < 0.05$) snacks, respectively, and decreased subjective liking for sweet (28% reduction, $p < 0.01$). The treatment effects on wanting all snacks, sweet-and-fat snacks in particular, were positively correlated to higher emotional eating scores ($p < 0.01$). The treatment effect of thylakoids on scores for wanting and liking were correlated to a reduced intake by treatment ($p < 0.01$ respectively), even though food intake was not affected significantly. In conclusion, thylakoids may be used as a food supplement to reduce homeostatic and hedonic hunger, associated with overeating and obesity. Individuals scoring higher for emotional eating behavior may have enhanced treatment effect on cravings for palatable food.

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Introduction

Eating is a great pleasure in life. However, snacking between meals, as well as ingestion of snacks and fast foods, has been associated with weight gain and obesity in all age groups (Garber & Lustig, 2011; Mesas, Muñoz Pareja, López García, & Rodríguez Artalejo, 2012). Since life in general has become more sedentary, overeating of palatable, energy dense foods causes a positive energy balance (Blundell & Cooling, 2000; Yeomans, Blundell, & Leshem, 2004) which may result in an increasing incidence of overweight and obesity worldwide (Blundell & Macdiarmid, 1997; Erlanson-Albertsson, 2005; Finkelstein et al., 2012; WHO, 2015).

The reason for overeating is that hunger has a homeostatic component but also a hedonic part, driven by the rewarding values of food (Berridge, 2009; Berthoud, 2011). Hedonic hunger favors energy-dense palatable food, rich in sugar and fat, for example snacks, pastries, desserts, baked confectionery and sweets – foods typically ingested in between meals and preferred by women (Drewnowski, 1997; Montmayeur, le Coutre, Drewnowski, & Almiron-Roig, 2010). While homeostatic eating is due to energy deficiency, hedonic eating is triggered by the anticipation of pleasure, regardless of energy status. The hedonic hunger is constituted by two components, wanting and liking (Finlayson, King, & Blundell, 2007; Mela, 2006). Wanting represents the anticipation phase, the motivation to eat a food item, and is triggered by cues. Liking is the hedonic reaction of the pleasure experienced through a rewarding orosensory stimuli.

Abbreviations: BMC, Biomedical Centre; E%, energy percentage; TFEQR18-V2, The Three Factor Eating Questionnaire, Revised 18-item, Version 2; VAS, visual analog scale.

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Even though the homeostatic and non-homeostatic pathways are separate, they affect each other (Finlayson et al., 2007). When the reward circuit interacts with the appetite-controlling neurons in the hypothalamus, this can result in up-regulated expression of hunger signals and blunting of satiety signals (Erlanson-Albertsson, 2005). Therefore, ingestion of palatable food, instead of terminating food intake, leads to a maintained drive to eat, with continued eating due to reward rather than energy deficit (Finlayson et al., 2007). Over-eating then becomes a possibility (Berthoud, 2006; Blundell & Macdiarmid, 1997). It has been suggested that individuals who are obese, dieting and/or under stress are more susceptible to hedonic eating (Garber & Lustig, 2011). In emotional eaters, who tend to develop overweight due to over-eating, food craving and consumption of food rich in carbohydrates and fat increase in response to stressors or negative affect (Cappelleri, Bushmakin, & Gerber, 2009; Raspopow, Abizaid, Matheson, & Anisman, 2010.

Obviously, there is a need to prevent overeating to avoid weight gain. One way is to attenuate the hedonic drive in those who experience increased cravings for palatable food.

Green-plant membranes, thylakoids, have been shown to reduce feelings of hunger as well as cravings for palatable food in human participants, during diet intervention and simultaneous weight loss in overweight women (Montelius et al., 2014; Stenblom et al., 2013, 2014). We found that these effects are connected to an altered secretion of appetite regulating hormones, including ghrelin, cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1), in rodents, pigs and humans (Köhnke, Lindbo et al., 2009; Köhnke, Lindqvist et al., 2009; Montelius, Osman et al., 2013; Montelius, Szwiec et al., 2013; Montelius et al., 2014). Based on these findings of suppressed hedonic hunger and increased levels of GLP-1, we were interested to deepen our knowledge of these effects on overweight middle-aged women in a similar group of participants but in a non-laboratory setting. In addition, since emotional eating is associated with overeating of snacks, we were interested to investigate any potential correlation between scores for eating behavior and the treatment effect of thylakoids.

Aim/primary objectives

The aim of this study was to investigate in a non-laboratory setting 1) how treatment with a thylakoid-rich spinach extract (from now on called thylakoids), served as a meal supplement before breakfast, affects subjective ratings of wanting and liking for snacks and sweets, as well as hunger and satiety, using visual analog scales (VAS), 2) to examine how thylakoid treatment affects intake of palatable food from an *ad libitum* buffet of the same sweets and snacks the participants rated their cravings for during the day, and 3) to investigate if the treatment effect of thylakoids is associated with scores for eating behavior.

Materials and methods

Participants

Thirty-two women were recruited through local advertising. The volunteers were assessed for eligibility through a screening procedure including questionnaires evaluating general health and subjective liking for different food products. During the screening process, 6 women were excluded and 26 women included in the study. Inclusion criteria were: women ages 40–70, normal weight or overweight. Exclusion criteria were: Diabetes, illnesses affecting appetite, food allergies or intolerance to food served in the study, or dieting during the last 3 months. Twenty-two participants completed the study and were included in the final analysis (Table 1).

Table 1
Baseline characteristics.

		Median	Interquartile range	Range (min–max)
Age (years)	(n = 22)	54.5	47.0–59.5	40–66
BMI (kg/m^2)	(n = 22)	25.3	24.5–29.4	22.4–36.2
Body fat (%)[a]	(n = 21)	37.2	31.5–40.4	26.9–47.1

[a] Body composition analyzer TANITA-BC 418 MA (Amsterdam, The Netherlands). One participant was excluded from this analysis due to failure of the body composition analyzer.

Eating behavior questionnaire

Prior to the trial days, the participants filled out the Three-Factor Eating Questionnaire Revised 18-item, Version 2 (TFEQ-R18V2) (Cappelleri et al., 2009). This is a revised version of the original Three-Factor Eating Questionnaire (TFEQ), a self-assessment scale used in studies of eating behavior. TFEQ-R18V2 contains 18 questions to measure three types of eating behavior: emotional eating, uncontrolled eating and cognitive restraint. Previous results from a large diverse sample of non-obese, as well as obese participants, showed a good reliability, and the domains were reported to be robust and stable (Cappelleri et al., 2009).

Study design

The study was a randomized, placebo-controlled, double blind, single-centered meal intervention study with a cross over design, conducted at the Biomedical Centre (BMC) at Lund University, Sweden. Each participant was tested on two days, separated by a wash out period of at least 1 week, receiving a 5 g supplement of thylakoids (Appethyl, Greenleaf Medical AB, Stockholm, Sweden) on one day and placebo on the other. The allocation to treatment or placebo was randomized and balanced between test days. The participants were not able to identify which drink contained the active component.

Participants arrived in the morning, fasted from 22:00 the night before (schedule, Table 2).

No alcohol or intense physical activity was allowed the days before or during test days.

Before breakfast, the participants filled out a questionnaire constructed as Visual Analog Scales (VAS) (Flint, Raben, Blundell, & Astrup, 2000) with questions regarding hunger, satiety and wanting for specific food products (Table 3). The questionnaire was constructed as a booklet with written instructions on the first page. All questions were followed by a 100-mm horizontal line, on which subjects marked their response. The line was anchored at each end, expressing the minimum value on the left: “Not at all”, and the

Table 2
Test day schedule, days 1 and 2.

07:30	Participants arrived at BMC	
07:45	VAS	Baseline; Time point 0
08:00	Blueberry drink, with/without 5 g thylakoids (treatment/placebo) Breakfast, including coffee/tea	
08:15	VAS	Time point 15 min
09:00	VAS	Time point 60 min
10:00	VAS	Time point 120 min
11:00	VAS	Time point 180 min
12:00	VAS	Time point 240 min
12:45	VAS	Time point 285 min
13:00	Lunch	Time point 300 min
13:20	VAS	Time point 320 min
14:00	VAS	Time point 360 min
15:00	VAS	Time point 420 min
16:00	Snack buffet served. VAS	Time point 480 min
16:45	VAS after snack buffet	Time point 525 min

Table 3
VAS-questions (translated from Swedish).

Hunger and satiety	
1.	How hungry are you right now?
2.	How satiated are you right now?
Wanting	
3.	How much do you want potato chips right now?
4.	How much do you want salted assorted nuts right now?
5.	How much do you want milk chocolate right now?
6.	How much do you want dark chocolate right now?
7.	How much do you want sweets right now?
8.	How much do you want "Dumlekola" right now?
9.	How much do you want "Ahlgrens bilar" right now?
10.	How much do you want "Gott & Blandat" right now?
11.	How much do you want "en chokladboll" right now?
12.	How much do you want a cinnamon bun right now?
13.	How much do you want some orange juice right now?
Liking	
1.	How much did you like the potato chips?
2.	How much did you like the salted assorted nuts?
3.	How much did you like the milk chocolate?
4.	How much did you like the dark chocolate?
5.	How much did you like the "Dumlekola"?
6.	How much did you like the "Ahlgrens bilar"?
7.	How much did you like the "Gott & Blandat"?
8.	How much did you like the "chokladboll"?
9.	How much did you like the cinnamon bun?
10.	How much did you like the orange juice?

maximum value on the right: "Extremely". Subsequently, a blueberry drink, with or without supplementation of thylakoids, was served followed by a standardized high carbohydrate Swedish breakfast. The VAS-questionnaire was filled out every hour as well as before and after lunch and the *ad libitum* snack buffet, served at 13:00 and 16:00 respectively.

Between breakfast and lunch, participants left the premises for work or free activities, and were reminded to fill out the VAS-questionnaires every hour via text messages. After lunch, the participants stayed in their allocated seats, sitting side by side with cardboard dividers between them, all facing a window. They were allowed to read or work on laptops until 16:00 when the snack buffet was served at the table on individual trays. A leaflet containing information about the products served accompanied the buffet describing the freshness of the foods. It included names of manufacturers and pictures of the packaging. The same pictures were used for the VAS-questionnaire. Before starting to eat from the snack buffet in front of them, the participants filled out the VAS-questionnaire once more. Then they were allowed to eat freely from the products on their trays, encouraged to follow their urges and ask for more should they run out. They were occupied by the snack buffet for 45 min, not doing anything else during this time. Afterwards, they filled out the VAS-questionnaire again, this time including questions regarding how much they liked the products they tasted that particular day, to enable evaluation of the rewarding properties of the items (Table 3). On the last page the participants were asked about adverse effects: "Have you experienced any new symptom or discomfort during the day, for example headache, rash, or nausea?"

Power and sample size

The number of participants required to achieve a power of 0.8 in this cross-over study was calculated using data from an earlier study (Stenblom et al., 2013). Based on these calculations, 15 participants were needed to detect a 10 mm difference in VAS-ratings of hunger. This number of participants is also sufficient for detecting differences in ratings for wanting palatable food (Flint et al., 2000). According to literature, 17 participants should be sufficient for detecting a difference in food intake of 500 kJ/120 kcal (Gregersen et al., 2008).

Ethics

The study was approved by the Ethics Committee for Human Studies in Lund (2006/361). The trial was conducted in accordance with the Declaration of Helsinki. All participants gave written informed consent before the study began. The participants did not receive any monetary compensation.

Thylakoids

All leafy green vegetables contain thylakoids. Five grams of extracted thylakoids is equivalent to 100 g of spinach. However, humans cannot utilize thylakoids in unprocessed vegetables, since the thylakoids are stacked inside non-digestible plant cell walls. Therefore, the only realistic way to achieve a dose compared to the one used in this study is to consume thylakoids as a supplement.

The thylakoids used in the present study were prepared from baby spinach leaves using the pH-method as previously described (Emek et al., 2010), followed by drum drying. One hundred grams of thylakoids contain 185 kcal, 26.1 g protein, 7.24 g fat, 48.7 g carbohydrate (including non-soluble and soluble fibers), 27.9 mg lutein, 730 μg zeaxanthin, 3.45 mg beta carotene, 21 μg vitamin A, 1330 μg vitamin K_1, 6.07 mg vitamin E and 166 μg folic acid.

