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2015

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### *Citation for published version (APA):*

Montelius, C. (2015). *Thylakoid membranes from green plants affect appetite and promote body weight loss*. [Doctoral Thesis (compilation), Department of Experimental Medical Science]. Department of Experimental Medical Science, Lund University.

*Total number of authors:*

1

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# Thylakoid membranes from green plants affect appetite and promote body weight loss

Caroline Montelius



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DOCTORAL DISSERTATION

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To be defended at LUX Hörsal nedre, Helgonav. 3, Lund.

Friday the 6th of February 2015 at 9 am.


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Karolinska Institutet

Organization LUND UNIVERSITY Faculty of Medicine, Dep. Of Experimental Medical Science, Appetite Regulation Unit Author(s): Caroline Montelius	Document name DOCTORAL DISSERTATION Date of issue 2015-01-15 Sponsoring organization
Title and subtitle <b>Thylakoid membranes from green plants affect appetite and promote body weight loss</b>	
Abstract: The incidence of overweight and obesity has reached alarming proportions. Today, overweight, obesity and the metabolic diseases cause more death than starvation. To counteract an increasing body weight gain, regulation of appetite and a controlled food intake is of greatest importance. Simply put, body weight is regulated by the amount of energy ingested, and the amount of energy expended. In todays sedentary lifestyle, we tend to have a much higher food intake than the amount of energy used by the body, resulting in body weight gain. Thylakoids are membranes extracted from chloroplasts of green leaves. They have previously been found to prolong the lipolysis and affect food intake and body weight gain in rodents, as well as affect appetite-regulating hormones in healthy volunteers. This thesis aims to explain mechanisms underlying the effects of thylakoids on food intake and body weight gain, as well as present longer time effects in humans. Results show that thylakoids promote body weight loss in humans after three months of daily supplementation. Several factors important for body weight regulation are affected by thylakoids. The digestion of dietary nutrients is prolonged after thylakoid supplementation, resulting in prolonged glucose and insulin responses. In the long run, fasting blood-glucose concentrations are also decreased. Thylakoids stimulate the release of the satiety promoting hormones cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1), while decreasing levels of the hunger promoting hormone ghrelin. Moreover, circulating levels of leptin and blood lipids are decreased after thylakoid supplementation for two and three months. Finally, thylakoids affect the subjective ratings of appetite and urge for specific food. The urge for energy dense, so called palatable food rich in fat and sugar, is specifically decreased after thylakoid intake. In conclusion, the results presented in this thesis show that thylakoids exert positive effects for body weight and appetite regulation and may thus be used as an effective tool for novel treatment of overweight, obesity and the metabolic diseases.	
Key words: Overweight, Obesity, Appetite Regulation, Thylakoids, Craving, Satiety	
Classification system and/or index terms (if any)	
Supplementary bibliographical information	Language English
ISSN and key title: 1652-8220	ISBN:978-91-7619-094-4
Recipient's notes	Number of pages 67 Price Security classification

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Cover pictures: Photographer Helge Rubin

Faculty of Medicine, Department of Experimental and Medical Science

ISBN 978-91-7619-094-4

ISSN 1652-8220

Lund University, Faculty of Medicine Doctoral Dissertation Series 2015:14

Printed in Sweden by Media-Tryck, Lund University

Lund 2015



KLIMATKOMPENSERAT  
PAPPER



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# Abstract

The incidence of overweight and obesity has reached alarming proportions. Today, overweight, obesity and the metabolic diseases cause more death than starvation. To counteract an increasing body weight gain, regulation of appetite and a controlled food intake is of greatest importance. Simply put, body weight is regulated by the amount of energy ingested, and the amount of energy expended. In today's sedentary lifestyle, we tend to have a much higher food intake than the amount of energy used by the body, resulting in body weight gain.

Thylakoids are membranes extracted from chloroplasts of green leaves. They have previously been found to prolong the lipolysis and affect food intake and body weight gain in rodents, as well as affect appetite-regulating hormones in healthy volunteers. This thesis aims to explain mechanisms underlying the effects of thylakoids on food intake and body weight gain, as well as present longer time effects in humans.

Results show that thylakoids promote body weight loss in humans after three months of daily supplementation. Several factors important for body weight regulation are affected by thylakoids. The digestion of dietary nutrients is prolonged after thylakoid supplementation, resulting in prolonged glucose and insulin responses. In the long run, fasting blood-glucose concentrations are also decreased. Thylakoids stimulate the release of the satiety promoting hormones cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1), while decreasing levels of the hunger promoting hormone ghrelin. Moreover, circulating levels of leptin and blood lipids are decreased after thylakoid supplementation for two and three months. Finally, thylakoids affect the subjective ratings of appetite and urge for specific food. The urge for energy dense, so called palatable food rich in fat and sugar, is specifically decreased after thylakoid intake.

In conclusion, the results presented in this thesis show that thylakoids exert positive effects for body weight and appetite regulation and may thus be used as an effective tool for novel treatment of overweight, obesity and the metabolic diseases.





# List of papers

This thesis is based on the following papers, which will be referred to in the text by the below given Roman numerals.

- I. Caroline Montelius, Karolina Gustafsson, Björn Weström, Per-Åke Albertsson, Sinan Cem Emek, Marilyn Rayner and Charlotte Erlanson-Albertsson, Chloroplast thylakoids reduce glucose uptake and decrease intestinal macromolecular permeability, *Brit J Nutr* 2011, 106; 836-844, doi: 10.1017/S0007114511001267
- II. Caroline Montelius, Nadia Osman, Björn Weström, Siv Ahrné, Göran Molin, Per-Åke Albertsson and Charlotte Erlanson-Albertsson. Feeding spinach thylakoids to rat modulates the gut microbiota, decreases food intake and affects the insulin response. *J Nutr Sci* 2013, 2:e20, doi: 10.107/jns2012.29
- III. Caroline Montelius, Katarzyna Szwiec, Marek Kardas, Liudmyla Lozinska, Charlotte Erlanson-Albertsson, Stefan Pierzynowski, Jens F Rehfeld and Björn Weström. Dietary thylakoids suppress blood glucose and modulate appetite-regulating hormones in pigs exposed to oral glucose tolerance test. *Clin Nutr* 2014, 33; 1122-1126, doi: 10.1016/j.clnu.2013.12.009
- IV. Eva-Lena Stenblom, Caroline Montelius, Karolina Östbring, Maria Håkansson, Sofia Nilsson, Jens F Rehfeld and Charlotte Erlanson-Albertsson. Supplementation of thylakoids to a high carbohydrate meal decrease feelings of hunger, elevate CCK levels and prevent postprandial hypoglycaemia in overweight women, *Appetite* 2013, 68: 118-23, doi: 10.1016/j.appet.2013.04.022
- V. 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

- VI. 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 in overweight women. *Appetite* 2014, 81: 295-304, doi: 10.1016/j.appet.2014.06.101

# Abbreviations

BIA	Bioelectrical impedance
BMI	Body mass index (kg/m <sup>2</sup> )
BMR	Basal metabolic rate
CCK	Cholecystokinin
CDC	Centers for Disease Control and Prevention
CFU	Colony-forming units
Chl	Chlorophyll
CVD	Cardiovascular disease
DEXA	Dual-energy X-ray absorptiometry
DNA	Deoxyribonucleic acid
E%	Energy percent (distribution of macronutrients)
EFSA	European Food Safety Authority
FFM	Fat free mass
FHM	Public Health Agency of Sweden (Folkhälsomyndigheten)
FITC-dextran	Fluorescein isothiocyanate labelled dextran
GI	Glycaemic index
GIP	Gastric inhibitory peptide
GI-tract	Gastrointestinal tract
GL	Glycaemic load
GLP-1	Glucagon-like peptide 1
HDL	High density lipoprotein

HFD	High fat diet
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
JBV	Swedish Board of Agriculture (Jordbruksverket)
LDL	Low density lipoprotein
NAFLD	Non-alcoholic fatty liver disease
NaTDC	Sodium dodecyl sulphate
OGTT	Oral glucose tolerance test
p-	Plasma
$P_{app}$	Apparent permeability coefficient
PCR	Polymerase chain reaction
rDNA	Ribosomal DNA
SCB	Statistics Sweden (Statistiska centralbyrån)
SUS	Skånes Universitets Sjukhus
TAG	Triacylglycerol
TNF-alpha	Tumour necrosis factor-alpha
T-RFLP	Terminal restriction fragment length polymorphism analysis
VAS	Visual analogue scale
VLDL	Very low density lipoprotein
WHO	World Health Organisation

# Populärvetenskaplig sammanfattning

Övervikt och fetma fortsätter öka världen över, och har redan nått enorma proportioner. Med dessa tillstånd följer en rad sjukdomar, så som diabetes typ 2, hjärt- och kärlsjukdomar och en del cancersjukdomar. Idag är det globalt sett fler människor som dör av fetma och de sjukdomar som är relaterade till övervikt, än som dör av undervikt och svält. Övervikt orsakas främst av att vi har ett högre energi-intag än den energimängd vi gör av med genom att röra på oss. Idag har många stillasittande jobb och väljer bilen eller bussen framför att promenera eller cykla. Vi prioriterar inte heller träning eller andra fysiska aktiviteter på fritiden. När det kommer till våra måltider består dessa ofta av extra "smakrik" mat, d.v.s. mat som innehåller en stor del fett och/eller socker. När vi sedan lagt på oss några extra kilon får vi ett ännu starkare sug för smakrik föda, så att dessa livsmedel fyller en ännu större del av vårt dagliga liv. Stressen kring olika dieter och kostråd gör tyvärr inte det ohälsosamma ätandet bättre. Trender som att äta flertalet mellanmål för att undvika att bli hungrig har lett till att vi idag har väldigt svårt att verkligen "känna" hur hungriga vi är och hur mätta vi blir efter en måltid. Detta gör att vi oftare äter större måltider och fler mellanmål. De medfödda hunger- och mättnadssignaler har blivit utslagna.

De läkemedel som idag finns tillgängliga för att motverka övervikt och fetma har oftast biverkningar, så som fettdiarré, huvudvärk, infektioner och hjärtklappning. Antalet fetmaoperationer, sk. gastric bypass, ökar även världen över.

I min forskning har vi funnit att ett membran från gröna blad, thylakoider, har positiva effekter hos både djur och människor. Tillsats av thylakoider leder till ett långsammare upptag av näringsämnen från mag- och tarmkanalen, vilket leder till ett lägre blodsockersvar efter måltid. Ytterligare förbättringar som vi funnit hos personer som ätit tillskott av thylakoider är bättre reglerad frisättning av insulin samt sänkta blodfetter. Vi har även funnit att thylakoider ger en ökning av de hormoner som påverkar mättnaden, och minskar de hormoner som gör oss hungriga, vilket gör att vi håller oss mätta och belättna en längre tid mellan måltiderna. Efter en måltid med thylakoider har vi även funnit att suget efter de extra smakrika produkterna med hög andel fett och/eller socker sjunker. Exempel på dessa smakrika produkter är choklad, kanelbullar och godis. Slutligen har vi visat i en studie hos människor att tillskott av thylakoider varje dag i tre månader resulterar i en vikttnedgång på ca 5 kg (ca 6% av kroppsvikten). Denna vikttnedgång kan verka blygsam, men den uppnås genom att endast ta thylakoid-tillskottet och i övrigt följa de svenska och nordiska rekommendationerna för födointag och fysisk aktivitet. Det är dessutom mer hälsosamt

med en långsam viktnedgång, som förhoppningsvis kan hållas stabil under resten av livet. Vid en sträng diet och snabb viktnedgång ändras kroppens ämnesomsättning för att ”spara” på den energi som kommer med födointaget, vilket är anledningen till den s.k. ”jojo-bantningen”. I våra studier med thylakoider har vi däremot sett att denna ändring i ämnesomsättning motverkas, vilket talar för att en varaktig viktnedgång bör vara möjlig med tillskott av thylakoider.

För att sammanfatta så påverkar tillskottet av thylakoider kroppen på ett fördelaktigt sätt. Thylakoider leder till en förbättrad reglering av blodsocker, insulin och blodfetter, påverkar hormoner som ökar mättnad och minskar suget efter ohälsosam mat. Tillskott av thylakoider har inte heller visat på några biverkningar. Sammanfattningsvis leder tillskott av thylakoider till viktnedgång. Vi fastslår därför att thylakoider kan vara en ny metod för att motverka och behandla övervikt, fetma och de metaboliska sjukdomarna.

# General introduction

The prevalence of overweight and obesity continues to increase worldwide, and has reached epidemic proportions, as obesity, and the medical complications that follow, cause more death today than underweight and starvation (WHO °311). Obesity is not only an adult disease, but also a health issue in children. In Sweden, approximately 50% (SCB) of the adult populations suffers from overweight or obesity, and approximately 18% of children 7-9 years old (1) are overweight or obese. The risk of obtaining a continuous overweight or obesity in adult life is 25% for overweight children below 6 years, and 75% for overweight adolescents (2). The simplest way of describing overweight is an increased intake of food and decreased energy expenditure every day (WHO °311).

Food rich in refined carbohydrates and/or fats is named palatable food. The intake of this food, and sugar sweetened drinks, compose approximately 20 % of the daily intake in the USA (3). In Sweden, the intake of sweet beverages, chocolate, sweet cakes and sweets in general, are approximately 17% of the total energy intake (JBV).

To decrease the excess intake of food, appetite control is crucial. Appetite is regulated by several hormones, as well as by the physical properties of the chosen food and the distension of the stomach. The physical properties of the food first affect the taste and mouth feeling, and second the digestion due to the composition of proteins, fats, refined carbohydrates and fibres. The physical properties also affect the secretion of the appetite regulating hormones. Moreover, the distension of the stomach affects the secretion of the same hormones, and as a feedback mechanism, some of these hormones affect the emptying of food from the stomach to the intestine. To complicate things even more, our cognitive functions play an important role in appetite control. If we believe our previous meal was smaller than we are used to, or smaller than we wanted it to be, we will feel hungry again earlier than we “should”, based on the caloric content of the meal (4-8).

Thylakoid membranes are derived from green leaves, and have previously been found to affect the digestion of lipids (9), food intake, body weight gain and appetite regulating hormones in rodents (10,11). Also, in normal weight and healthy humans, thylakoids have been shown to affect appetite regulating hormones in a dose dependent way during a one meal study (12).

This thesis describes the effects of thylakoid supplements in one *in vitro* study, two animal studies and three human studies. Thylakoid supplementation to overweight



humans have been studied, both during mealtime interventions and in the longer perspective of two and three months. Also, the effects on glucose metabolism and interactions with the gut microbiota have been investigated.

# Background

## Thylakoid membranes

Thylakoids are the most abundant membranes on earth, present in all green plant cells as a part of the chloroplast (figure 1). They host the membrane bound components in the light harvesting cycle, as photosystem I and II, and are thus responsible for the photosynthesis. Thylakoids contain hundreds of different membrane proteins, galactolipids, sulpholipids, various vitamins (A, E and K) and polyphenols/antioxidants, such as chlorophyll, carotenoids, lutein and zeaxantin (13). Thylakoids have a flat, coin-like structure and are stacked on each other in a formation called grana (figure 1).

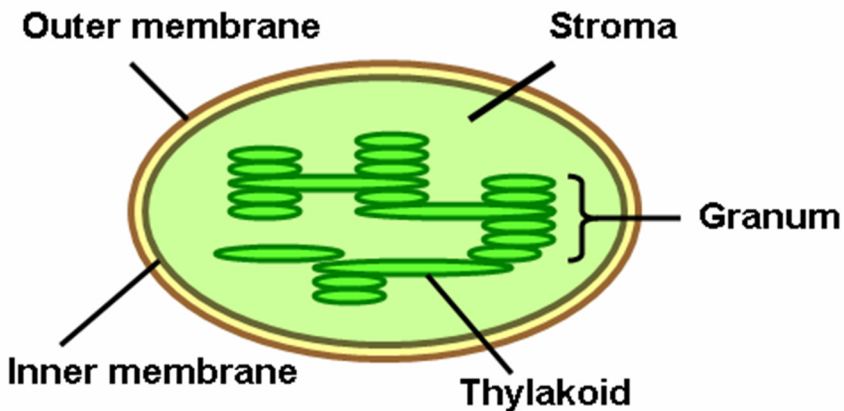
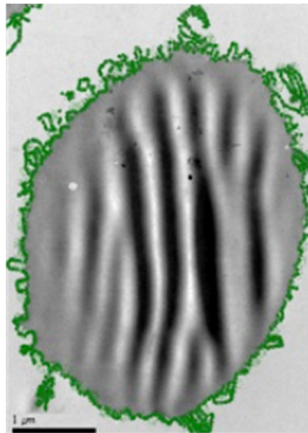


Figure 1. Schematic overview of thylakoids, stacked as coins in granums, inside a chloroplast.

Previously, thylakoids have been found to retard the fat digestion by binding to both dietary fat, lipase and co-lipase *in vitro* (9). The interaction between thylakoids and lipids has been studied with electron microscopy, and it has been concluded that thylakoids cover the entire surface of lipids *in vitro* (figure 2). When thylakoids bind to lipids, and to lipase/co-lipase, the result is a prolonged lipolysis (9).



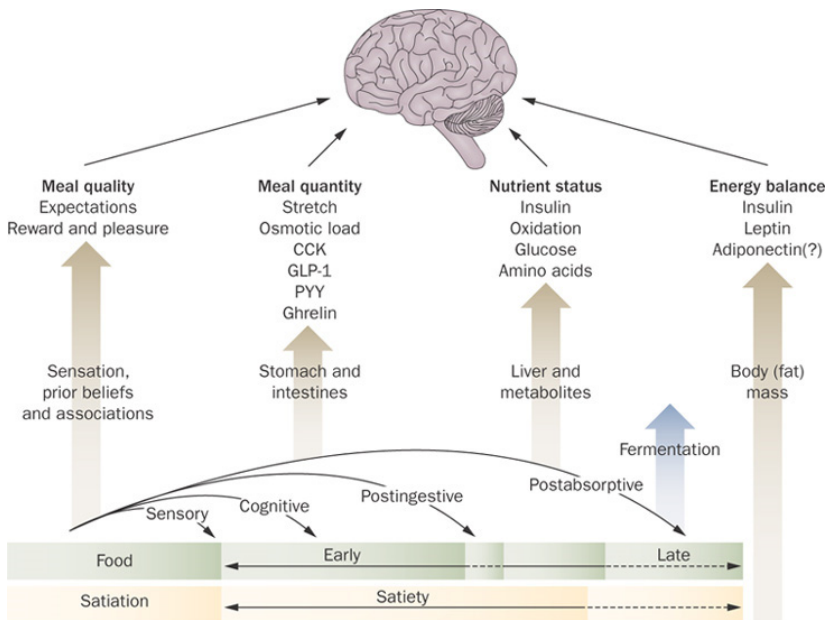
**Figure 2.** Electron microscopy picture of a lipid droplet covered by thylakoids. Thylakoids are colored green in photoshop (done by Sinan C Emek) (9).

Animal studies in rat and mouse have shown that the addition of thylakoids is associated with a decreased food intake and a decreased amount of body fat (10,11)(unpublished data). Moreover, in a single meal study in healthy volunteers, thylakoids were suggested to increase the satiety hormone cholecystokinin (CCK) and decrease the hunger hormone ghrelin (12). The secretion of insulin has also been shown to decrease after supplementation of thylakoids (12,14).

The thylakoid membranes used in my work have all been derived from spinach leaves (*Spinach oleracea*). Thylakoids are prepared from fresh spinach leaves (or dried leaves for the Appethyl product), as described earlier (11). Three different thylakoid powders have been used; prepared by ourselves, prepared by SwePharm AB (Södra Sandby, Sweden) and the product Appethyl produced by Green Leaf Medicals AB (Stockholm, Sweden). 100 g of Appethyl contain 365 kcal, and the composition of macronutrients is 26.1 g protein, 7.2 g fat and 48.7 g carbohydrate. The content per 100 g of powder etc. is 5750 IU vitamin A (4760 µg beta-carotene), <1 mg vitamin C, 1330 µg vitamin K<sub>1</sub>, 6.07 mg vitamin E, 166 µg folic acid, 27.9 mg lutein, 730 µg zeaxanthin, 2910 mg calcium, 115 mg iron and 274 mg sodium.