Based on a previous dose–response study, a single dose of 5 g thylakoids was used in this study (Stenblom et al., 2013). The thylakoids were served in a cold blueberry drink, mixed with 2.5 g rapeseed oil (Zeta, Di Luca & Di Luca AB, Stockholm, Sweden) and 92.5 g blueberry soup (Ekströms original, Procordia Food AB, Eslöv, Sweden), served immediately before breakfast on treatment test days. On control test days, the participants were served a placebo drink, which consisted of 2.5 g rapeseed oil mixed with 92.5 g blueberry soup. The blueberry drinks contained 73 kcal with thylakoids and 64 kcal without.

Meals

Breakfast on both test days was a standardized Swedish high carbohydrate breakfast with yogurt and muesli, one slice of white bread with butter, cheese and sweet pepper, coffee or tea. Total energy content was 509 kcal. The energy distribution was 31.2 percent of total energy (E%) fat, 15.4 E% protein and 53.4 E% carbohydrates. Sugars constituted 22.6 E%. The lunch served on both test days was a slice of thick crust pizza, made from sourdough, with tomato sauce, ham, onion, mushrooms and cheese, served with boiled broccoli and tap water. Coffee or tea following lunch was optional, but subjects were required to have the same on both test days. Energy distribution of the lunch was 45.9 E% carbohydrates, 32.5 E% fat and 21.6 E% protein. Total energy content was 480 kcal. In addition, participants were allowed one cup of coffee/tea, without sugar or sweetener, between breakfast and lunch, and one cup of coffee/tea after lunch. Calculations of energy distribution were made using Dietist Net Pro (Kost och Näringsdata AB, Bromma, Sweden).

Snack buffet

A product questionnaire was constructed for this study as a tool for choosing well-suited products to measure cravings and snack intake in a Swedish population. It contained specific questions about snacks of the most popular brands in Sweden: potato chips, nuts, chocolate, sweets, baked confectionery, buns, fruit juice and soft drinks. Questions were asked about how often the responders consumed the palatable foods, how much they liked the products on a nine point hedonic scale and which product in each category they

Table 4
Components of the snack buffet, energy content per 100 g.

Products	Calories (kJ/kcal)	Protein (g/E%)	Fat (g/E%)	Carbohydrate (g/E%)	Sugar (g/E%)	Salt (g)
Salty						
Potato chips "Sourcream & Onion" (Estrella, Sweden)	2200/525	6.0/4.6	32.5/56.4	50.5/39	3.6/2.8	1.7
Assorted nuts "Vår klassiska nötmix" (OLW, Sweden)	2450/590	25.0/16.8	48.0/72.5	16.0/10.7	4.2/2.8	1.3
Sweet						
Sweets "Ahlgrens bilar" (Cloetta, Sweden)	1450/350	6.0/6.8	0.3/0.8	81.0/92.4	55.0/62.7	0.25[a]
Sweets (gummies) "Gott&Blandat" (Malaco, Sweden)	1450/340	0.0/0	0.0/0	85.0/100	61.0/71.8	0.25[a]
Orange juice "Apelsin juice" (Kiviks Musteri AB, Sweden)	180/40	0.6/5.9	<0.5/11	8.5/83.1	8.5/83.1	0.01[a]
Sweet-and-fat						
Milk chocolate "Marabou mjölkchoklad" (Marabou, Sweden)	2290/550	4.8/3.5	32.0/53	59.0/43.4	58.0/42.7	0.25[a]
Dark chocolate "Lindt Excellence 70% dark" (Lindt & Sprüngli, Sweden)	2180/520	8.0/6.1	40.0/68.7	33.0/25.2	28.0/21.4	0.15[a]
Chocolate covered toffee "Dumlekola original" (Fazer, Sweden)	1980/470	3.6/3.1	21.0/40.4	66.0/56.5	53.0/45.4	0.4[a]
Chocolate pastry "Delicatoboll" (Delicato, Sweden)	2000/480	5.0/4.2	29.0/54.7	49.0/41.1	30.0/25.2	0.4
Cinnamon bun "Kanelbulle" (Bonjour, VAASAN Sverige AB, Sweden)	1350/320	6.2/8	9.6/27.8	50/64.3	15/19.3	0.5

[a] Salt content initially expressed as sodium content has been converted to amount of salt by multiplying the sodium value by 2.5, as recommended by the Swedish National Food Agency (Livsmedelsverket).

preferred. Based on the responses, a range of snacks, sweets and confectionery was chosen for the snack buffet (Table 4). These products were grouped in three different categories: salty, sweet, and sweet-and-fat snacks. Snacks with salty taste included potato chips and salted assorted nuts; products with sweet taste, containing sugar, included two different kinds of sweets and orange juice; products with sweet taste containing both sugar and fat were: milk chocolate, dark chocolate, chocolate covered toffee, chocolate pastry, and cinnamon bun. Each participant received a tray with the products served in separate containers, weighed before and after consumption. The amount of sweets and snacks was large enough to allow the participants to consume as much as they liked while preventing all of the food from being consumed. Coffee, tea and water were served with the snacks in a limited amount. The drinks were optional, but the participants had to have the same drinks on both test days.

Statistics

All statistical analyses were done using the Prism version 6, statistical software (GraphPad Software, Inc, San Diego, CA, USA). Normal distribution of the paired parameters and the calculated differences between treatment and control conditions was computed by d'Agostino and Pearson omnibus normality test, verified by boxplot and histogram analysis and comparison between mean and median values. Variations in VAS-ratings over time were analyzed with a two-way repeated measures (RM) ANOVA in order to test time, treatment, and time by treatment interaction. Analyses of VAS-ratings at individual time points as well as differences in total area under the curve (tAUC) for VAS-ratings, food intake and ratings of liking were performed using Wilcoxon matched-pairs signed rank test. Reported p-values in text and figure legends were analyzed with Wilcoxon matched-pairs signed rank test if not otherwise stated.

Treatment effect of thylakoids on feelings of hunger, satiety and urge for specific food products and liking respectively was calculated by subtracting VAS-ratings for treatment day from control day values, except for satiety, which was calculated the opposite way. Treatment effect on food intake was calculated as difference in caloric intake between control and treatment days. Calculating delta values for the treatment effects was required to account for paired observations. These delta values were then correlated to eating behavior scores, as measured by the TFEQR18-V2 questionnaire, divided into the three domains: emotional eating, uncontrolled eating and cognitive restraint. Correlations were also calculated between eating behavior scores, BMI and body fat percentage (Table 1) as well as between treatment effects on wanting and liking respectively versus food intake. All correlations were computed by Pearson correlation coefficient. Correlation coefficients (r), and coefficients of determination, (R^2), equal to and above 0.3 were considered fairly strong and have been included in the manuscript. In both figures and text, data are expressed as mean +/− SEM if not otherwise stated. p-values <0.05 were considered statistically significant, and p-values <0.10 of interest.

Results

VAS-ratings on hunger and satiety

Treatment with thylakoids reduced subjective ratings of hunger compared to control throughout the day ($F(1,21) = 6.237$, $p < 0.05$, Fig. 1A). tAUC for hunger decreased by 21% between control and treated conditions ($p < 0.05$, Fig. 1B). The difference in hunger ratings between control and treatment was most pronounced prior to lunch and before the snack buffet, reaching highest significance at time points 285 min and 480 min after beginning of breakfast (Fig. 1A).

Treatment with thylakoids also increased ratings of satiety compared to control (Fig. 1C, D). Satiety-scores were generally higher during the whole day in the treated condition ($F(1,21) = 8.745$, $p < 0.01$, Fig. 1C), tAUC for satiety being increased by 14% following treatment ($p < 0.01$, Fig. 1D). Significant differences in satiety between control and treatment groups were observed at 60 and 120 min, as well as immediately before the snack buffet at time point 480 min (Fig. 1C).

VAS-ratings on wanting palatable foods

Treatment with thylakoids decreased subjective feelings of wanting all kinds of palatable food compared to control (Fig. 2). VAS-ratings were consistently lower during the treatment test day for all categories of snacks (salty, sweet and sweet-and-fat) as well as total wanting for all snacks.

Ratings for wanting salty snacks in the treated and control conditions are presented in Fig. 2A. Treatment with thylakoids reduced cravings for salty snacks continuously during the day ($F(1,21) = 6.110$, $p < 0.05$). tAUC decreased by 30% between control and treated conditions ($p < 0.01$, Fig. 2B). Specifically, the urge for salty snacks was lower in the treated group at time points 60, 120 and 360 minutes compared to the control (Fig. 2A).

Effect of treatment on ratings for wanting sweet snacks is presented in Fig. 2C ($F(1,21) = 8.412$, $p < 0.01$). Wanting for sweet

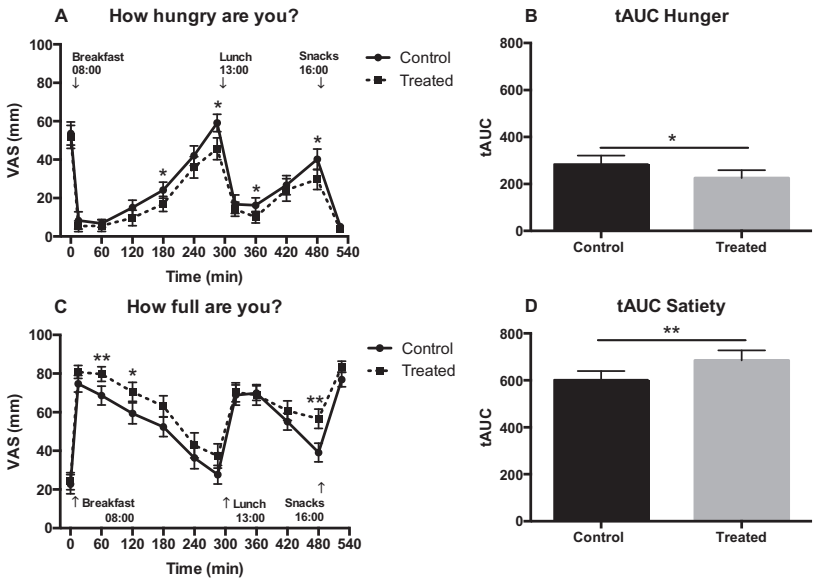

Fig. 1. Hunger- and satiety-ratings following intake of thylakoids or placebo. Treatment with thylakoids decreased ratings of hunger (A, B) and increased ratings of satiety (C, D) compared to the control. $^{*}p < 0.05$, $^{**}p < 0.01$. A and C: Two-way RM ANOVA followed by Wilcoxon signed rank test for individual time points. B and D: Wilcoxon signed rank test.

decreased 38% between control and treated conditions ($p < 0.001$, tAUC, Fig. 2D). Furthermore, there was a tendency toward a time by treatment interaction in wanting sweet snacks, i.e. the time impact on VAS-ratings depended on whether the participant received treatment or placebo ($F(11,231) = 1.803$, $p = 0.05$). The urge for sweet snacks was lower in the treated group compared to control at all time points between breakfast and lunch, starting already at 15 minutes after breakfast. It was also lower in the afternoon at time point 420 minutes (Fig. 2C).

Treatment with thylakoids also reduced ratings for wanting sweet-and-fat snacks as shown in Fig. 2E ($F(1,21) = 5.198$, $p < 0.05$). Wanting for sweet-and-fat decreased 36% in the treated group compared to control ($p < 0.05$, tAUC, Fig. 2F). The urge for sweet-and-fat snacks was lower in the treated group compared to control at time points 60, 120, 180 minutes and also right before lunch at 285 minutes (Fig. 2E).

Similar to the effects on the separate categories of snacks respectively, the ANOVA analysis of total wanting of all snacks revealed an effect of treatment ($F(1,21) = 7.364$, $p < 0.05$, Fig. 2G). Subsequent analysis of individual time points showed that treatment decreased total wanting at 15, 60, 120, 180, 285 and 420 minutes after breakfast ($p < 0.05$). Wanting for all snacks combined decreased by 36% in the treated group compared to the control ($p < 0.05$, tAUC, Fig. 2H).

Food intake from ad libitum *snack buffet*

Of the 22 participants, everyone ate something from the snack buffet on both test days. Median total intake in the control group was 912 kcal (lower and upper quartiles 619–1081 kcal) and median intake in the thylakoid group was 810 kcal (lower and upper quartiles 598–1019 kcal), which corresponds to a reduced intake in the treated condition by 11% (Fig. 3). This reduction, however, was not statistically significant.