# Appetite regulation

Appetite is regulated by different cascades of events occurring in the body, and can be divided into the physical and hormonal events. The physical factors are the distension of the stomach post-ingestion, and the taste and texture of the food. Hormonal factors can be divided into episodic hormones, such as ghrelin, CCK and glucagon-like peptide 1 (GLP-1), and tonic hormones, as insulin and leptin (15). The difference between satiation and satiety was described already in 1987, as satiation is the progress to stop eating after a meal, and satiety is the long-term regulation acting between the meals (4,16)(figure 3).



**Figure 3.** The satiety cascade describing the phases of food intake leading to satiation; end of food intake, and the period between meals; satiety (16).

Satiation is first regulated by sensory factors, to inhibit the continuation of the meal. Secondly, cognitive factors inhibit further intake of food, as we know we have just eaten and shouldn't be hungry. The cognitive effects are very strong and people are more prone to eat more frequently if they believe the previous meal was smaller than usual, despite the nutritional and caloric value of that meal (8). Also, neural inputs from the stomach to the brain, acting via the vagus nerve or hypothalamus, results in the secretion of several hormones, such as CCK and GLP-1 (17). These hormones signal to the brain that food is being digested and nutrients absorbed. Finally, energy is being utilized and/or stored, before hunger signals returns, and hormones such as ghrelin

inform the brain that new energy is needed, thus a new cascade begins (5,17,18). Macronutrient composition, sensory qualifications, energy density and the structure of the food are all factors affecting the duration of the time phase “satiety” (5).

The incretin hormones, mainly GLP-1 and gastric inhibitory peptide (GIP) are hormones that stimulate the release of insulin after food intake and thereby decrease the magnitude of the blood-glucose response. Other effects of the incretin hormones are a slower gastric emptying and decreased food intake (19).

Moreover, appetite and food intake are regulated by different food components that trigger the release of macronutrient specific hormones. The “sensory-specific satiety”, was first described by Rolls et al (7). The implications for this is that the satiation for a specific food does not affect the wanting of another food with a different taste, i.e. even when completely full after a savoury and salty meal, a sweet and palatable piece of chocolate-cake can still be desirable.

## **Ghrelin**

Ghrelin is secreted from specific enteroendocrine cells in the gastric mucosa, and is the only known circulating peptide hormone acting to initiate food intake (20-22). Ghrelin receptors are localized in the part of the hypothalamus known to regulate food intake (23,24), and in reward-linked areas as the ventral tegmental area of the brain (25,26). Circulating ghrelin concentration is elevated in the fasting state, and decreased within one hour after a meal. In an energy deficient state, as in starvation and anorexia nervosa, circulating levels are highly increased, whereas circulating levels are suppressed in severe obesity (23). No differences in basal ghrelin levels have however been found in moderate overweight/underweight and otherwise healthy subjects (21). However, as body weight loss occurs, basal circulating ghrelin levels increase, and with weight gain levels decrease (21).

Ghrelin stimulates gastric emptying in both animals and humans (23), and a strong correlation between gastric emptying and ratings of hunger have been found (27). The decreased secretion as a response to food intake appears to be influenced by the nutrients, and not by gastric distension. Thus, an oral glucose load decrease levels of circulating ghrelin but the same volume of pure water does not (28). Glucose and lipids, but not proteins, have been shown to decrease the postprandial ghrelin levels (29). As to the stimulation of food intake, intravenous infusions of ghrelin have been found to effectively increase the amount of food intake following a free buffet (20,21). Obese subjects were even more sensitive to the intravenous infusions of ghrelin as their voluntary food intake was more profound than in normal weight individuals (21). Ghrelin levels have also been shown to increase more rapidly after intake of a meal in overweight and obese subjects, as compared to in lean subjects (29).

## CCK

Cholecystokinin (CCK) is secreted from endocrine I-cells in the small intestine as a response to nutrients (22,30), specifically fat (31,32) and proteins (33,34). As CCK is secreted it binds to specific CCK-receptors (1 and 2), acting to stimulate gallbladder contraction and inhibit gastric emptying. Moreover, CCK stimulates the release of pancreatic enzymes and decrease the intestinal motility. The results of these events are an increased satiety and a reduction in food intake (22,35,36). The CCK-specific effect on appetite is mediated through the binding to CCK<sub>1</sub>-receptor on the vagus nerve (22). The early effects of satiety, “cease eating”, is regulated by the distension of the stomach, activating secretion of CCK from the hypothalamus (37). The in-between meals satiety-signal from CCK is a combined effect of the prolonged gastric emptying and the presence of nutrients in the small intestine, further stimulating CCK secretion (37). Although CCK stimulate satiety, studies investigating the effect of CCK on weight loss have not seen any clear results. CCK appears to promote a short-term satiety, but not to suppress the total daily food intake since the inter-meal time have been shown to be shortened (22,38-40).

## GLP-1

Glucagon-like peptide 1 (GLP-1) is an incretin hormone, and is the cleavage product from pre-proglucagon. It is secreted rapidly after eating, mainly from intestinal L-cells, but also from the brainstem (22,41). All macronutrients stimulate GLP-1 release, but fats, and specifically monounsaturated fats, stimulate the release to a larger magnitude than proteins and carbohydrates (22). The release of GLP-1 occurs in two phases, an early secretion occurs already minutes after food intake and last about 30 minutes, and a later secretion occurs approximately one hour after food intake (41). The early secretion might be due to the distension of the stomach signalling via the vagus nerve to mediate the release of GLP-1 (42), or via the L-cells present in the duodenum (43). GLP-1 acts already before the ingestion of the meal, in response to anticipation for the food. After food intake, GLP-1 delays gastric emptying, gut motility and affect the ileal break, acting to optimize the digestion and absorption of nutrients. GLP-1 enhances satiety in a synergistic way, both affecting the satiety regulating areas in the brain and through the direct gut-regulating effects mentioned above (41). Moreover, GLP-1 enhances insulin secretion and inhibits glucagon release (44), and is therefore used for treating diabetic patients. In regard to body weight, GLP-1 promotes body weight loss (41,45-47), and has been shown to specifically target visceral and truncal adipose tissue (46).

## Leptin

Leptin has a crucial role in energy homeostasis as it is secreted from adipose tissue as a response to the adipose tissue status. Lean and obese subjects thereby have different circulating concentrations of leptin. As long as subjects are weight stable, either overweight or lean, the metabolic effects of leptin does not affect the subject. However, if subjects loose weight, the metabolic effects of leptin are to restore the adipose status to the set point. Circulating levels of leptin are thereby decreased with body weight loss, leading to several effects, as decreased energy expenditure and increased reward value of food. Increased levels of leptin in weight stable subjects have however not been found to effect the metabolism, hence increased leptin concentrations does not initiate body weight gain (48). The role of leptin is mainly to preserve the reproduction capacity and to maintain the adipose storage volume for survival reasons (49). A decreased body weight and amount of adipose tissue would in an evolutionary approach threatening the survival and reproduction. However, in the present obesity epidemic, the role of leptin to regain body weight and keep adipose tissue levels stable, are not favourable.

## Glucose and Insulin

Concentrations of blood-glucose and insulin are in healthy subjects well controlled. Glucose levels increase after food intake, as nutrients are digested and absorbed from the intestine. As glucose-levels increase, the secretion of insulin from beta-cells in the pancreas is stimulated. Dietary carbohydrates are digested to simple glucose or saccharide chains. The rate of absorption into the blood stream is due to the length of the saccharide chain, thus a longer chain results in a prolonged blood-glucose curve and simple glucose and shorter chains results in a fast absorption and a higher peak in blood-glucose. Food resulting in the prolonged absorption and lower blood-glucose curve have a low glycaemic index (GI), and food that results in a fast absorption rate and high peak in blood-glucose have a high GI (50). The glycaemic index is defined as the incremental area under the obtained blood-glucose curve for a specific food, as compared to the curve obtained after intake of pure glucose or white bread (51). In general, refined carbohydrates generate a high blood-glucose response (i.e. have a high GI value), and non-starchy carbohydrates, as legumes and vegetables generate low blood-glucose responses (have a low GI value). The total glycaemic load (GL) is also important for the blood-glucose response. The GL is the sum of the GI-value and the dietary carbohydrate content of the specific food (52).

In healthy individuals, blood-glucose levels are strictly regulated by homeostatic regulatory systems. Concentrations under 2.2 mmol/L (hypoglycaemia) may cause coma, seizure or death. Concentrations above 11.1 mmol/L may occur after intake of a high glucose load, but long-time fasting levels exceeding 11.1 mmol/L may cause kidney failure, cardiovascular damage, neurological damage, damage to the retina and damage to feet and legs (50,53)(WHO Diabetes definitions). Normal fasting blood-

glucose levels should be <5.6 mmol/L, 5.7-7 mmol/L is defined as being glucose or insulin impaired, and fasting blood-glucose concentrations exceeding 7 mmol/L is defined as having diabetes (WHO Diabetes definitions, Diabetes Reference guide).

Increased blood-glucose levels after food intake, together with elevated GLP-1 concentrations, promote secretion of insulin from the pancreatic beta-cells, and inhibit the release of glucagon from the alpha-cells. Insulin promotes glucose uptake by muscles and adipose tissue. High insulin/low glucagon results in a decreased circulating level of glucose, stimulation of glycogenesis and lipogenesis, and suppression of gluconeogenesis and lipolysis. Approximately 2-4 hours following a meal, most nutrients are absorbed from the intestine. After a high GI meal, the high insulin/low glucagon ratio continues for a longer time and blood-glucose levels then usually fall below the fasting levels, thus resulting in hypoglycaemia. When consuming a low GI meal, the absorption rate from the intestine is prolonged, and thus the hypoglycaemia after 2-4 hours usually does not occur (50). On the contrary, decreased blood-glucose concentrations promotes secretion of glucagon, epinephrine, cortisol and growth hormones to promote a normalisation of the blood-glucose levels (50).

Intake of high GI/GL food/meals elicits a high demand on the beta-cells for producing and secreting more insulin. The beta-cell function might thereby be impaired (54). A decreased glucose and insulin sensitivity in the muscle and adipose tissues might occur after an increased pressure on the homeostatic regulatory system for a long time (54). The long-term consumption of a high GI/GL diet thus result in a greater risk of diabetes, CVD, obesity, cancer and hyperlipidaemia (52) (55).

## Palatable food

Food rich in fat and/or refined carbohydrates are named palatable food. These kinds of food play a significant part of today's daily food intake in the Western society. Overweight and obese subjects have been shown to have an increased liking for such food (56). The increased access, and thereby intake of these food, have been stated as a possible factor for the increased bodyweight seen worldwide (57,58). Food products with high amounts of refined sugar and fat are usually inexpensive, produced in big volumes, served in large portions sizes and distributed to all corners of the world (58). As these food have a high GI value the result after intake is a rapid increase of blood-glucose, followed by hypoglycaemia in the later postprandial state (50). Very high fluctuations in blood-glucose concentrations, and thus high demands on beta-cell function to release high concentrations of insulin, is not beneficial in the long run, leading to impaired beta-cell function and increased incidence for diabetes and obesity (54).



In animal studies, the long-term intake of palatable food has been shown to increase the overall food intake and promote binge-eating. Moreover, palatable food increase stress and alterations in reward pathways (57,59,60). In humans, it has been suggested that palatable food can cause an addiction, with neurological and physiological effects just as the ones seen for substance addictions (59). A high intake of palatable food is correlated to a higher food reward sensitivity (61). On the contrary, a long term intake of palatable food may lead to insensitivity of the reward value, thus increased intake is needed to get the same feeling of reward (62).

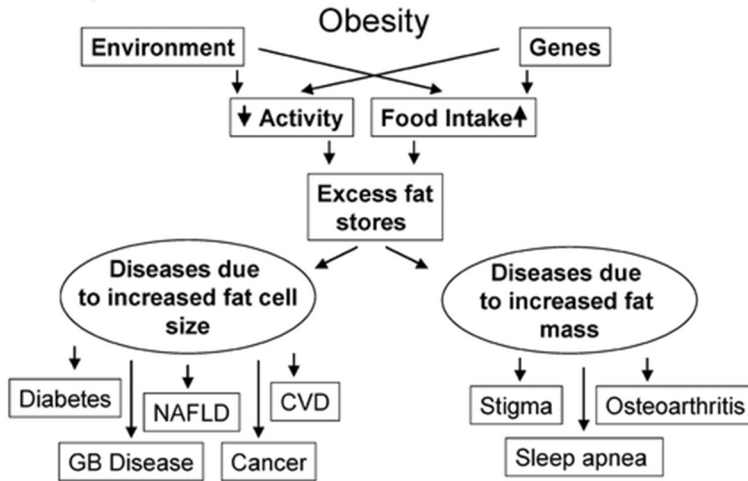
## Metabolic Syndrome

The worldwide obesity-epidemic trend continuous to increase (WHO factsheet n°311). Today, over 1.4 billion adults are classified as overweight, i.e. a body mass index (BMI) between 25 kg/m<sup>2</sup> and 29.9 kg/m<sup>2</sup>, and more than 300 million adults are obese, i.e. BMI over 30kg/m<sup>2</sup> (WHO °311) (58). With overweight and obesity many other diseases follow, such as diabetes type II, hyperlipidaemia, atherosclerosis, some cancers, non-alcoholic fatty liver disease (NAFLD), and cardiovascular diseases (CVD), together named the metabolic syndrome. WHO has stated that 3.4 million adults die every year as a consequence of overweight or obesity (WHO 311). The National Cholesterol Education Program has defined what parameters that should be measured to establish if a patient suffers from the metabolic syndrome or not. These parameters include measurements of waist circumference, circulating levels of triacylglyceride (TAG) and high-density lipoprotein (HDL), fasting blood-glucose levels and the blood-pressure. Three or more of these parameters should be elevated to classify patients as suffering from the metabolic syndrome (63).

## Obesity

Obesity should be considered a chronic disease, and is the result of an imbalance between energy intake and energy expenditure (64). As more energy is ingested, the storage in fat cells increase, resulting in enlarged fat cells and/or increased number of fat cells. These fat cells then secrete several peptides and fatty acids that in turn are responsible for the metabolic syndrome diseases (64). Environment, genes, food intake and physical activity all influence the incidence of obesity (figure 4).

## Pathogenesis of Health Problems Associated with Obesity



**Figure 4.** Health problems and consequences of obesity. Both environment, genes, physical activity and food intake are responsible for obesity. Obesity can lead to diabetes type II, gallbladder (GB) disease, non-alcoholic fatty-liver disease (NAFLD), various cancers, cardiovascular diseases (CVD), the stigma of being obese (the consequences of an increased body mass in daily life activities), sleep apnea and osteoarthritis. The later three consequences are due to the increased body mass itself, whereas the first five diseases mentioned are due to the increased size and/or number of fat cells (64).

Overweight and obesity are usually measured by calculating an individual's BMI ( $\text{kg}/\text{m}^2$ ), waist circumference and body fat mass. The BMI ranges are 18.5-24.9 for normal weight, 25-29.9 for overweight and  $\geq 30$  for obese (63). The waist circumference should be  $\leq 88$  cm for women and  $\leq 102$  cm for men (63). The percentage of body fat varies between age, sex and ethnicity, but on average a woman should have a body fat % of 20-35% and men 8-14% (65). There are several techniques for measuring body composition, where dual-energy X-ray absorptiometry (DEXA) and bioelectrical impedance (BIA) are the most commonly used. The DEXA scan is more reliable than the BIA, but the BIA is more easily used and not as expensive as the DEXA (66).

Today, more than 10% of the global population suffers from obesity (WHO 2011). When looking at children under the age of 5 years, more than 40 million have been classified as overweight or obese (WHO 2011). The USA is usually referred to as the most "obesogenic" country. Approximately 69% of the adult USA population are overweight or obese, 35% are obese (CDC), and the average BMI value for adults was  $28.7 \text{ kg}/\text{m}^2$  in 2010 (67). In Sweden, approximately 40-50% of the population are overweight or obese and 14% are obese (SCB, FHM). The average BMI for the adult population was 25.1 in 2011 (SCB).

The causes for overweight and obesity are, as described earlier, the combination of an increased intake of energy-dense food, decreased physical activity and a more sedentary lifestyle. These changes have been described as the result of environmental and social changes in urban planning, food processing and education, all factors for a changed lifestyle (WHO °311).

## **Diabetes type II**

There is a strong correlation between overweight/obesity and the risk of developing diabetes type II in both genders (68,69) (WHO °312). Decreased insulin sensitivity and impaired glucose tolerance is the pre-diabetic state, where fasting levels of circulating insulin and blood-glucose are elevated. The pre-diabetic state is reversible, as is the first stages of diabetes, before the beta-cell function is totally destroyed. The fully developed diabetes, when insulin-injections are needed for survival, is however irreversible (70). Diabetes type II comprises more than 90% of all people suffering from diabetes mellitus worldwide (WHO °312). Diabetes increase the risk of several of the other diseases included in the metabolic syndrome, i.e. app. 50% of patients with diabetes eventually die from CVD (WHO °312).

## **Dyslipidaemia**

Dyslipidaemia is the collective name of increased levels of total cholesterol, low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL), TAG and decreased levels of HDL. Increased levels of TAG, total cholesterol, LDL and VLDL combined with a decreased HDL concentration are individual risk factors for CVD (71)(National Cholesterol Education Program). Total cholesterol concentrations should be <4.5 mmol/L, LDL <2.5 mmol/L, TAG <2 mmol/L and HDL >1 mmol/L (Health care advice-1177). To patients with dyslipidaemia, lifestyle changes including changing the diet, increasing the physical activity and body weight loss are first recommended. The dietary recommendations include decreased intake of sweet and fatty food, red meat, alcohol and salt, increased intake of vegetables, fruits, fish and low fat meats, and cessation of smoking (Health care advice-1177, National Cholesterol Education Program). Finally, if the lifestyle changes are not enough, therapeutic agents as statins can be prescribed (71) (Health care advice-1177).

## Body weight regulation

The regulation of body weight can easily be described by the amount of energy ingested and the energy expended. There are several equations how to calculate the amount of energy an individual require to sustain their body weight mass, all of them based on the basal metabolic rate (BMR). The BMR is the amount of energy needed to “stay alive”(72), and the calculations are based on gender, body weight mass, height and age. One of the most commonly used equations is the Harris Benedict Equation (72-74). To calculate the amount of energy an individual require for weight stability, the BMR is used together with an estimated factor for the total amount of energy expended during one day, i.e. not only physical activity as going to the gym, but also the amount of energy expended when standing, sitting, preparing food etc. (72).

In general, men more easily loose weight compared to women, due to the larger amount of lean body mass. Women have a larger amount of fat mass, and thus the energy expenditure, which is mostly based on muscles and lean mass, is smaller in women than in men (75,76).

Body weight loss in the short term is quite easily obtained, but weight loss maintenance is not as easy. Several studies have reported that loosing weight and sustained weight loss of around 10% decrease the energy expenditure, decrease circulating levels of leptin, delay satiation of a meal, increase the reward value of food, decrease the perception of the amount of food eaten and decrease food restraint (48,77-79). All of these events occur to initiate body weight gain, and even one year after weight loss has occurred, this metabolic struggle continues.

The WHO has defined three keynotes to reduce overweight and obesity (WHO °311; WHO definitions of unhealthy diets), and these recommendations are in line with the Nordic Nutritional Recommendations as well; limitation of energy intake from fat and sugar, increased consumption of fruits, vegetables, legumes, whole grains and nuts, and increased physical activity of 60 min/day for children and 150 min/week for adults.

## Present strategies for tackling obesity

The different events of the satiety cascade (4, 16) (figure 3, page 19) can all be the target for pharmaceutical drugs, functional food or other dietary components acting to increase the intensity or duration of satiety. There are several diet regimes and strategies concerning the difference in nutrient content. It has been established that the three macronutrients show big differences in their satiating power, where proteins are the most satiating. Next comes carbohydrates and least satiating are fats (80). Carbohydrates can have very big deviations in what satiating power they possess, mostly depending on their GI and GL values (51).