There was a tendency toward a reduced caloric intake of salty food items following treatment with thylakoids compared to control, with a median difference of 65 kcal ($p = 0.0547$, Fig. 3). This corresponds to a reduction in intake of salty snacks by 26% between control and treated conditions, since median intake of salty snacks in the control group was 248 kcal (lower and upper quartiles 111–322 kcal) and median intake in the thylakoid group was 183 kcal (lower and upper quartiles 80–294 kcal).

There were no significant differences in the intake of sweet or sweet-and-fat snacks between treatment and control conditions.

Liking for palatable food, measured after consumption from the snack buffet

Only the participants who tasted an item were allowed to rate their liking for it. For each specific food product to be included in the analysis of liking scores, participants had to taste and rate their liking for the product at both control and treatment days. One participant was excluded from the analysis due to failure to fill out the form correctly. Treatment with thylakoids produced a lower liking of sweet products after consumption, compared to control days ($p < 0.01$, Fig. 4). Median rating for liking sweet was 32 in the control group (lower and upper quartiles 21–52) and 23 in the thylakoid group (lower and upper quartiles 9–40). This corresponds to a reduction in liking sweet by 28%. Median liking for all snacks together was 27 in the control group (lower and upper quartiles 16–37) and 17 in the treated group (lower and upper quartiles 15–34). This however was not significant ($p = 0.15$, Fig. 4). Liking for salty and sweet-and-fat products was not significantly changed between treatment and control days.

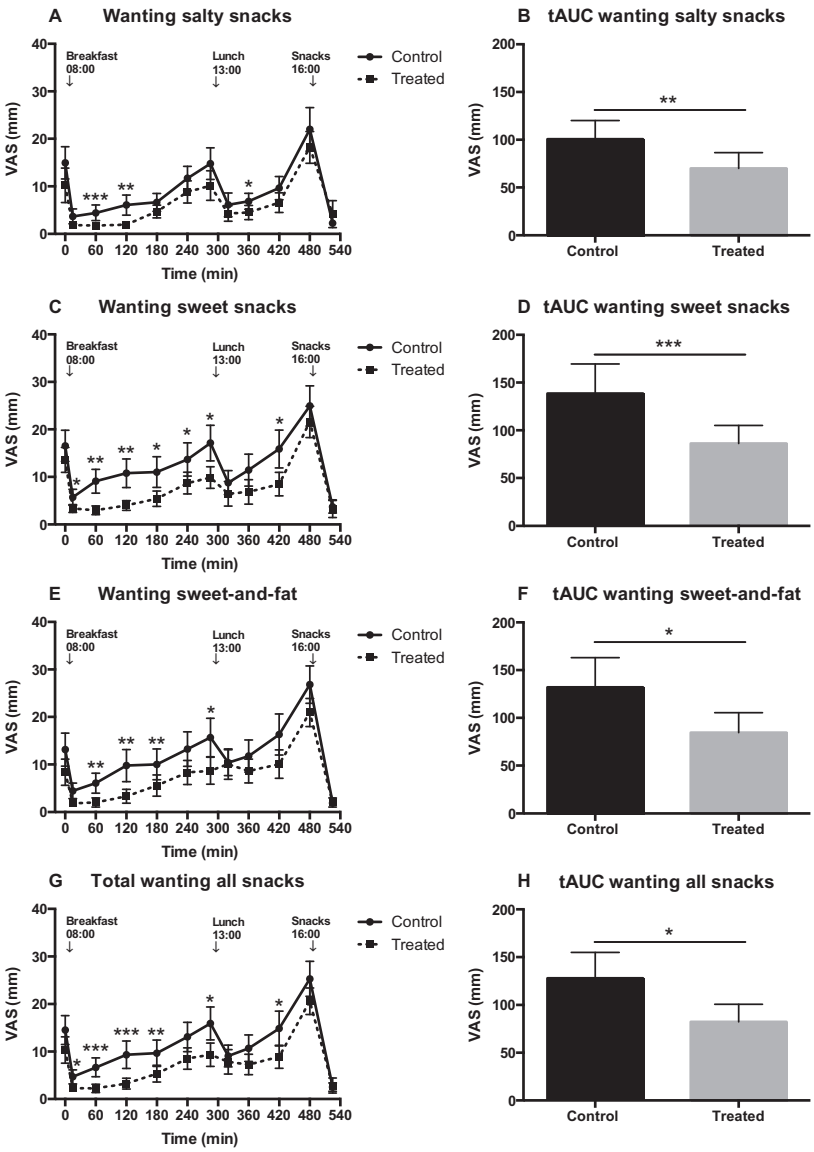

Fig. 2. VAS-ratings of wanting salty, sweet, sweet-and-fat snacks and all snacks combined, comparing treatment and placebo conditions (control). Treatment with thylakoids reduced wanting for all categories of snacks compared to the control (A–H). Presented data are an average of the ratings for the individual items of palatable food, presented per category (Table 3). For all parameters, effect of treatment was analyzed by tAUC and by two-way RM ANOVA followed by analysis of differences in individual time points using Wilcoxon matched-pairs signed rank test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

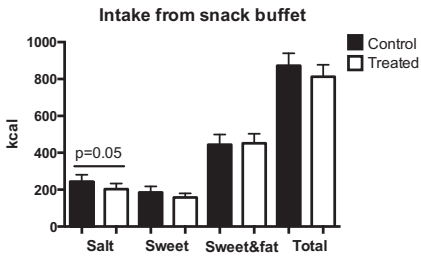

Fig. 3. Energy intake from the afternoon snack buffet. Supplementation with thylakoids produced a tendency toward reduced caloric intake of salty snacks on treatment day compared to control ($p = 0.0547$, Wilcoxon matched-pairs signed rank test, n = 22).

Correlations between treatment effects on wanting versus treatment effects on food intake

The treatment effect on wanting sweet-and-fat snacks was positively correlated with the treatment effect on intake of sweet-and-fat snacks, so that greater reduction in wanting sweet-and-fat snacks during the course of the day correlated with a reduced intake of sweet-and-fat snacks at the *ad libitum* snack buffet in the afternoon ($p < 0.01$, $r = 0.54$, Fig. 5A). Similarly, there was a positive correlation between the treatment effect on wanting all kinds of snacks and the treatment effect on intake of all kinds of snacks ($p < 0.01$, $r = 0.60$, Fig. 5B). The correlation plots show that participants with higher treatment effect on wanting, i.e. the greatest reduction in ratings for wanting in the treated condition compared to control, also have greatest reduction in food intake between control and thylakoid trial days. There was also a positive correlation between the reduction in wanting sweet-and-fat and the difference in total intake between control and treated conditions ($p < 0.01$, $r = 0.64$).

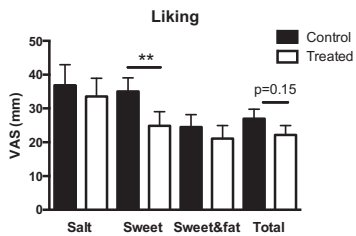

Fig. 4. Liking for specific food products after consumption, with and without treatment with thylakoids. Liking of products (VAS) scored immediately after the *ad libitum* snack buffet, presented in categories and as total liking for all products, divided by the number of products in each category to produce an average score. Treatment with thylakoids decreased the liking of sweet products between control and treatment days ($p < 0.05$, Wilcoxon matched-pairs signed rank test, n = 18 for liking salt, 18 for sweet, 20 for sweet-and-fat, and 21 for total liking).

Correlations between treatment effects on liking versus treatment effects on food intake

The treatment effect on liking for sweet was positively correlated with the treatment effect on intake of sweet, so that participants with greatest reduction in intake of sweet snacks between control and treated conditions rated lower liking for sweet after consumption from the *ad libitum* snack buffet ($p < 0.05$, $r = 0.58$). Similarly, there was a positive correlation between the treatment effect on liking sweet-and-fat snacks and the treatment effect on intake of sweet-and-fat snacks ($p < 0.01$, $r = 0.58$, Fig. 6A), liking all kinds of snacks versus total snack intake ($p < 0.01$, $r = 0.65$, Fig. 6B), liking all kinds of snacks versus intake of sweet-and-fat snacks ($p < 0.01$, $r = 0.57$), and liking sweet-and-fat snacks versus total intake of all snacks, control minus treated conditions ($p < 0.01$, $r = 0.58$).

Eating behavior measured by the three-factor eating questionnaire (TFEQ-R18V2)

Participants responded to each of the 18 questions about statements characterizing eating-related behaviors on a four-point Likert scale (Likert, 1932). Responses were coded on a four-point scale (1–4) with higher values indicating more of the behavior. Mean values for each of the three behavioral categories were: emotional eating domain 2.27 (+/– 0.15), cognitive restraint domain 2.52 (+/– 0.15) and uncontrolled eating domain 2.26 (+/– 0.10).

Correlations between treatment effect on wanting and scores for eating behavior

The treatment effect on wanting sweet-and-fat snacks as well as all kinds of snacks was positively correlated with emotional eating

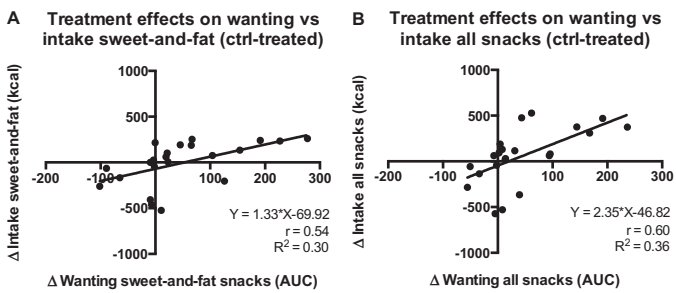

Fig. 5. Correlations between treatment effects on wanting and treatment effects on food intake. Graphs show correlation plots of the treatment effect on VAS-ratings for wanting sweet-and-fat snacks versus the treatment effect on intake of sweet-and-fat (A) and all kinds of snacks together (B). VAS-ratings and caloric intake are presented as delta values, i.e. control day minus treatment day values in each category, the difference representing the treatment effect of thylakoids. The treatment effect on wanting sweet-and-fat snacks was positively correlated to a reduction in food intake ($p < 0.01$, A), so was the treatment effect on all categories of snacks together ($p < 0.01$, B).

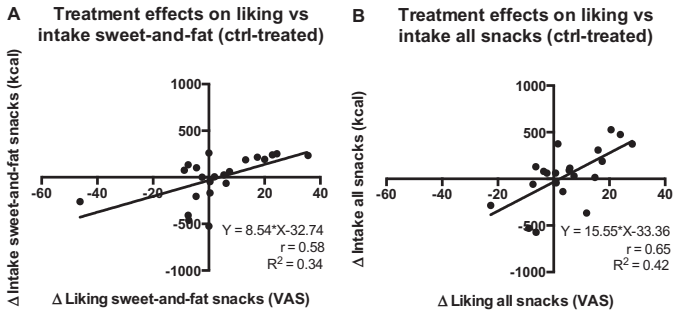

Fig. 6. Correlations between treatment effects on liking and treatment effects on food intake. Graphs show correlation plots of the treatment effect on VAS-ratings for liking sweet-and-fat (A), and all snacks (B), versus treatment effect on intake of the corresponding categories of snacks. VAS-ratings and caloric intake are presented as delta values, i.e. control day minus treatment day values in each category, the difference representing the treatment effect of thylakoids. The treatment effect on liking sweet-and-fat snacks was positively correlated to a reduction in intake of sweet products ($p < 0.01$, A), so was the treatment effect on all categories of snacks together ($p < 0.01$, B).

scores, so that higher scores for emotional eating behavior was correlated to a greater treatment effect of thylakoids on ratings for wanting sweet-and-fat foods ($p < 0.01$, $r = 0.62$, Fig. 7A) and all categories of palatable foods ($p < 0.01$, $r = 0.55$, Fig. 7B). Scores for cognitive restraint and uncontrolled eating behavior were not correlated with treatment effects on wanting.

Additional correlations analyzed

There were no statistically significant correlations between the different eating behavior scores and the treatment effect of thylakoids on hunger, satiety, food intake or liking. Neither were there any correlations between BMI or body fat percentage and scores for eating behavior, nor any correlations between the different eating behavior scores.

No serious adverse events or effects were reported. Mild cases of nausea during or after the snack buffet occurred at three occasions in the placebo group and at two occasions in the treated group. Headaches were reported at two occasions in the placebo group and at one occasion in the treated group.