The pharmaceutical drugs available globally today can be divided into two groups; the ones approved especially for obesity and the ones that affect body weight but are prescribed for other complications originating from obesity. The drugs prescribed for obesity specifically are divided into long-term and short-term use, and these include orlistat, lorcaserin, phentermine/topiramate, naltrexone/bupropion and phentermine, benzphetamine, diethylpropion and phendimetrazine (81) (table 1).

Table 1: Overview of the currently available drugs for treating obesity.

Drug	Prescribed/ Sold as	Effects	Body weight loss	Side effects
<b>Prescribed for long term treatment of obesity</b>				
Orlistat (81)	Xenical Alli	Blocking the lipolysis	3 m: ~6% 1 year: ~11%	GI symptoms as steatorrhea
Lorcaserin (82,83)	Belviq	Target serotonin-2C receptor to reduce food intake	1 year: ~ 5%-11%	Headache, nausea, dizziness and upper respiratory infections  Small risk of damage to heart valves (in animals brain tumours have been found (81))
Phentermine/ Topiramate (81,84)	Qsymia	Increase norepinephrine and affect the $\gamma$ -aminobutyric acid receptors to reduce appetite	3 m: ~7% 1 year: ~10-12%	Upper respiratory infections, constipation, paraesthesia and dry mouth
Naltrexone/ Bupropion (81,85)	Contrave	Affect adrenergic and dopaminergic receptors to reduce food intake	3 m: ~3% 1 year: ~5-7%	Nausea and increased heart rate
<b>Prescribed for short term use only (<math>\leq 12</math> weeks) for treating obesity</b>				
Phentermine (81,86)	Adipex-P Oby-Cap Suprenza T-Diet Zanryl	Stimulate norepinephrine release to suppress appetite and increase energy expenditure	~5%	Dry mouth, insomnia, palpitation, dizziness, headache, constipations, hypertension
Benzphetamine (81,86)	Didrex			
Diethylpropion (81,86)	Tenuate			
Phendimetrazine (81,86)	Adipost Bontril PDM Bontrol Slow release Melfiat			

The drugs prescribed for other diseases but are also affecting body weight are metformine, pramlintide, GLP-1 analogues (such as Liraglutide) and Gliflozins for patients suffering from diabetes, and bupropion and topiramate for patients suffering from neurobehavioral disorders (81). The pharmacological agents prescribed for treating other diseases, but also affecting body weight, results in ~ 2,5-10% reduction of body weight. Side-effects vary, but include urinary tract infections, hypoglycaemia, difficulties with concentration, dizziness and headache (81).

## Microbiota

The gut microbiota has been suggested to be one of the environmental factors affecting body weight (87). The human microbiota is composed of trillions of cells, from a very large quantity of different bacteria, but only a few phyla (88). It can be viewed as an organ of itself, having great effects on the host's physiology and well-being. The functions of the microbiota are many, such as metabolic and barrier effects (88). To achieve the best effects a symbiosis between the different bacteria is the optimum. An impairment of the composition of the microbiota has been associated with several health concerns and diseases such as allergies, inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), diabetes type II and obesity (89). Some of the bacteria present in the GI-tract can produce toxins as well as invade the gut mucosa and cause inflammations or worse, translocation of bacteria from the GI lumen into mesenteric lymph nodes, liver, spleen or blood (90). Other bacteria are commonly called the "good" bacteria, or the commensal bacteria. These bacteria have in recent years been the target for use in probiotic products.

There are several strains of bacteria present in the gut, with a very high variability between individuals. The two phyla of Firmicutes and Bacteroidetes are however numerically dominant, despite the uniqueness of individuals. Obese individuals have been shown to have more Firmicutes and less Bacteroidetes, as compared to lean subjects (91).

The major part of the gut microbiota is present in the ileum and colon (87). One of the abilities of the various bacteria present is to digest and utilize energy from otherwise indigestible food products, as plant polysaccharides and other fibres (92). The industrialised food today are more or less digested and absorbed in the upper part of the intestine (87), which might be related to the increased incidences of diseases coupled to a dysbiosis or altered composition of the microbiota, such as obesity.

# Aims and hypotheses

The general aims of this thesis were to investigate the effects of thylakoids on body weight and appetite regulation. Further to follow-up and investigate previous results from research on thylakoids. The studies prior to mine have shown that thylakoids affect digestion *in vitro*, body weight and food intake in rodents and appetite regulating hormones in one meal-study in healthy volunteers. Below are the individual aims and hypothesis of the papers included in my thesis.

- In paper I the aim was to study the *in vitro* uptake of glucose across the intestinal wall with addition of thylakoids in a dose-dependent way. The hypothesis was that thylakoids would affect the passage and permeability of the intestinal wall.
- In paper II the aim was to study the effect of thylakoids on the microbiota. The hypothesis was that the addition of thylakoids would affect the microbiota since thylakoids contain a large variety of polyphenols and non-digestible components. These components could serve as potential nutrients to the bacteria.
- In paper III the aim was to study the *in vivo* uptake of glucose with an oral glucose tolerance test in pigs. The hypothesis was that the addition of thylakoids would result in a retarded glucose uptake and possibly change the response of metabolic parameters.
- In paper IV the aim was to investigate thylakoids effects in humans consuming a high carbohydrate meal with or without thylakoid supplementation in a cross-over design. The hypothesis was that thylakoid supplementation would result in a prolonged glucose uptake, as well as have effects on other metabolic parameters including appetite.
- In paper V the aim was to investigate the effect of a daily supplementation of thylakoids for two months. The thylakoid supplementation was given together with a restrained diet and exercise regime to overweight women. The hypothesis was that thylakoids would promote decreased body weight and improved metabolic parameters.



- In paper VI the aim was to investigate the effects of thylakoids over three months in overweight women. Further, an intervention was made the first and last days of the study to investigate the direct versus the long-term effects of thylakoids on appetite regulating hormones and ratings of fullness, hunger and urge for specific food. The hypothesis was that a daily intake of thylakoids for three months would result in an improved regulation of appetite, with less urge for palatable food. The secondary hypothesis was improved metabolic parameters and body weight loss.

# Methodology

The aim of this chapter is to give an overview of the techniques and methods used in the thesis, as well as a discussion and reflection of the chosen methods. Details of the different methods are given in papers I-VI.

## Animal models (paper I-III)

The use of animal models; rats (paper I and II) and pigs (paper III), was approved by the Ethical Committee of Animal Experiments at Lund University. National guidelines for the care and use of the animals, and the European Community's regulations concerning the protection of experimental animals, have been followed. All three studies have been carried out at the Department of Biology, Lund University, Lund, Sweden.

In paper II and III, high-fat diets (HFD) have been used. These diets have been prepared in the lab, to have the best control of what ingredients the diets consisted of. There are several commercially available diets one can use, but the diet components in the normal chow versus the experimental purified chows usually differ, i.e. different fats and sources of carbohydrates are used. My goal was to investigate the role of thylakoids in the different diets, without other components affecting the results, and therefore we chose to prepare the experimental diets ourselves.

Taken together, working with animal models is a good way to closely monitor the effects, without too many environmental issues. The animals are kept in isolated environments, with controlled light-cycles, humidity and feeding times. The confounding factors in experimental animal studies are mostly if, and when, the animals consume the experimental diet.

## Ussing chambers (paper I)

In paper I, intestinal segments from rats were mounted in Ussing diffusion chambers, consisting of two connecting half-cells (93). Ussing chambers have been used since the 1950s in both physiological and pharmacological contexts to measure passage of compounds from one side of a membrane to another. The rat intestinal segments were

rinsed and mounted in the chambers, with the mucosal side of the intestine facing one chamber and the serosal side facing the other. The connecting area between the chambers had an exposed intestinal area of 1.78 cm<sup>2</sup>. The Ussing chambers were filled with Krebs buffer, kept at 37°C and connected to a carbogen supply. The intestinal segments were considered viable for a minimum of two hours after mounted in the chambers, i.e. the length of the experiment. A test-solution was added to the mucosal side of the intestine, consisting of modified Krebs buffer, oleic acid, NaTDC, methyl-D-glucose, FITC-dextran and ovalbumin. Krebs buffer was added to the serosal side. The passage of the markers in the test-solution, from the mucosal side of the intestine to the serosal side, was measured. “Pure” thylakoids (11) prepared in the lab, of different concentrations, were added together with the marker molecules to the mucosal side of the intestine. Confounding factors when working with Ussing chambers are the individual handling technique when mounting the tissue in the chambers, as well as different part of the intestine being more or less prone to leak. The intestine needs to be stretched during the mounting in the chambers, but if not handled with care a lesion could easily happen with leakage as a result. Thus, chambers were mounted with several proximal and distal segments of the intestine from every rat (8 rats in total), to achieve as many individual results as possible and to minimize the risk of leaking chambers to interfere with the results.

## **Gut microbiota (paper II)**

The rats in paper II were fed two different diets; the experimental diet was administered in the evening for consumption during the night, and normal rat chow was given for *ad libitum* eating during the day. The experimental diet was a high-fat diet with the energy distribution of 25E% carbohydrates, 60E% fat and 15E% proteins, with or without addition of 4 g (132 mg chlorophyll) thylakoid powder (prepared in the lab (11)). The experimental diet was composed of normal rat chow and rapeseed oil, and produced in the lab. The rats were kept on this schedule for 10 days and both total food intake and the voluntary intake of the normal chow, as well as body weight change was recorded. The last day an oral glucose tolerance test (OGTT) was performed and blood-glucose concentrations were analysed at time points 0, 15, 30, 45, 60, 90 and 120 minutes. At 120 minutes blood was collected for analysing the insulin concentrations. Following the OGTT, rats were anaesthetized and tissues from ileum, caecum and colon were collected for microbial analysis. Moreover, faecal samples were collected both first and last day of the experiment for analysing both the microbial and fat content. The fat content was analysed to investigate thylakoids effect on the fat uptake, i.e. whether or not thylakoids caused steatorrhea. Several bacterial groups were analysed, both by counting of colony-forming units (CFU), 16S rDNA sequencing, quantitative PCR analysis and terminal restriction fragment length polymorphism analysis (T-RFLP). Confounding factors were whether or not, and at what time prior to the OGTT, the animals consumed the experimental diet. However, all experimental

diet was consumed, but at what time the experimental diet was consumed was not monitored. This could affect the results of the OGTT. Another confounding factor was that the microbiota was solemnly analysed at the last day, and thus the found differences could have been present between the groups also before treated with thylakoids and the HFD.

### **Oral glucose tolerance test (paper III)**

The six pigs used in paper III were fed a high-fat diet with the energy distribution of 50E% carbohydrates, 36E% fat and 14E% protein. The diet was composed of conventional pig chow, fresh milk cream and rapeseed oil, and was produced in the lab. Pigs were given 4% of their body weight per day of the diet, and consumed the diet for one week before the experiments started. Pigs were fasted for 18h prior to the oral glucose tolerance test (OGTT) was performed. The study was a crossover study where pigs were given two glucose-loads, one with thylakoids (Appethyl, 0.5g/kg body weight) and one serving as control. The glucose-loads were infused directly via a syringe. Blood samples for analysing blood-glucose, p-insulin, p-CCK and p-ghrelin, were taken at time points 0, 15, 30, 45, 60, 90, 120, 180, 240 and 360 minutes. Pigs can be difficult to work with as they grow fast and have strong wills. We still managed to infuse the entire glucose-load to each pig. The intake of the high fat diet was problematic for some of the pigs, as they had difficulties consuming the entire amount every day.

### **Clinical studies (paper IV-VI)**

I have performed three experimental studies where women have been test subjects, one meal-study and two interventional studies of eight and twelve weeks respectively. All three studies have been conducted at the Overweight and Diabetes Unit, and at the Division of Occupational and Environmental Medicine, Skånes University Hospital (SUS), Lund, Sweden. The studies have been approved by the Ethical Committee of Lund University, and have been conducted in accordance with the Declaration of Helsinki. All subjects have given their written and oral consent before the start of the studies. Subjects for all studies were recruited through advertisement in the local community.

In the longer interventional studies (V and VI) screening processes were performed before participants were enrolled in the studies. This was done to achieve normal distribution throughout the groups, with regard to body weight, BMI and fasting concentrations of blood-glucose, p-insulin, p-TAG and p-cholesterol (total and LDL). Exclusion criteria for participation in any of the clinical studies were diabetes, food

allergies or intolerances, irritable bowel syndrome, recent use of antibiotics, vegetarians/vegans and if the subject had followed any diet the last three months. All studies were designed as single-blinded studies, i.e. the participants have not been aware of what group they have been enrolled in, but I have been the one randomizing the participants into a thylakoid or control group, as well as the one being on charge of the studies. The clinical personnel have however not been aware of which participant being in which group. At the end of the studies participants were asked what group, or order of meals (study IV), they thought they were enrolled in. From this, we can conclude that all three studies were successful in blinding the active versus placebo product. Thus, the aim of having blinded participants with successful placebo products has been implemented.

When working with human subjects, many confounding factors can be found. In our meal-studies the subjects were kept in an isolated area where most environmental factors was controlled. However, there were some factors hard to control, such as if the participants follow the regulations regarding what is allowed to do and not. As the participants were answering questions regarding their appetite reading magazines that could contain food recipes was not allowed. We had to allow the participants to use laptops for work, as well as reading books and having conversations. These factors could have affected the appetite ratings. The participants were told to relax, but not to fall asleep, which can be hard while just sitting for several hours. In the longer interventional studies (V and VI), other confounding factors can be found. These include following the diet and exercise regimens, and if they remembered to take their daily supplementation. Most participants believed they followed the recommendations completely, but after consultation it was concluded that this was not the case for some of them. Therefore, the lesson learned is that it is crucial to keep track on the participants with regular individual meetings. One limitation in both studies V and VI were the number of participants. The resources in personnel and our small premises and clinics were the limiting factors.

### **Meal-study (paper IV)**

Twenty healthy women were included in a cross-over meal-study where the effect of thylakoids (SwePharm AB) in a high carbohydrate meal, with the energy distribution of 71E% carbohydrates, 11E% fat and 18E% protein, was investigated. Thylakoids were administered in a blackcurrant jam. Blood-samples for analysing blood-glucose, p-insulin, p-CCK, p-ghrelin and p-TNF-alpha were taken at time points 0, 30, 60, 90, 120, 180 and 240 minutes. At the same time points, ratings of hunger, fullness, urge to eat and thoughts of food were analysed by VAS questionnaires. The study included three visits at the clinic, where a carbohydrate-rich breakfast with the addition of a low or a high dose of thylakoids vs. no thylakoids (control), were served. A limitation of the meal-study was the small number of participants. However, since this study was a

crossover meal-study where every participant consumed the three different breakfasts, the differences between the breakfasts could easily be monitored.

### **Two-month diet intervention study (paper V)**

In this study, 26 overweight women were randomized into a thylakoid (n=12) and control group (n=14), and followed a restricted diet and exercise regime for two months. Thylakoids (SwePharm AB) were administered in a blueberry drink, consumed every morning before breakfast. Every second week the participants arrived in the clinic for measurements of body weight, waist- and hip-circumferences and body-composition. Fasting blood-samples were taken for analyses of blood-glucose, p-insulin, p-HbA1c, p-TAG, p-cholesterol (both total and LDL), p-Apo B1 and p-leptin. The first and last days of the study, participants were instructed to answer visual analogue scale (VAS) questionnaires at given time-points the entire day.

The diet regime was a calorie-restricted three-meals per day diet, where the participants were given a selection of recipes to choose from. The daily energy restriction was -15E%. Participants were instructed to accomplish 60 minutes of low/medium intensity exercise each day. Limitations of the study were the limited number of participants and the compliance to the study protocol. Totally 30 women were enrolled and all finished the study, but four participants had to be excluded in the data analysis due to incompliance with the diet and exercise regime. Meeting the participants every second week was a good way to keep track on and motivate the participants individually. Moreover, participants were asked to answer questions in a diary every day and to use a pedometer. These tools were used to measure the compliance. A major drawback of this study was the limited time of two months, and for further studies we decided to keep the interventional studies at a minimum of three months.

### **Three-month diet intervention and meal-study (paper VI)**

38 women were enrolled in a twelve-week interventional study, divided in one placebo group (n=19) and one thylakoid group (n=19). First and last days of the period a complimentary meal-study was performed with or without supplementation of 5 g thylakoids (Appethyl). The participants were monitored for six hours, with blood-samples taken at time points 0, 15, 30, 45, 60, 90, 120, 180, 240, 300 and 360 minutes. Blood-samples were analysed for concentrations of blood-glucose, p-insulin, p-ghrelin, p-TAG, p-cholesterol (total, LDL and HDL) and p-leptin. VAS questions rating feelings of hunger, satiety and wanting for specific food were answered at the same times as the blood-sampling, as well as at time points 420, 480, 540, 600 and 660 minutes. Standard breakfast, lunch and dinner were given in the meal-study days.

During the twelve-week intervention, participants were given a daily blueberry shot, with or without supplementation of 5 g thylakoids (Appethyl), to be consumed before breakfast. The diet regime was set at three-meals-per-day, and no snacking in between the meals was allowed. There was however no energy restriction. Participants were instructed to carry out 30 minutes of low-intensity physical exercise every day. Every third week participants arrived in the clinic for anthropometric measurements and fasting blood-samples for analysing blood-glucose, p-insulin, p-leptin and p-cholesterol (total, HDL and LDL). Of the 38 women enrolled in the study two individuals were excluded from the data set due to incompliance with the recommendation of three-meals-per-day and the exercise regime. Confounding factors in this study were the question of whether or not the participants followed the recommendations of three-meals-per-day and the recommended exercise of 30 minutes. A voluntary diary was used, however not many of the participants chose to use the diary. The last day of the study participants were given a questionnaire with questions regarding their compliance, and all rated their individual efforts at a maximum level. Still, the possibility of participants not taking the daily supplementation or not following the diet and exercise recommendations cannot be disregarded.

# Results and Discussion

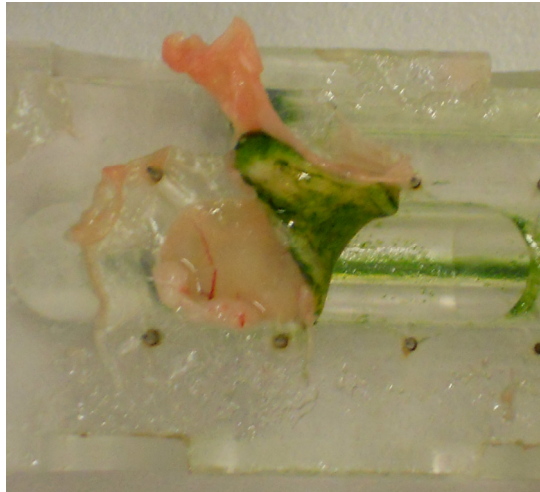
In this section the results and discussion from the individual papers will be presented.

## Paper I

### *Chloroplast thylakoids reduce glucose uptake and decrease intestinal macromolecular permeability*

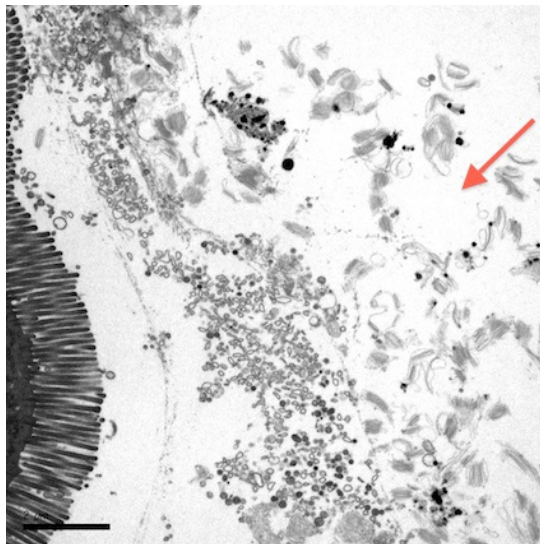
The passage of methyl-glucose over the rat intestine in Ussing chambers decreased in a dose-dependent way after addition of three concentrations of “pure” thylakoids; 1.2, 2.9 and 5.8 mg chlorophyll/ml. Significant differences were found between all concentrations versus the control, between 1.2 and 2.9 mg chl/ml and between 1.2 and 5.8 mg chl/ml. The permeability was calculated by the apparent permeability coefficient ( $P_{app}$ ), showing a significant reduction of the trans-mucosal transport of methyl-glucose as thylakoids were added to the lumen of the intestine. As the thylakoids were pre-treated with trypsin, to mimic the *in vivo* degradation normally occurring as food passes through the GI-tract, the passage of methyl-glucose was even further decreased. Moreover, as larger molecules were investigated, FITC-dextran of 4000 Da and the protein ovalbumin of 45 000 Da, the permeability over the intestinal wall showed significant dose-dependent reductions as thylakoids were added to the mucosal side. After the experiments were terminated, the mucosal side of the intestine was visually covered with a green layer (figure 5).