Discussion

The present study demonstrates that a single dose of thylakoids prior to breakfast increased subjective ratings of satiety and decreased ratings of hunger as well as cravings for snacks and sweets during the day. Intake of thylakoids also decreased subjective liking for sweet, scored directly following consumption. There are strong correlations between the treatment effect on wanting and actual food intake between control and thylakoid conditions, as well as between the treatment effect on liking and food intake, even though there were no significant differences in food intake per se. When correlated to eating behavior scores, treatment effect of thylakoids on wanting was positively correlated to emotional eating for all snacks, sweet-and-fat foods being specifically targeted.

The promotion of satiety by thylakoids found in this study is an entirely novel finding. This started 60 minutes after breakfast, suggesting enhanced early satiety signaling. In addition, ratings for satiety in the treated group were also higher several hours after lunch, compared to placebo. Both the acute and late effects of thylakoids to enhance satiety may be explained by increased secretion of satiety hormones CCK and GLP-1 (Köhnke, Lindbo et al., 2009; Montelius et al., 2014; Stenblom et al., 2013).

Both CCK and GLP-1 are known to have a biphasic pattern of secretion. Through gastric distension, as well as by calories entering the small intestine, CCK is released, first in the hypothalamus, and later peripherally, which induces satiety (Pappas, 1992). GLP-1 secretion has a similar pattern, the early phase being mediated by the vagus nerve and the second phase by direct contact of luminal nutrients with the L-cells in the distal small intestine (Baggio & Drucker,

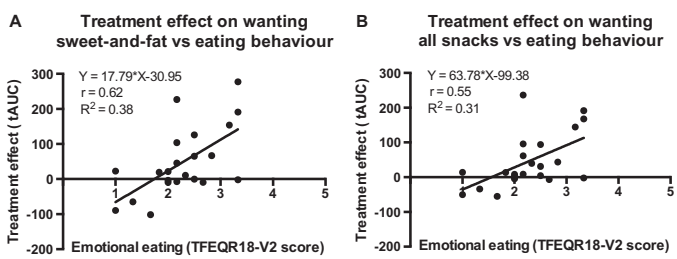

Fig. 7. Correlation between treatment effect of thylakoids on wanting palatable food and emotional eating behavior scores. Graphs show correlation plots of the treatment effect on VAS-ratings for wanting versus scores for emotional eating measured by the TFEQR18-V2 (A and B). VAS-ratings are presented as delta values, i.e. control day minus treatment day values for the tAUC in each category, the difference representing the treatment effect of thylakoids. The treatment effect on wanting sweet-and-fat snacks was positively correlated to emotional eating ($p < 0.01$, A). So was the treatment effect on all categories of snacks together ($p < 0.01$, B).

2007; Flint, Raben, Ersbøll, Holst, & Astrup, 2001; Näslund & Hellström, 2007; Wishart, Horowitz, Morris, Jones, & Nauck, 1998).

Treatment also had an attenuating effect on hunger ratings throughout the day. In comparison to ratings for satiety, the difference in hunger ratings reached significance later, at 180 minutes after breakfast in comparison to 60 minutes for satiety. The present findings on hunger verify previous reports on decreased hunger ratings following treatment with thylakoids (Stenblom et al., 2013, 2014). The suppressed hunger may both be related to reduced ghrelin-levels (Köhnke, Lindbo et al., 2009) and an indirect effect of increased circulating levels of the satiety hormones CCK and GLP-1. Indeed, the late appearance of hunger suppression suggests an indirect effect by thylakoids on promotion of satiety hormones. GLP-1 in physiological levels has been shown to inhibit gastric emptying and reduce ratings of hunger (Flint et al., 2001). A reduction in ghrelin levels would cause a similar effect, since ghrelin accelerates gastric emptying and stimulates eating (Cummings, Frayo, Marmonier, Aubert, & Chapelot, 2004).

Besides increasing satiety and reducing hunger, in this study, treatment with thylakoids also reduced wanting for all categories of palatable snacks: salty, sweet, sweet-and-fat and all snacks together, compared to control. This is a novel finding. In comparison, decreased urge for sweet and sweet-and-fat foods has previously been reported in long-term studies (Montelius et al., 2014; Stenblom et al., 2014), but never in a single dose meal study using within-subject comparisons, which is more sensitive and accurate in measuring appetite scores (Flint et al., 2000). Interestingly, in the three-month study (Montelius et al., 2014), on the first day of treatment, ratings for wanting sweets and wanting chocolate were significantly lower in the treated group compared to control in the afternoon. In contrast, in the present study, treatment suppressed wanting for sweets and snacks already before lunch and this effect lasted throughout the day. The difference between the two studies lies in the experimental setting. In the three-month study, participants stayed in the laboratory for blood sampling between breakfast and lunch. During this time, they did not score high for wanting. In the afternoon however, in their natural environment, the control group scored higher, and the differences in wanting appeared. In the present study, participants went back to their everyday circumstances between breakfast and lunch. Consequently, they were exposed to food cues such as colleagues having coffee and cake at the midmorning break. In comparison, on day 90 of the three-month study, cravings for sweets and chocolate were significantly lower both before lunch and after lunch in the treated group, showing the potentiation of the effect due to the repeated administration of thylakoids on wanting.

The present results demonstrate that treatment with thylakoids have immediate effects on wanting. This may be an effect of altered levels of appetite regulating hormones, since both GLP-1 and ghrelin have been implicated in food reward (Egecioglu et al., 2010; Skibicka, 2013; Skibicka, Hansson, Egecioglu, & Dickson, 2012).

The suggested mechanism, according to previous studies in humans, may be the increased release of GLP-1 following thylakoid supplementation (Montelius et al., 2014). GLP-1 analogs have been shown to decrease the rewarding value of sweet (sucrose) and sweet-and-fat (chocolate) food, decrease food intake and shift food preference from candy to chow in rat (Dickson, Shirazi, Hansson, Bergquist, & Nissbrandt, 2012; Raun et al., 2007).

In the present study, participants who scored higher for emotional eating behavior experienced greater effect of thylakoids on reducing wanting for palatable food, particularly sweet-and-fat foods. Emotional eating behavior is associated with increased consumption of sweet-and-fat foods, especially in response to stressors and negative emotions (Cappelleri et al., 2009; Epel, Lapidus, McEwen, & Brownell, 2001; Keskitalo et al., 2008). Therefore, the participants in this study scoring high for emotional eating behavior were more susceptible to the effects of thylakoids to reduce the rewarding properties of sweet-and-fat foods. Since the correlation between eating behavior types and BMI is strongest for emotional eating behavior, it is important to target overeating in emotional eaters specifically to avoid weight gain in this group (Karlsson, Persson, Sjöström, & Sullivan, 2000).

Measuring food intake is difficult due to the complex nature of eating behavior (Blundell et al., 2010). Accordingly, despite the increased satiety and decreased hunger and wanting during the day, food intake was not significantly affected in the experimental setting of the present study. There was however a tendency toward decreased intake of salty snacks by treatment, which is a new finding. Measuring food intake alone is not reliable, since measurement of food is constrained by the experimental design. Instead, food intake is best used in conjunction with measurements of motivation to eat (Stubbs et al., 2000). Visual analog scales (VAS) are reliable tools for this assessment (Drapeau et al., 2007; Flint et al., 2000). In the present study, correlation analyses showed that treatment effects on VAS-scores for wanting and liking correlated positively with a reduction in food intake between control and thylakoid conditions. These findings indicate that individuals who experience the greatest reduction of VAS-ratings for wanting and liking also have the greatest reduction in food intake of the corresponding products. Though measurements of consumption of the foods tested did not show statistically significant differences between treated and controls in this single dose study design, the correlation analyses reveals treatment effects of thylakoids that are likely to influence food consumption in repeated dose situations as shown in a previous three-month study with daily supplementation of thylakoids (Montelius et al., 2014). Thus, ratings of wanting may be more accurate in predicting eating behavior in a real-life situation compared to food intake alone. This emphasizes the importance of the reported findings that thylakoids suppress hunger and wanting. However, although we demonstrated a depressed hunger and wanting by thylakoids in this study we cannot assure that this guarantees a decreased food intake.

After the afternoon snack buffet, ratings of liking for sweet food were suppressed in the treated group compared to placebo. Liking is generally considered to be related to opioid signaling (Berridge, 2009). The preference for sweet taste is regulated by endogenous opiates (endorphins), which are also implicated in food cravings and drug reward. Consistently, opiate antagonists have been shown to reduce rated pleasure for sweet (Fantino, Hosotte, & Apfelbaum, 1986; Yoshida et al., 2010). Enterostatin, released during fat digestion, acts like an opioid antagonist in animal models, in particular when the fat digestion is retarded (Berger, Winzell, Mei, & Erlanson-Albertsson, 2004; Ookuma, Barton, York, & Bray, 1997; Takenaka et al., 2008). Consequently, one could argue that thylakoids through its retardation of fat digestion, as previously demonstrated (Albertsson et al., 2007), may cause an increased release of enterostatin and thus induce an opiate-blocking effect, which affects liking for sweet in this study. Leptin has also been demonstrated to stop the liking of sweet food (Yoshida et al., 2010). In a previous publication (Köhnke, Lindbo et al., 2009), there was an increased release of leptin at time point 360 min after intake of breakfast with thylakoids compared to control. Consequently, leptin may also assist in the suppression of liking sweet caused by thylakoids.

Findings on reduced liking are important. Indeed, overweight women have been shown to have an increased liking for sweet (Ettinger, Duizer, & Caldwell, 2012). Furthermore, liking of sweetness influences wanting of sweet-tasting products (Drewnowski, Mennella, Johnson, & Bellisle, 2012). Thus, a reduced liking may, over time, cause a reduction in wanting for sweet. As a consequence, this may reduce snacking in between meals and hence overeating. In this study, besides reducing liking for sweet after consumption,

treatment reduced wanting for sweet to a greater extent compared to other kinds of snacks. There was also an earlier onset; wanting for sweet was reduced from 15 minutes following breakfast and onwards, in comparison to wanting for salty and sweet-and-fat snacks, which were reduced from 60 minutes respectively. This implies that treatment with thylakoids has a particular effect on liking and wanting sweet. The mechanism for this targeting is not known. Further studies are needed to understand the effects of thylakoids to suppress the liking for sweet taste, diminish the urge for palatable food and its particular effect in emotional eaters, including measurement of gut hormones.

Conclusion

Supplementation with thylakoids in the morning affects subjective ratings of appetite during the rest of the day. It reduces feelings of hunger and increases feelings of satiety. It also reduces wanting for palatable food, and this effect is enhanced in emotional eaters. Furthermore, the treatment effect on wanting and liking is correlated to reduction in food intake. In addition, liking for sweet is reduced after consumption. We suggest that these effects are due to altered secretion of appetite regulating hormones, induced by the thylakoids, affecting reward-related areas in the brain.

Even though analysis of appetite regulating hormones could have made our study stronger, we deliberately did not take any blood samples in this study. Since laboratory settings have been shown to interfere with thoughts about food and food intake, a natural surrounding was preferred in order to achieve more accurate results on VAS-ratings and food consumption (Blundell et al., 2010). Another possible limitation to this study was the small number of participants even though the cross over design increased the power.

With these limitations in mind, we have shown that treatment with thylakoids attenuate hunger, homeostatic and hedonic, including wanting for palatable food and liking for sweet. Reducing wanting is important, since wanting is a major cause of hedonic eating, which contributes to overconsumption and obesity (Blundell & Gillett, 2001; Koenders & van Strien, 2011; Yeomans et al., 2004). In addition, individuals who are obese and/or dieting are even more susceptible to hedonic hunger (Garber & Lustig, 2011). Therefore, reducing cravings for palatable food is necessary, both to control appetite, prevent weight gain and to facilitate a permanent weight loss. Supplementation by thylakoids may help prevent eating between meals, hence over-eating and in the long run, weight gain.

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Paper V

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Dietary thylakoids reduce visceral fat mass and increase expression of genes involved in intestinal fatty acid oxidation in high-fat fed rats

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[1]*Department of Experimental Medical Science, Appetite Regulation Unit, Faculty of Medicine, Lund University, Lund, Sweden;* [2]*Physiology and Behavior Laboratory, ETH Zürich, Schwerzenbach, Switzerland;* [3]*AstraZeneca, R&D Gothenburg, Mölndal, Sweden; and* [4]*Department of Biology, Lund University, Lund, Sweden*

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Stenblom E-L, Egecioglu E, Montelius C, Ramachandran D, Bonn B, Weström B, Mansouri A, Langhans W, Erlanson-Albertsson C. Dietary thylakoids reduce visceral fat mass and increase expression of genes involved in intestinal fatty acid oxidation in high-fat fed rats. *Am J Physiol Regul Integr Comp Physiol* 311: R618–R627, 2016. First published August 3, 2016; doi:10.1152/ajpregu.00212.2016.—Thylakoids reduce body weight gain and body fat accumulation in rodents. This study investigated whether an enhanced oxidation of dietary fat-derived fatty acids in the intestine contributes to the thylakoid effects. Male Sprague-Dawley rats were fed a high-fat diet with ($n = 8$) or without thylakoids ($n = 8$) for 2 wk. Body weight, food intake, and body fat were measured, and intestinal mucosa was collected and analyzed. Quantitative real-time PCR was used to measure gene expression levels of key enzymes involved in fatty acid transport, fatty acid oxidation, and ketogenesis. Another set of thylakoid-treated ($n = 10$) and control rats ($n = 10$) went through indirect calorimetry. In the first experiment, thylakoid-treated rats ($n = 8$) accumulated 25% less visceral fat than controls. Furthermore, fatty acid translocase (*Fat/Cd36*), carnitine palmitoyltransferase 1a (*Cpt1a*), and mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase 2 (*Hmgcs2*) genes were upregulated in the jejunum of the thylakoid-treated group. In the second experiment, thylakoid-treated rats ($n = 10$) gained 17.5% less weight compared with controls and their respiratory quotient was lower, 0.86 compared with 0.91. Thylakoid-intake resulted in decreased food intake and did not cause steatorrhea. These results suggest that thylakoids stimulated intestinal fatty acid oxidation and ketogenesis, resulting in an increased ability of the intestine to handle dietary fat. The increased fatty acid oxidation and the resulting reduction in food intake may contribute to the reduced fat accumulation in thylakoid-treated animals.

fat metabolism; energy expenditure; plant extracts; steatorrhea; food intake

OVERWEIGHT AND OBESITY DEVELOPS when energy intake chronically exceeds energy expenditure. The abnormal accumulation of body fat in obesity can lead to impaired health (15). Fat accounts for 35–40% of the calories ingested in Western countries (5, 6) even though, according to common recommendations, it should not exceed 30% (43). To find new ways to treat obesity, it is, therefore, essential to target both the excessive intake of dietary fat and the enhanced fat accumulation. Peroxisome proliferator receptor activator-α (PPAR-α) is a nuclear hormone receptor expressed in tissues with a high energy demand, including liver, heart, brown adipose tissue (BAT), and small intestine (19). As a transcription factor PPAR-α regulates biological processes by altering the expression of its numerous target genes. PPAR-α increases the expression of enzymes that promote lipolysis, fatty acid oxidation, and ketogenesis (3). PPAR-α-ligands such as fibrates are currently used for the treatment of hypertriglyceridemia (3, 19). PPAR-α-activation also has therapeutic effects on insulin resistance, glucose homeostasis, and atherosclerosis. Furthermore, pharmacological activation of PPAR-α has been shown to reduce food intake. This eating-inhibitory effect was associated with a decrease in the respiratory quotient (RQ) and with evidence of an enhanced intestinal fatty acid oxidation and ketogenesis (17, 18).

Thylakoids are biological membranes, derived from green-leaf chloroplasts, that have been shown to affect eating and energy metabolism (11), and they may do so, in part, by stimulating the oxidation of diet-derived fatty acids in the intestine. When added to the diet, thylakoid membranes bind to pancreatic lipase and colipase and, thereby, slow down the digestion of fat in the intestine (1, 8). They also form a transient barrier covering the intestinal mucosa, which prolongs nutrient uptake (29). Although all macronutrients, including the dietary fat and the thylakoids themselves, are ultimately digested and absorbed, thylakoids slow down this process substantially (8). Further studies have shown that dietary thylakoids decrease ghrelin levels in man (21) and pig (31) and reduce hunger and cravings for palatable food in man (28, 33, 37–39). Thylakoids have also been shown to increase circulating levels of satiation peptides and decrease food intake, body weight, and fat mass in various species, including humans (1, 9, 21, 22, 28, 30, 31, 39). Specifically, reduced hip circumference in combination with a lower circulating level of leptin in response to thylakoids suggest that they lead to a reduction of body fat also in humans (38). Together with the reduced blood lipid levels found in rodents (1, 22) and man (28, 38), these findings suggest that thylakoids increase fat oxidation.

One site of increased fatty acid oxidation may be the intestine (17, 35). Because thylakoids reduce the rate of fat digestion and fat absorption, they have the possibility to stimulate the fatty acid transport and oxidative systems in the intestine for a longer time. The aim of this study was, therefore, to investigate the effects of long-term treatment with dietary thylakoids in the rat on fatty acid oxidation, examining gene expression levels corresponding to enzymes involved in fatty acid transport and fatty acid oxidation in the intestine and liver. Body composition, body fat pads, blood metabolites, total body energy expenditure, nutrient-substrate utilization, activity lev-

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els, body temperature, and fecal fat content were also measured, as well as the activity of drug-metabolizing enzymes in the liver.

MATERIALS AND METHODS

Thylakoids and Diets

Chlorophyll containing green-plant thylakoid membranes used in the study were prepared from spinach leaves using the pH method, as previously described (9), followed by drum drying. One-hundred grams of thylakoids have an energy content of 1,528 kJ and contain 26.1 g protein, 7.2 g fat, 48.7 g carbohydrate, 0.27 g sodium, 2.0 g chlorophyll, 27.9 mg lutein, 0.7 mg zeaxanthine, 3.5 mg β-carotene, 0.021 mg vitamin A, 1.3 mg vitamin K, 6.0 mg vitamin E, and 0.17 mg folic acid. A control high-fat diet (HFD) and a thylakoid-enriched HFD (thylHFD) were used in the experiments (Research Diets, New Brunswick, NJ). The diets were based on the D12451 high-fat diet from Research Diets. The thylHFD contained 33% wt/wt spinach extract (Appethyl, Green Leaf Medical AB, Stockholm, Sweden), which yielded a dose comparable to previous studies in mouse and rat with shown effects on body weight and food intake (1, 22). The energy content was 1,930 kJ/100 g for the HFD and 1,808 kJ/100 g for the thylHFD, the difference was due to the ash and water content of the thylakoid powder. The energy distribution for both diets was 46 E% fat, 18 E% protein, and 36 E% carbohydrates.

Animals and Housing

The study was conducted according to the European Communities regulations concerning protection of experimental animals. Male Sprague-Dawley rats (Charles River) were used, weighing on average 260 g at randomization. The animals were housed in a specific pathogen-free environment, maintained on a 12:12-h light-dark cycle with lights on at 0700, at stable temperature (21 ± 2°C) and relative humidity (50 ± 10%). They had ad libitum access to a standard chow (R36; Lantmännen, Kimstad, Sweden) and tap water until the start of the experiments. The first experiment was performed at the Department of Biology, Lund University, and the animal experiment protocol was approved by the Lund University Ethical Review Committee for Animal Experiments (no. M108-13). The second experiment was performed by the Centre for Physiology and Bio-Imaging, Core Facilities, Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden. The animal experiment protocol was approved by Gothenburg University Ethical Review Committee for Animal Experiments (no. 327-2012).

First experiment: Intestinal Fat Oxidation

Sixteen rats were used for the first experiment, investigating the effects of dietary thylakoids on gene expression of enzymes involved in fatty acid oxidation in the intestine and the liver, body weight, body fat mass, food intake, plasma metabolites, and cytochrome *P*-450 (CYP) activity in the liver. After 7 days of acclimatization, the rats were housed individually and randomized on the basis of body weight to receive control HFD or thylHFD. To standardize the intake of thylakoids, a fixed amount of 15 g HFD or thylHFD was given to the animals in the afternoon for 13 consecutive days for consumption during the dark period. During the light period, all animals had ad libitum access to HFD without thylakoids. Food intake and body weight were measured daily.

Termination of experiment and sampling procedure. In the afternoon on *day 13*, the rats received 3 g of either HFD or thylHFD, after which they were kept fasted until the morning, when they received another 3 g of the same feed 1 h prior to termination of the experiment. The animals were sedated with isoflurane (Baxter Medical AB, Kista, Sweden) and were euthanized by heart puncture and exsanguination. Blood was collected in EDTA tubes and was centrifuged at 3,000 *g* for 10 min at 4°C. Plasma was collected in cryotubes, frozen, and stored at −80°C for later use. Individual fat pads, mesenteric, epididymal, retroperitoneal, and inguinal subcutaneous white adipose tissue (WAT), interscapular BAT, and livers were dissected and weighed. The mesenteric fat was dissected by stripping the intestine of the fat using blunt dissection with forceps. The epididymal fat pads were removed in their entirety. The retroperitoneal fat pads, extending laterally from the kidneys, were removed from each side. Inguinal subcutaneous fat pads were dissected bilaterally. Livers were cut in pieces and immediately frozen on dry ice and stored at −80°C for later use. Intestinal segments were dissected, each 10 cm in length: duodenum, from the pylorus to the ligament of Treitz, proximal jejunum, next to this segment, and distal ilium, proximal to the cecum. The intestinal segments were washed in ice-cold PBS and were immediately placed on an ice-chilled glass plate, and the mucosa was scraped off using microscope slides, then frozen on dry ice and stored at −80°C for later analysis.

Analyses of plasma metabolites. Plasma levels of nonesterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) were analyzed at the Department for Clinical Chemistry, Sahlgrenska University Hospital, Gothenburg, Sweden. NEFA were analyzed by an enzymatic colorimetric method using a NEFA-HR kit (Wako Pure Chemical Industries, Osaka, Japan). BHB was analyzed using a locally produced reagent 3-hydroxybutyrate dehydrogenase, which, together with NAD^+, converts BHB to acetoacetate, NADH, and H^+. The measured amount of NADH is proportional to the amount of BHB. TG levels were analyzed using a Cobas 6000 analyzer (Cobas, Roche, Switzerland) at the Department for Clinical Chemistry, Lund University Hospital, Lund, Sweden.

Quantitative real-time polymerase chain reaction. RNA was extracted from all intestinal tissues with the TRIzol reagent (15596-018; Ambion by Life Technologies, Zug, Switzerland), treated with DNase (79254; Qiagen, Hombrechtikon, Switzerland), and quantified. One microgram of RNA was used to synthesize cDNA with the high-capacity cDNA RT kit (4368814; Applied Biosystems, Zug, Switzerland). All samples were analyzed by qPCR using FAST SYBR Green master mix (4385617; Applied Biosystems) and the ViiA 7 Real-Time PCR System (Applied Biosystems) (Table 1). Each sample was run in triplicate, analyzed with the $2^{-\Delta\Delta C_T}$ method (24), and normalized against β-actin (*Actb*) as a reference gene.

Cytochrome P-450 activity. Livers were analyzed for determination of cytochrome *P*-450 (CYP) activity. Probe reactions for CYP1A2 (phenacetin-*O*-deethylation), CYP2C9 (diclofenac-4′-hydroxylation), CYP2D6 (bufaralol-1′-hydroxylation), and CYP3A4 (midazolam-1′hydroxylation) (36) were studied in liver subcellular fractions (microsomes) from thylakoid-treated and control animals. The preparation of microsomes was carried out as previously described (12). Final protein concentrations were determined in triplicate with a Pierce

Table 1. *List of genes and corresponding primers*

Gene Name	Primer Sequence 5′ to 3′
3-hydroxy-3-methylglutaryl-CoAsynthase 2 (*Hmgcs2*)	F - CTGCCTCCCTCTTCAACG R - CACAGACCACCAGGGCATA
Carnitine palmitoyltransferase 1a (*Cpt1a*)	F - TGATCCCTCAGAGCCACAG R - GGTCTGCCGACACTTTGC
Actin, beta (*Actb*)	F - CTAAGGCCAACCGTGAAAAG R - GCCTGGATGGCTACGTACA
Fatty acid binding protein 2 (*Fabp2*)	F - AACTCGGCGTCGACTTTG R - CCAACAAGTTTATTTCCCTCCAT
Acyl-CoA- dehydrogenase, long chain (*Acadl/Lcad*)	F - GCAGTTACTTGGGAAGAGCAA R - GGCATGACAATATCTGAATGGA
CD36 molecule (*Fat/Cd36*)	F - GCGACATGATTAATGGCACA R - TGGACCTGCAAATGTCAGAG
Peroxisome proliferator-activated receptor, α (*Ppara*)	F - CCTCGAACTGGATGACAGTG R - CCCTCCTGCAACTTCTCAAT

BCA Protein assay kit (Thermo Scientific, Waltham, MA) using a BSA standard curve.