**Figure 5.** Rat intestine mounted in an Ussing chamber, displaying the mucosal side of the intestine covered with thylakoids (green), and the serosal side of the intestine (pink).

The green layer was further investigated with electron microscopy and thylakoid structures were found to cover the mucosal layer of the microvilli (figure 6).



**Figure 6.** Electron microscopy picture of the mucosal side of the intestine. Microvilli are seen on the left, and all the structures on the right are thylakoids (see arrow).

Interactions between methyl-glucose, FITC-dextran and ovalbumin to thylakoids were also measured. All three molecules showed affinity to thylakoids in various degrees. FITC-dextran had an affinity of 22%, methyl-glucose had an affinity of 17% and ovalbumin had an affinity for thylakoids of 12%. This concludes that even though some of the marker molecules are bound directly to thylakoids, the main fraction were still present as free thylakoid molecules in the Ussing chambers. The effects of a decreased passage over the intestine could thereby not only be explained by the affinity between marker molecules and thylakoids.

The proposed mechanism describing the findings of the reduced passage of marker molecules over the intestinal wall are thylakoids binding effect to the mucosal layer. Hence, thylakoids create a network slowing down the transport of the molecules from the lumen to the intestinal wall where the uptake and passage of molecules occur. This conclusion was based on thylakoids native properties, and how thylakoids act in the mixture added to the mucosal side of the intestine in the Ussing chambers. Thylakoids expose both hydrophilic and hydrophobic properties. As bile salts and oil was added to the mixture in the present study, thylakoids swell and partly unfold, resulting in a larger thylakoid-area and net negative charge of the thylakoid surface. This allows a larger coverage of the mucosal area. Moreover, treating thylakoids with trypsin results in swelling of the membranes with an even larger exposed area (94), hence the decreased uptake and passage observed with the trypsin-treated thylakoids further supports this hypothesis. The affinity analysis showed that there is a small affinity between the marker molecules and thylakoids. This affinity might affect the passage of marker molecules from the lumen of the intestine to the endothelial cells. Thus, the present study indicate a combined effect of 1) thylakoids covering the intestinal wall and 2) an affinity between thylakoids and the marker molecules, acting together to prolong the uptake and passage over the intestinal wall.

The importance of a prolonged passage and uptake across the intestinal wall is the possibility of preventing hyperglycaemia and to reinforce the intestinal barrier. A reinforced intestinal barrier has many positive health effects, such as protection against harmful bacteria, avoidance of allergies and avoidance or treatment for IBD/IBS. Important to note is that the decreased passage and uptake of molecules will only occur until digestive enzymes break down thylakoids.

## Paper II

*Feeding spinach thylakoids to rats modulate the gut microbiota, decreases food intake and affect the insulin response*

Rats were given a high-fat diet with or without supplementation of thylakoids for 10 days. The thylakoid supplementation resulted in a decreased intake of standard chow during daytime, compared to control. No differences in body weight over the 10-days

intervention were found. The last day an OGTT was performed but no differences between thylakoid and control groups in blood-glucose concentrations was found. 120 minutes after the oral glucose-load was infused, p-insulin concentrations were measured, and found to be significantly lower in the thylakoid group versus control. Also the fat content in the faeces was analysed, resulting in no differences between the thylakoid and control groups. This concludes that thylakoid supplementation does not cause steatorrhea.

The bacterial quantification in faeces and intestinal tissues showed increased appearance of Lactobacilli in ileal mucosa and decreased appearance in the faeces in the thylakoid group compared to control. Specifically *Lactobacillus reuteri* was increased and *Lactobacillus johnsonii* were decreased in the ileal mucosa in the thylakoid group. A mixture of *Staphylococcus*, *Kocuria*, *Bacillus simplex* and some Bifidobacteria were decreased in faeces, colon mucosa and in caecum mucosa in the thylakoid group versus control. No differences over the ten days period were found within each group when looking at *Enterobacteriaceae* and Bifidobacteria. The bacterial composition showed no differences between the groups in the tissue samples. There was however a difference between the groups in the total microbial composition of the faeces. Also, the thylakoid group had a more homogenous composition in the faeces as compared to the control group.

The decreased intake of normal rat chow after addition of thylakoids in a high-fat diet during the night indicate an increased satiety, supported by earlier feeding studies in rodents (10). These results might be an effect of the increased concentrations of satiety promoting hormones found previously (10,12). The deviated microbiota presented in this paper could also have affected the food intake. There are several studies showing strong interactions between microbiota and body weight (95-97). The decreased insulin concentrations 120 minutes after an OGTT could be due to a prolonged uptake of glucose every day during the complete diet intervention in the thylakoid group, resulting in increased insulin sensitivity. The composition of the microbiota could also explain the increased insulin sensitivity. Several studies have shown that different bacterial strains, and the composition of the bacteria, may have anti-diabetic effects such as reducing insulin levels (98-101).

The increased amount lactobacilli in ileal mucosa and less lactobacilli in faeces in the thylakoid group indicate that thylakoids promote an increased colonisation of lactobacilli in the gut. Especially *Lactobacillus reuteri*, known for having health-promoting effects (102) was increased by the thylakoid supplementation, and harmful bacteria as *Staphylococcus*, *Kocuria* and *Bacillus simplex* was decreased. *Lactobacillus reuteri*'s health-promoting effects are decreased abdominal fat and age-related adiposity, and decreased inflammation (102).

The mechanisms for how thylakoids affect the microbiota can so far only be on the speculative level. Thylakoids might directly influence growth of commensal bacteria on the molecular level, and/or thylakoids could be energy substance for the bacteria. The

effects might also be indirect, through thylakoids effect on appetite and food intake affecting the bacterial composition and growth.

## Paper III

### *Dietary thylakoids suppress blood glucose and modulate appetite-regulating hormones in pigs exposed to oral glucose tolerance test*

The OGTT with supplementation of thylakoids to pigs improved the blood-glucose response compared to control. The expected peak in blood-glucose concentration was decreased and no postprandial hypoglycaemia was observed. P-insulin concentrations followed the blood-glucose response, with slightly decreased p-insulin secretion after the thylakoid supplementation compared to control. For p-CCK, the supplementation of thylakoids resulted in a prolonged secretion with the highest level measured at 30 minutes after the OGTT. Moreover, the p-CCK levels were elevated for three hours after the thylakoid supplementation. The control OGTT resulted in a p-CCK peak after 15 minutes, where after the levels dropped fast. P-ghrelin levels decreased after the oral glucose-load in both the control and thylakoid experiment. The thylakoid supplementation did suppress the p-ghrelin secretion from 60 minutes, not reaching the same levels as the control OGTT until the termination of the experiment, i.e. six hours after the glucose infusion.

Food with a high glycaemic index usually result in a rapid and high increase of blood-glucose concentrations, and eventually hypoglycaemia (50). This study showed that thylakoids have the ability to prolong the glucose-uptake also *in vivo*. The addition of thylakoids counteracted the late phase hypoglycaemia, which was observed with the control OGTT. Whereas the prolonged uptake of glucose can be explained by the thylakoids forming a steric hindrance in the intestine shown previously (paper I) remains unknown. The slightly decreased insulin concentration after the thylakoids supplementation, compared to control, was most likely the result of the improved blood-glucose concentrations. This combined effect of an improved glucose- and insulin-homeostasis needs to be verified in human subjects (investigated in papers IV, V and VI), and if verified could be promising for decreasing the incidence of pre-diabetes and diabetes type II.

The increased secretion of CCK after addition of thylakoids to the OGTT could be explained by a prolonged gastric emptying as thylakoids forms a “swollen” network when exposed to bile acids, trypsin and pancreatic juice (94). Thylakoids have been found to prolong the passage of molecules over the intestinal wall, as well as bind to glucose itself (paper I). This could theoretically result in a slower release of sugars to the intestines, and subsequently an prolonged stimulation of the CCK-secreting I-cells (103). The same reasoning can explain the results of a postprandial suppression of ghrelin, since ghrelin is primarily secreted from endocrine cells in the gastric fundus

(104), thus a prolonged gastric emptying would result in a prolonged secretion also of ghrelin. Control of the gut hormones, as CCK and ghrelin, is of greatest interest when designing new concepts to promote weight loss, prevent weight gain and thus decrease the incidence of the metabolic diseases.

## Paper IV

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

The participants were served three different breakfasts; one control without thylakoids and two breakfasts with two different doses of thylakoids. No difference between the two doses of thylakoids could be found, and the material was therefore treated only as with or without thylakoids. The thylakoid-supplemented high-carbohydrate breakfast resulted in a slightly decreased rating of the concept of hunger motivation (in the article  $p < 0.05$  is stated, but an errata has been submitted to the journal stating this  $p$ -value should be 0.07). Hunger motivation was the combined ratings of hunger, thoughts of food and urge to eat. The blood-glucose and  $p$ -insulin concentrations were also affected by the thylakoid supplementation. Thylakoids counteracted hypoglycaemia with a second peak two hours after intake. The  $p$ -insulin levels followed the blood-glucose curve. Further, the  $p$ -CCK concentration was elevated after the thylakoid-supplemented breakfasts, compared to the control breakfast. A significantly elevated level in  $p$ -CCK was observed in the later postprandial phase of 180-240 minutes. Both the thylakoid supplemented and the control breakfasts resulted in a decreased  $p$ -TNF- $\alpha$  concentration four hours after intake, but no difference between thylakoid and control was observed.