Microsomes (0.5 mg/ml) from four thylakoid-treated and four control animals were mixed with phosphate buffer (0.1 M, pH 7.4) and incubated in duplicate with a cocktail of phenacetin (30 μM), diclofenac (10 μM), bufuralol (5 μM), and midazolam (3 μM) at 37°C. After 10 min of preincubation, the reactions were initiated with the addition of NADPH (1 mM). Aliquots were taken at 0, 7, 15, 20, 30, and 60 min and quenched with two parts acidified acetonitrile. The samples were centrifuged at 2,737 *g* for 20 min at 4°C, and the supernatant was diluted 1:2 in H_2O prior to analysis. The amounts of *O*-deethyl-phenacetin, 4′-hydroxy-diclofenac, 1′-hydroxy-bufuralol, and 1′-hydroxy-midazolam formed were determined (see below), and the rates of formation were calculated as picomoles per minute per milligram protein.

LC-MS/MS analysis of CYP probe substrates. The analysis of the corresponding metabolites from the CYP probe substrates (*O*-deethyl-phenacetin, 4′-hydroxy-diclofenac, 1′-hydroxy-bufuralol, and 1′-hydroxy-midazolam) was done with LC-MS/MS on a Waters Synapt HDMS mass spectrometer (Waters, Milford, MA) operating under positive electrospray ionization conditions. Leucine-enkephaline was used as a lock mass (*m/z* 556.2771) for internal calibration. Chromatographic separations were performed on a Waters Acquity UPLC system (Waters) using an Acquity UPLC BEH C18 column (2.1 × 100 mm). The mobile phases consisted of H_2O/0.1% formic acid and acetonitrile/0.1% formic acid, and the LC gradient was 10–70% acetonitrile/0.1% formic acid in 6 min at a flow rate of 500 ml/min. All MS data were processed in Metabolynx (Waters, Milford, MA), and the metabolites were quantified with authentic standard curves.

Second Experiment: Indirect Calorimetric Measurements

Twenty rats were used for the second experiment, investigating the effects of dietary thylakoids on energy expenditure, nutrient substrate utilization, and body composition. Upon arrival at the animal facility, the rats were housed together in pairs. After a minimum of 7 days, the rats were sedated with isoflurane (Baxter Medical AB, Kista, Sweden), received the NSAID rimadyl as an analgesic (5 mg/kg ip), and telemetry devices (G2 E-Mitter, MiniMitter, Sunriver, OR) were implanted intraperitoneally, according to the protocol provided by the manufacturer for later measurements of body temperature and activity counts. After 1 wk of recovery, the rats were randomized on the basis of body weight to either a HFD ($n = 10$) or thylHFD ($n = 10$) for 14 days with ad libitum access. New food was offered twice a week, and food consumption per cage was monitored. Body weight was measured at baseline (*day 0* of HFD-feeding), *day 7*, *day 12* (prior to the indirect calorimetric measurements), and at the end of the study (*day 14*).

Measurement of RQ. On *day 12* of HFD feeding, the animals were put individually into sealed chambers (SOMEDIC Metabolic System, INCA, Hörby, Sweden) for the measurement of oxygen consumption ($\dot{V}O_2$) and carbon dioxide ($\dot{V}CO_2$) production at room temperature for 48 h. The indirect calorimetric measures were combined with the MiniMitter telemetry system recording activity and core body temperature (14). $\dot{V}O_2$, RQ, body temperature, and activity were analyzed for the whole 48-h period, as well as during the second day of the measurements, divided in 6-h bouts, starting at lights on. Oxygen consumption and CO_2 production data were collected every 2 min, and the remaining variables were measured every minute. Energy expenditure was calculated from Weir's equation (42).

Body composition analysis. Immediately following the indirect calorimetry, the animals were injected with pentobarbital sodium intraperitoneally, and a local anesthetic (Lidocaine 0.1 ml and 0.2 ml sc in the neck and stomach). Telemetric probes were removed, and body composition was measured using a minispec LF110 whole body composition analyzer (Bruker's, Doubravnik, Czech Republic).

Fecal analysis. Fecal droppings were collected from all the rats ($n = 10$ rats/group) during the 48 h of indirect calorimetry and were dried for later analysis. The fecal samples were analyzed for fat content using acid hydrolysis (Eurofins Food & Feed Testing Laboratory, Linköping, Sweden).

Statistical Analysis

Data were analyzed using the Prism statistical software, version 6 (GraphPad Software, San Diego, CA). Normal distribution was assessed using Shapiro-Wilk normality test. Nonparametrical statistical methods were used for all analyses except for body weight, body weight gain, BCA, indirect calorimetry, and fecal fat content, which were normally distributed and analyzed by *t*-test, as well as a two-way ANOVA with time and treatment as fixed factors followed by a Bonferroni corrected multiple-comparison test of all individual time points. Linear regression was used to test for differences in slopes or intercepts. In figures and text, data are expressed as median and interquartile range and differences computed by Mann-Whitney *U*-test, if not otherwise stated. P values < 0.05 were considered statistically significant.

RESULTS

First Experiment: Intestinal Fat Oxidation

Food intake. All rats but one in the HFD Group finished the 15 g of food administered during the night, every night. In the thylHFD Group, three rats did not finish their 15 g of food on more than one occasion. The thylHFD-fed rats had a lower caloric intake than HFD control rats during the dark hours ($P < 0.0001$, Fig. 1*B*), whereas during the daytime, thylakoid rats increased their food intake compared with controls ($P < 0.001$, Fig. 1*C*). Overall, supplementation of thylakoids did not affect food intake compared with control measured over the entire experiment ($P = 0.72$, Fig. 1*A*). During 24 h, the thylHFD-fed and HFD-fed control rats consumed on average 348 and 360 kJ (median, $P = 0.15$, Fig. 1*D*), respectively.

Body weight. There were no differences in body weight between thylHFD and HFD control rats either on *day 0* or *day 14* ($P = 0.63$, Fig. 1*G*). Neither were there any differences between the groups in body weight gain over time ($P = 0.81$ Fig. 1*F*) nor total body weight gain per MJ ($P = 0.81$, Fig. 1*E*).

Adipose tissue: WAT and BAT. Thylakoids decreased visceral fat pad weight (i.e., the intra-abdominal mesenteric, epididymal, and retroperitoneal fat pads) by 25% compared with control ($P < 0.05$, Fig. 1*H*). Thylakoids also decreased the percentage of visceral WAT per body weight compared with control ($P < 0.05$, Fig. 1*I*). The weight of inguinal subcutaneous WAT was not significantly different between the groups; the thylakoid group had 3.25 g of inguinal subcutaneous WAT (median, lower, and upper quartiles 3.05–3.67 g) and the control group had 3.71 g of inguinal subcutaneous WAT (lower and upper quartiles 2.87–4.59 g). Neither was there any significant difference between the weights of the interscapular BAT; the thylakoid group had 0.38 g interscapular BAT (median, lower, and upper quartiles 0.33–0.43 g), and the control group 0.37 g (lower and upper quartiles 0.36–0.43).

Intestinal fat oxidation genes. All genes were normalized to *Actb* for analysis. Thylakoids induced a strong gene expression of *Hmgcs2* ($P < 0.05$, Fig. 2*E*), *Cpt1a* ($P < 0.05$, Fig. 2*F*), and *Fat/Cd36* ($P < 0.05$, Fig. 2*I*) in the jejunum compared with control. There was also a tendency toward increased expression of *Lcad* ($P = 0.05$, Fig. 2*G*), while the expression of

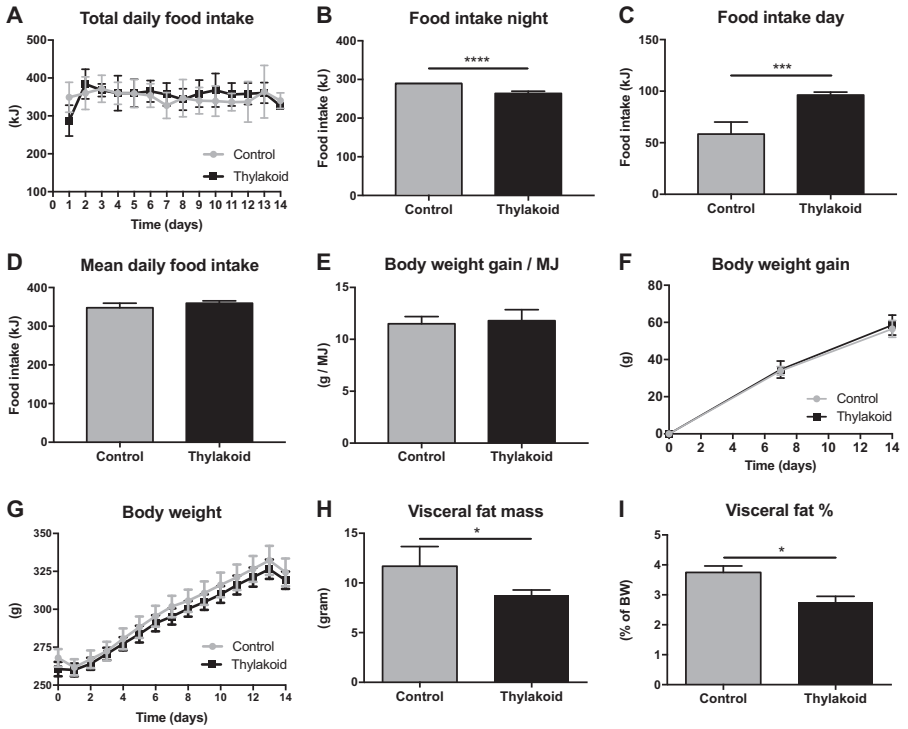

Fig. 1. Thylakoids decreased visceral fat. Food intake and body weight were unaffected. Food intake (*A–D*): Thylakoids did not affect the overall energy intake during the 14-day experiment compared with controls (*A*) nor mean daily food intake (*D*). The reduced caloric intake at night (*B*) was fully compensated by an increased intake during daytime (*C*). Body weight (*E–G*): Thylakoids did not affect body weight (*G*), or body weight gain over time (*F*). Neither was there any difference between the groups regarding body weight gain per ingested energy (*E*). Visceral fat (*H–I*): Thylakoid treatment decreased visceral body fat mass (*H*) and BW percentage (*I*) compared with control. Group data are expressed as median and interquartile range for food intake and visceral fat, and as means ± SE for body weight (n = 8 rats/group). *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$.

Fabp2 (Fig. 2*H*) and *Ppara* (Fig. 2*J*) was not affected in the jejunum. No differences were seen in the duodenum (Fig. 2, *A–D*), the ileum (Fig. 2, *K–N*), or the liver (Fig. 2, *O–T*), except for a reduced expression of *Ppara* in the liver ($P < 0.05$, Fig. 2*T*).

Plasma metabolites. Thylakoids did not affect levels of BHB, TG, or NEFA in plasma taken 70 min after the test meal (Table 2).

Liver weights and CYP activity. Thylakoids did not affect liver weights compared with controls. There were no differences in formation rate regarding either phenacetin-*O*-deetylation (CYP1A2), diclofenac-4′-hydroxylation (CYP2C9), bufuralol-1′-hydroxylation (CYP2D6), or midazolam-1′-hydroxylation (CYP3A4) (data not shown) between the thylakoid group and control group.

Second Experiment: Indirect Calorimetric Measures

Food intake. Thylakoids decreased total caloric intake over the course of the study compared with control treatment ($P < 0.01$, total area under the curve, Fig. 3*A*). During 24 h, the thylHFD-fed rats consumed 371 kJ (median value) compared with the HFD-fed control rats that consumed 466 kJ ($P < 0.01$, Fig. 3*B*).

Body weight. With ad libitum intake of thylHFD, thylakoids decreased body weight gain over time compared with the HFD-fed control group [effect of treatment: F (1, 18) = 7.2, $P = 0.01$, Fig. 3*E*]. On *day 12*, thylakoids had decreased body weight gain compared with controls by 17.5% ± 3.9 ($P < 0.001$, Fig. 3*E*). A significant interaction between time and treatment was found for absolute body weight [F (2, 36) = 6.0, $P < 0.01$, Fig. 3*D*], but no differences were found at any individual time point. Total body weight gain per MJ was not affected (Fig. 3*C*).

Body composition. Thylakoids decreased total fat mass, as shown by the body composition analysis performed on *day 14*, even though this difference did not reach statistical significance ($P = 0.10$, Fig. 3*F*). There were no treatment differences in body fat percentage (Thylakoid Group: 3.6 ± 1.6 g, Control

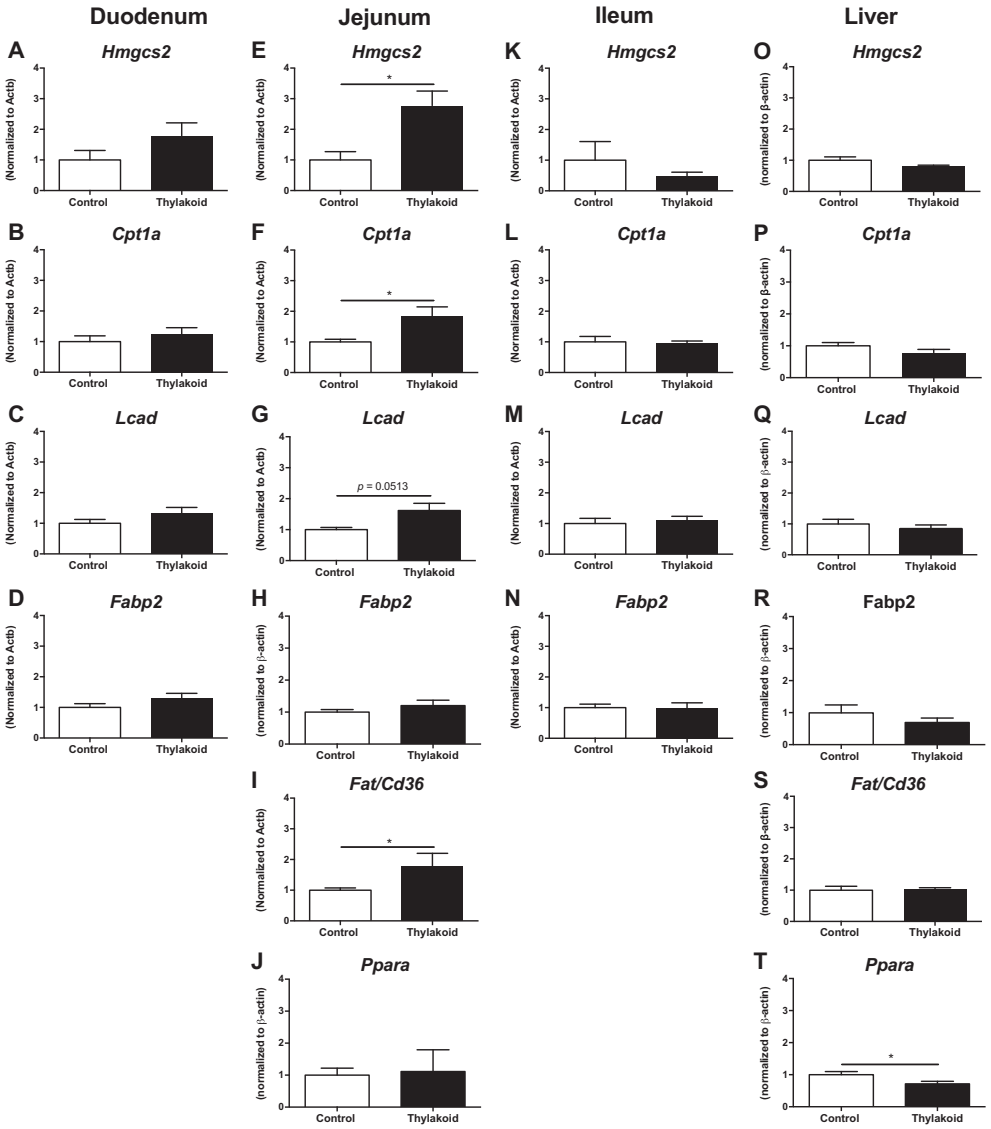

Fig. 2. Thylakoids increased gene expression in the jejunum; however, duodenum, ileum, and liver were primarily unaffected. Duodenum (*A–D*): Thylakoids did not affect gene expression of fat metabolism-related genes in the duodenum compared with control ($n = 5$ control and 7 thylakoid rats). Jejunum (*E–J*): Thylakoids induced a strong expression of fat metabolism-related genes in the jejunum compared with control: *Hmgcs2* (*E*), *Cpt1a* (*F*), and *Fat/Cd36* (*I*). In addition, there was a tendency toward increased expression of *Lcad* (*G*), while *Fabp2* (*H*) and *Ppara* (*J*) were not affected ($n = 6$ control and 7 thylakoid rats). Ileum (*K–N*): Thylakoids did not affect gene expression of fat metabolism-related genes in the ileum compared with control. ($n = 4$ control and 4 thylakoid rats). Liver (*O–T*): Thylakoids did not affect gene expression of fat metabolism-related genes in the liver compared with control, except for a reduced expression of *Ppara* (*T*) ($n = 8$ control and 8 thylakoid rats). Data are presented as fold change in gene expression and expressed as means ± SE. *$P < 0.05$.

Table 2. *Plasma metabolites*

	Control Group Median (Lower-Upper Quartiles)	Thylakoid Group Median (Lower-Upper Quartiles)
BHB, mmol/l	0.16 (0.13–0.28)	0.17 (0.04–0.24)
TG, mmol/l	1.45 (1.40–1.75)	1.65 (1.43–2.03)
NEFA, mmol/l	0.22 (0.00–0.24)	0.21 (0.00–0.21)

BHB, β-hydroxybutyrate; TG, triglycerides; NEFA, nonesterified fatty acids.

Group: 5.1 ± 2.4 g), total lean mass (Thylakoid Group: 317.4 ± 17.4 g, Control Group: 328.0 ± 19.7 g) or lean mass percentage (Thylakoid Group: 84.0 ± 2.6 g, Control Group: 83.9 ± 2.5 g).

Measurement of RQ. Thylakoids decreased the RQ during the dark period compared with controls, 0.86 vs. 0.91 ($P < 0.05$, Fig. 4*A*), whereas no differences in RQ were found during the light period. When analyzed according to the relative cumulative frequency method (34), an overall decrease in RQ over the whole 48-h period was found in the Thylakoid Group compared with controls, 0.84 vs. 0.88 ($P < 0.05$, Fig. 4*B*). Analysis of the Hill slope coefficient revealed an increased adaptive metabolic capability to HFD feeding in the thylakoid-fed group, the Hill slope being steeper than in controls, i.e., 1.0 vs. 0.8 ($P < 0.05$, Fig. 4*B*). Thylakoids had no effect on whole body oxygen consumption during the indirect calorimetric measurements when normalized to absolute body weight or lean mass (data not shown). There were no differences in core body temperature (Fig. 5*C*) or activity levels (Fig. 5*B*) between the thylHFD and HFD rats. Finally, there were no differences either in the total energy expenditure over 48 h (Fig. 5*A*) or the total energy expenditure as a function of fat-free mass, where no differences in either the intercepts ($P = 0.42$) or the slopes ($P = 0.25$) were found (data not shown). Similarly, energy expenditure data for the dark hours were not significantly different between the groups (data not shown).

Fecal fat analysis. Thylakoids did not increase the fecal fat content compared with control. Analysis of fecal droppings collected during the 48 h of indirect calorimetry showed no difference in total fat content between thylakoid and control groups ($P = 0.64$, Fig. 6).

DISCUSSION

In this study, we report entirely novel findings, suggesting that daily intake of thylakoids together with a high-fat diet, increases intestinal fatty acid oxidation in the rat. The decreased RQ of rats consuming thylHFD indicates a shift in whole body substrate utilization toward an increase in fatty acid oxidation, which may start already in the intestinal enterocytes. The intestine, being a major metabolic organ accounting for ~25% of total body oxygen consumption, requires a large amount of energy for enterocyte nutrient absorption (23, 41). As such, the intestine is able to use different sources of energy according to availability (23). Earlier studies

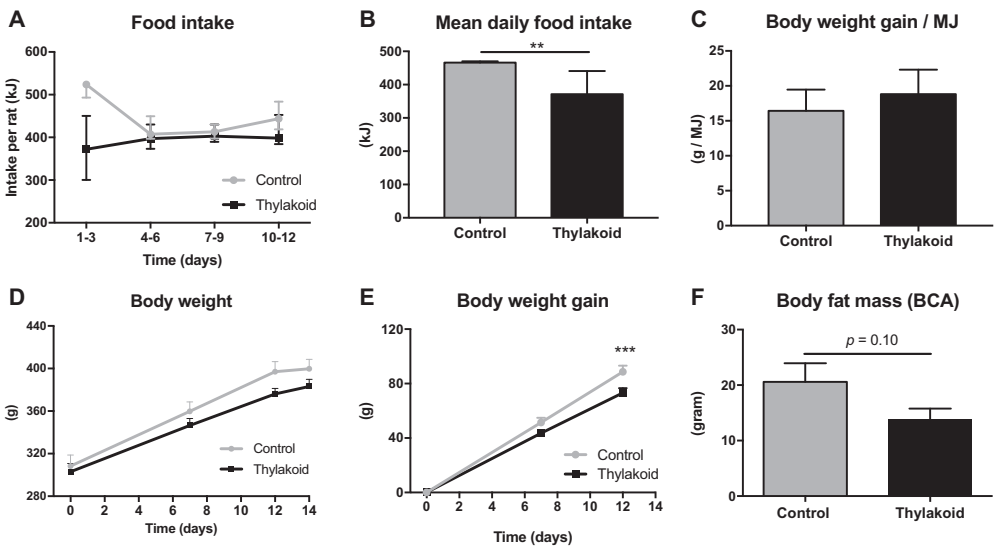

Fig. 3. Thylakoids decreased food intake, body weight gain, and body fat mass. Food intake (*A* and *B*): Thylakoids decreased food intake compared with control treatment. The graphs show average food intake per rat and day over time (*A*) and average food intake per rat during 24 h (*B*), data are expressed as median and interquartile range. Body weight gain (*C*–*E*): Thylakoids decreased body weight gain over time (*E*), reaching a significant difference in body weight between ThylHFD and HFD on *day 12* ($P = 0.01$ and $P < 0.001$, two-way ANOVA followed by Bonferroni post hoc test). There were no significant differences between the groups in absolute body weight at any time point (*D*) or in body weight gain per ingested MJ (*C*). Group data are expressed as means ± SE. Body composition analysis (*F*): Thylakoids tended to decrease body fat mass compared with control treatment, but the difference did not reach statistical significance. Data are expressed as means ± SE ($n = 10$ rats/group). $^{**}P < 0.01$, $^{***}P < 0.001$.

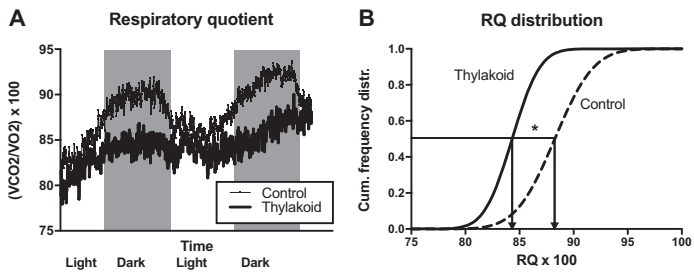

Fig. 4. Thylakoids decreased nighttime RQ. *A*: mean values during 48 h show a decrease in RQ in the Thylakoid Group during the dark hours ($P < 0.05$). *B*: frequency distribution of RQ values, presented as relative cumulative frequency. Statistical comparisons of the curves are based on the 50th percentile values, showing a decrease in the Thylakoid Group. In addition, the Hill slope for the thylakoid-treated group was steeper than in controls, indicating increased adaptive metabolic capability. Data are expressed as means ($n = 10$ rats/group). *$P < 0.05$.

have shown that when animals are fed a HFD, fatty acid oxidation and ketogenesis are induced (7, 20, 23, 40). This occurs through an increased activation of PPAR-α-dependent signaling in the intestine and liver, increasing the expression of enzymes that promote fatty acid oxidation, such as fatty acid translocase (FAT/CD36), fatty acid binding protein (FABP), and CPT1. PPAR-α also stimulates ketogenesis via HMG-CoAS2. Our findings suggest that adding thylakoids to a HFD further enhances these effects, helping the animals adapt to the increase in fatty acid substrate load. Analysis of the cumulative frequency distribution of the RQ and the Hill slope coefficient of the cumulative frequency distribution curves support this interpretation, indicating that thylakoid treatment enhances metabolic flexibility, i.e., the ability to switch between fuel sources depending on availability.

The decrease in RQ appeared during the dark period when animals are active, eat more, and normally tend to utilize more carbohydrates than fat (26). The increase in fat utilization over longer periods of time may, at least in part, explain the decrease in body weight and change in body composition seen both in the present study and in previous studies (11). The total energy expenditure was only measured for 48 h, which may not have been long enough to detect an increased energy expenditure representative for the whole 14-day experiment, corresponding to the reduced body weight gain that was found over the longer time span. It is important in this context that there was no fat loss via the feces, indicating that fat is, indeed, absorbed when thylakoids are present. This finding also supports previous studies stating that thylakoids prolong fat digestion reversibly (1, 8) and do not cause steatorrhea (30). One limitation to this study was that macronutrient oxidation could not be calculated since urinary nitrogen extraction was not measured (13).

Gene expression levels of the fatty acid oxidative enzymes *Hmgcs2*, *Cpt1a*, and *Fat/Cd36* in the intestinal mucosa samples from the jejunum of rats were significantly higher in the thylakoid-treated group compared with the control group. There were, however, no differences in expression of *Ppara* in the intestine. These findings indicate that ingestion of thylHFD upregulated fatty acid oxidation and ketogenesis in enterocytes, but that this upregulation was not due to increased *Ppara* expression. Nevertheless, because all of the upregulated genes are normally regulated by PPAR-α-activity, there may still be a posttranscriptional activation of PPAR-α with dietary thylakoids in the intestine, thylakoids acting as a PPAR-α-agonist.

With PPAR-α being a key regulator of fatty acid uptake, activation of fatty acids and intracellular binding, mitochondrial, and peroxisomal fatty acid oxidation, ketogenesis, triglyceride turnover, and lipid droplet biology (19), the enzymes corresponding to the genes upregulated in this study are vital for different steps in fatty acid absorption and metabolism. FAT/CD36 is a key transporter of long-chain fatty acids across the cell plasma membrane into the enterocyte (4) and, therefore, important for the absorption of fatty acids. CPT1, a key regulatory enzyme of β-oxidation, catalyzes the rate-limiting step in the transport of activated fatty acids across the inner membrane of the mitochondria (3, 32), and HMGCS2 is the rate-limiting enzyme of ketogenesis (32). All of the observed changes in gene expression are, therefore, consistent with the interpretation that thylakoids enhanced the oxidation of diet-derived fatty acids in enterocytes, in particular, in the jejunum.

The hypothesis that thylakoids act as a ligand for PPAR-α is consistent with both the findings in the present study, as well as with earlier studies on thylakoids, demonstrating the lowering effects of thylakoids on blood lipid levels (1, 21, 22) and body fat accumulation (10, 22). An increase in intestinal fatty acid oxidation could also contribute to the reduced body weight and body fat in this study, as well as in earlier thylakoid studies in rodents (1, 10, 22) and humans (28). These findings are supported by the fact that there was no difference in fecal fat content between thylakoid-treated and control animals. mRNA translation is, however, regulated by many different factors. An upregulated gene expression, therefore, does not automatically translate into increased protein synthesis, and the effects of thylakoids on protein levels must be explored in future studies.

Karimian Azari et al. (17, 18), demonstrated that administration of the PPAR-α-agonists oleoyl ethanolamide or Wy-14643 stimulated fatty acid oxidation and ketogenesis, respectively, in the intestine and, in particular, in the jejunum, but not in the liver (17). Wy-14643 also caused an acute and transient decrease in RQ, and both compounds reduced food intake and increased hepatic portal vein BHB levels (18). These findings suggest that activation of PPAR-α may, indeed, lead to results similar to the ones found in the present study. In another study, an inhibition of diacylglycerol-*O*-acyltransferase and, hence, of the final step in triacylglycerol resynthesis in the enterocyte, also caused similar results, indicative of enhanced intestinal fatty acid oxidation and ketogenesis, and a shift toward whole body fat oxidation, as well as a decrease in food intake in rats fed a HFD (35), suggesting that any manipulation of intestinal fatty acid metabolism that may cause a shift in intestinal fat handling from reesterification to oxidation may cause similar effects.

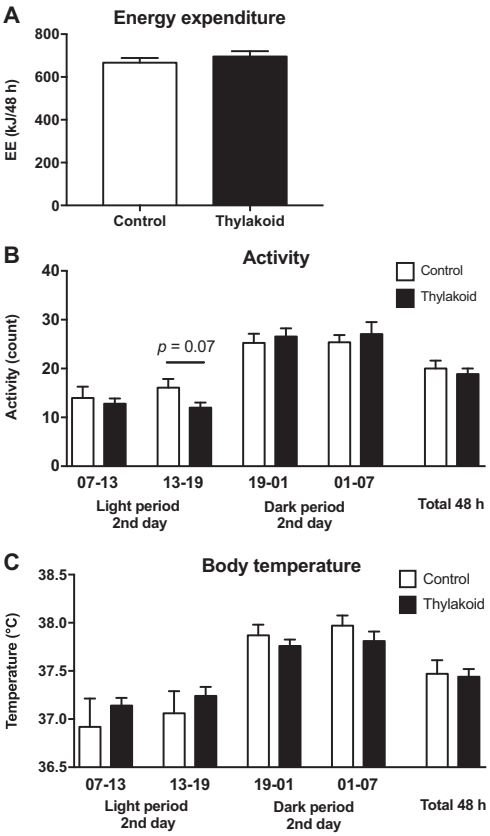

Fig. 5. Thylakoids did not affect energy expenditure, activity levels, or body temperature. Energy expenditure (*A*): There was no difference between groups regarding total energy expenditure during the 48 h of indirect calorimetry. Activity levels (*B*): There were no significant differences in activity counts between the groups, measured during the indirect calorimetry. Body temperature (*C*): There were no differences between groups regarding core body temperature measured during the indirect calorimetry. Data are expressed as means ± SE (n = 10 rats/group).

With gene expression level changes indicating increased ketogenesis, one might expect increased levels of ketone bodies in plasma in the thylHFD-treated animals compared with the control animals. Dietary thylakoids did, however, not affect plasma levels of TG, NEFA, or ketone bodies at the selected time point. Nevertheless, this does not exclude a possible increase in ketogenesis and, in particular, in intestinal ketogenesis. Because the blood samples were taken more than 60 min after the test meal, there is the possibility that a peak in ketone body levels at an earlier time point was missed. In an earlier study, BHB levels peaked at 35 min and had already normalized at 60 min (17). Furthermore, in this earlier study, blood samples were taken from the hepatic portal vein, which might have yielded higher plasma levels of ketone bodies, if these were mainly produced in the intestine, compared with cardiac blood samples collected in the present study.

In the present study, we replicated previous results showing decreased body weight gain and body fat mass (9, 22), as well as reduced food intake (22, 30), in response to thylakoid treatment. Looking at food intake over time in the second experiment (Fig. 3*A*), HFD-fed animals ingested more than the thylHFD animals during the first 3 days, suggesting that the HFD was more palatable than the chow they switched from. There is no indication that the thylHFD was less palatable than the regular chow, because the food intake in the Thylakoid Group was stable over time (27). Had there been a palatability issue, the food intake would have been decreased during the first few days (2), which was not the case in this study nor in any previous studies with thylakoids in mice and rats (1, 9, 22, 30). In addition, palatability of thylakoids has been tested in conjunction with earlier studies and shown to have no appetite-suppressive effect (22). Therefore, we suggest that the reduced caloric intake in the thylakoid-fed animals, in spite of ad libitum access to food, may be explained by increased satiety due to enhanced fatty acid oxidation, which has been found associated with decreased food intake before (17, 23, 35). In previous studies with thylakoids, reduced food intake has been attributed to an increased release of satiation signals, such as CCK and GLP-1 (1, 21, 22, 28, 31, 39). Since appetite-regulating hormones were not analyzed in the present study, no conclusions can be drawn about the possible effect of satiety hormones in this study.

Thylakoids decreased body weight gain compared with controls in the second but not the first experiment. This may be due to the different feeding schedules. In the first experiment, the fixed amount of food at night may have limited the rats' total food consumption and, consequently, also attenuated their body weight gain (Fig. 1*F*), as opposed to ad libitum feeding during the whole 24 h in the second experiment (Fig. 3*E*). Seeing that the thylHFD-fed rats consumed the same daily amount of energy in both experiments (Figs. 1*D* and 3*B*), while the HFD-fed rats consumed more during the ad libitum condition (Fig. 3*B*), the Control Group was probably more affected by the night time restriction than the Thylakoid Group, which is reflected in the augmented body weight during the second experiment. This is confirmed by the fact that the thylakoid rats did not always finish the food given at night, while the control rats did. There were no differences in body weight gain per

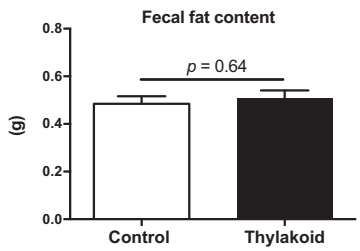

Fig. 6. Fecal fat analysis. The total fat content of the feces collected during the 48-h indirect calorimetry was not different between groups. Data are expressed as means ± SE, (n = 10 rats/group).

ingested MJ between groups in either experiment, suggesting that thylHFD does not increase energy expenditure compared with HFD, but changes the source utilized. Calculations of energy expenditure confirmed that there was indeed no difference between the groups. The calculation of energy expenditure was determined on the basis of $\dot{V}O_2$ and $\dot{V}CO_2$ alone, which involves an error of about 4% in the fasting condition, however, decreasing with the metabolic rate (13).

Thylakoid treatment did not affect liver weights and CYP enzyme activity. Such an effect is important, considering that the number of patients being treated with multiple drugs continues to increase (16). Active components of fruits and vegetables can have inhibitory effects on CYP450 and drug metabolism, thereby causing serious adverse effects (25). Spinach juice has previously been reported to inhibit CYP1A2 in vitro and affect the metabolism of heterocyclic aromatic amines. Thylakoid treatment did not cause any differences in CYP activity compared with control animals for the CYPs examined in this study; however, the direct inhibitory effect of thylakoids remains to be addressed.

In conclusion, we found that compared with a control HFD, thylakoids together with a HFD in the rat stimulate intestinal fatty acid oxidation at the gene expression level—which has never been shown before—indicating that thylakoids may stimulate intestinal fatty acid oxidation by the upregulating of associated genes and, possibly, through activation of PPAR-α. The fact that thylakoids reduced the RQ during indirect calorimetry is also an entirely novel finding. Together with the fact that no difference in fecal fat was found, the increased fatty acid oxidation may contribute to the known effects of thylakoid treatment, such as body weight loss, reduced body fat, and blood lipids, as well as increased circulating levels of satiety hormones.

Perspectives and Significance

The present findings suggest that thylakoids are a promising natural additive that may be ingested for beneficial effects stimulating fat oxidation, starting already in the intestine. Further studies are necessary to examine whether the effect of thylakoids is, indeed, mediated through activation of PPAR-α.

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DISCLOSURES

C. Erlanson-Albertsson is scientific advisor for Greenleaf Medical AB, as well as part owner and board member of Thylabisco AB.

AUTHOR CONTRIBUTIONS

E.-L.S., E.E., C.M., B.R.W., A.M., W.L., and C.E.-A. conception and design of research; E.-L.S., E.E., C.M., D.R., B.B., and B.R.W. performed experiments; E.-L.S., E.E., D.R., and B.B. analyzed data; E.-L.S., E.E., D.R., B.B., A.M., W.L., and C.E.-A. interpreted results of experiments; E.-L.S., E.E., and D.R. prepared figures; E.-L.S., E.E., D.R., and B.B. drafted manuscript; E.-L.S., E.E., C.M., D.R., B.B., B.R.W., A.M., W.L., and C.E.-A. edited and revised manuscript; E.-L.S., E.E., C.M., D.R., B.B., B.R.W., A.M., W.L., and C.E.-A. approved final version of manuscript.

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