The deviations in blood parameters after addition of thylakoids was shown also in the previous study (paper III), and in studies by Köhnke et al (10,12). The novelty of this study was thus the subjective ratings of hunger and satiety, using VAS questionnaires. Supplementation of thylakoids resulted in a slightly decreased rating of hunger motivation compared to control. The difference between thylakoid and control was found in the later postprandial time phase, as thylakoids promoted a decreased hunger from three hours after the intake until the test terminated. All blood parameters were affected in the later postprandial time phase, and it can then be speculated if thylakoids affect the gastric emptying. A prolonged gastric emptying would result in a prolonged secretion of CCK, a decreased secretion of ghrelin, prevention of hypoglycaemia and also a decreased hunger motivation. In turn, the increased CCK will cause an even slower rate of gastric emptying (37). The secretion of CCK is mostly dependent on the hydrolysis of fat, even though the previous study (paper III) demonstrated a deviated CCK also response after an OGTT. Albertsson et al (9) described already in 2007 that

thylakoids retard fat digestion by binding to both lipase/colipase and to lipid droplets, thereby explaining the prolonged secretion of CCK seen in the present paper.

We hypothesised that the addition of thylakoids would promote a decreased secretion of TNF-alpha following the test-meal, due to the high content of bioactive compounds in thylakoids, as bioactive compounds have been found to increase plasma antioxidant capacity (105). However, no differences between the meals could be found. The TNF-alpha concentrations were however decreased after both the thylakoid and the control meals. This could be explained by the relatively high fibre content in the test-breakfasts, 8 g (4.5%) fibre for the thylakoid meals and 8.5 g (4.7%) in the control meal, as several studies have shown that the addition of various fibres decrease inflammatory markers, such as TNF-alpha (106-108).

## Paper V

### *Decreased urge for palatable food after a two-month dietary intervention with green-plant membranes in overweight women*

The two-months restrained diet with or without thylakoid supplementation resulted in a decreased body weight and body fat-mass in both groups. However, no differences in body weight or fat-mass between the groups were found. The analysis of the metabolic and anthropometric parameters weekly change over the two-month period showed a significant difference between the thylakoid and control groups, when all parameters were analysed together. Specifically hip-circumference, p-leptin, p-LDL-cholesterol, p-ApoB1 and blood-glucose were significantly more reduced per week in the thylakoid group versus the control group.

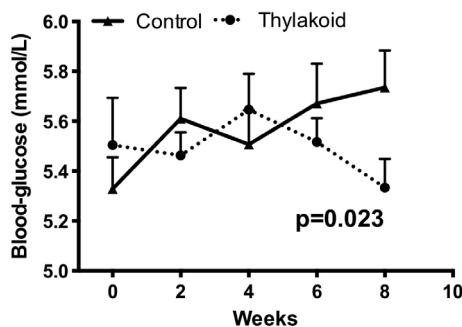
In the VAS-questionnaires answered first and last days of the study, thylakoid supplementation significantly reduced the rating of hunger the last day compared to the first day. There were no differences in the control group or between the groups. The rated urge for chocolate and carbohydrate rich snacks were decreased in the thylakoid group when comparing the first and last days. The urge for chocolate was significantly reduced in the thylakoid group versus the control group both the first and last days (ANOVA analysing time and treatment effects). Specifically, the thylakoid group rated their urge for chocolate significantly lower in the afternoon the last day of the study. The ANOVA analysis (time and treatment effect) for the urge for carbohydrate rich snacks showed a significantly lower urge in the thylakoid group versus control only on the first day of the study. The urge for a carbohydrate rich snack was specifically decreased prior to lunch and in the afternoon in the thylakoid group, versus the control group.

The restrained diet in the present study probably explains why there were no differences seen between the thylakoid and control groups regarding body weight loss or fat mass

loss. Still, since the subjective ratings of hunger was decreased during the two months in the thylakoid group we speculate that the strict diet regime was easier to follow with the supplementation of thylakoids. Decreased hunger is a strong predictor of successful weight loss (109), and the common outcome when losing weight is an increased hunger. This is partly due to decreased leptin-levels, signalling to the brain to consume more energy to regain the body weight status (48). In a weight-loss intervention the leptin-associated hunger is not beneficial for preventing weight regain. In the present study p-leptin was decreased in the thylakoid group indicating a loss of body fat mass, even though this could not be found with the bio-impedance scale used. Still, hunger ratings were decreased in the thylakoid group. Thus thylakoids seems beneficial for promoting body weight loss and body fat-loss by acting against the leptin-associated increased hunger.

The circulating levels of p-LDL-cholesterol and p-Apo-B1 are strongly associated with each other; every LDL particle have one Apo-B molecule attached (110). There is current evidence reporting that analysing ApoB is a stronger predictor of CVD (110,111). The present study showed a reduction in circulating levels of both LDL and Apo-B1 after supplementation of thylakoids, compared to control. This indicates that thylakoids can be beneficial for decreasing the risk of CVD. However, further studies are needed to substantiate these possible effects.

Further, blood-glucose was decreased in the thylakoid group over the two-month period (Figure 7).



**Figure 7.** Fasting blood-glucose levels during the two-months study in overweight women. Blood-glucose levels are decreased in the thylakoid group, while slightly increased in the control group. There is a significant difference between thylakoid and control groups when comparing the weekly change in fasting blood-glucose levels.

This can be explained by a prolonged uptake of glucose following the thylakoid supplementation. Thus, thylakoids improve blood-glucose homeostasis in healthy, overweight subjects. This may be beneficial for preventing or treating patients suffering

from pre-diabetes and diabetes type 2. More studies are however needed to further investigate these results.

## Paper VI

### *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*

The most important finding of this study was the significantly decreased body weight over the three-month period in the thylakoid group compared to the control group. This was seen already after six weeks of thylakoid supplementation. This is the first time a significant change in body weight by supplementation of thylakoids was found in humans. The total body weight loss in the present study was 6.2% in the thylakoid group versus 4.4% in the control group over the three-month period. The magnitude of weight loss can be compared to the pharmacological agents for treating obesity, as liraglutide (prescribed for treating diabetes) (47), orlistat (112) and phentermine/topiramate ER (Qsymia TM) (81). In both the thylakoid and control groups significant reductions in BMI, FFM, body fat mass (kg and %), and waist circumference was found, but no differences between the groups were seen.

Further, the supplementation of thylakoids resulted in a decreased concentration of p-total cholesterol and p-LDL-cholesterol, compared to the control group. The concentration of p-leptin was significantly reduced in both the control and thylakoid groups, but no difference between the groups was found. Fasting blood-glucose levels was slightly more decreased in the thylakoid group ( $-0.24 \pm 0.46$ ) versus control ( $-0.08 \pm 0.26$ ). However, these changes in fasting blood-glucose, nor in p-insulin levels, were not significantly different between thylakoid and control groups during the three-months intervention.

Participants were closely monitored the first and last days of the study, as two single-meal studies were carried out these days. Thylakoid supplementation in a blueberry drink resulted in a prolonged absorption of glucose over the intestinal wall. The peak in blood-glucose curve was delayed in the thylakoid group versus the control group both the first and last days of the study. Secretion of p-insulin followed the glucose-curve, thus resulting in a slightly lower concentration after food intake with thylakoid supplementation. The thylakoid effect was strongest the first day, resulting in significantly lower values of both blood-glucose and p-insulin already 15 minutes after food intake. In the last day, the control group peaked in blood-glucose at 15 minutes while the thylakoid group peaked at 30 minutes after food intake, hence the prolonged glucose uptake was still present after three months of daily thylakoid supplementation in the thylakoid group. The secretion of p-GLP-1 was also affected by the thylakoid supplementation, as elevated concentrations were observed compared to the control group both first and last days. The difference in p-GLP-1 was more pronounced the



last day of the study, compared to the first day. This suggests that thylakoid supplementation augment both the direct neuronal stimulation of GLP-1, and the stimulation of L-cells in the proximal and distal intestine. Usually when body weight is decreased, the postprandial GLP-1 secretions are decreased as well (113), but in the present study this was not observed in either the control or the thylakoid group. Important to note is that the baseline concentration of p-GLP-1 was not affected in neither the thylakoid nor the control group, i.e. only the endogenous postprandial secretion of GLP-1 is affected by thylakoid supplementation. No differences in or between the thylakoid and control groups were found in postprandial or fasting p-ghrelin levels.

The supplementation of thylakoids significantly reduced the urge for sweet snacks (candy) and for chocolate, compared to the control group. This was seen both the first and last days of the three-month study. The urge for a carbohydrate rich snack (sandwich) or a salty snack (chips/peanuts) did however not differ in or between the groups. The subjective ratings of hunger and satiety did not deviate between the groups neither the first nor the last day of the study. These results implicate that thylakoids effectively suppress the urge for palatable sweet food. The combined effect of a reduced urge for palatable food and the increased postprandial secretion of GLP-1, suggests that thylakoids strongly affect satiety regulating pathways as well as the reward value of food-intake. This results in a successful reduction of body weight, even without a restrained diet and exercise regime.

# General discussion

To summarize, in my research I have found that thylakoids prolong the uptake of nutrients from the mucosal side of the intestine, and that they affect the gut microbiota. Addition of thylakoids to the diet, affect circulating levels of blood-glucose, insulin, blood-lipids and appetite regulating hormones, such as CCK, ghrelin, GLP-1 and leptin. These effects occur both after a one-time ingestion, and after a longer time period of daily ingestions of thylakoids. Moreover, thylakoids affect food intake in rodents and have been found to enhance the feeling of satiety and urge for specific food in humans. Finally, thylakoids promote body weight loss in overweight women.

Important to note is that the decreased uptake of nutrients from the intestine will only occur until thylakoids are broken down by digestive enzymes. The digestion of thylakoids is retarded at least two hours, due to the high content of pigments (94). The decreased urge for specific food after thylakoid supplementation found in paper V and VI does however have the largest effects in the afternoon, even though the thylakoid supplement was taken with breakfast, i.e. effects are seen approximately 8 hours after intake. The mechanism for this is not clear, but is probably due to a prolonged gastric emptying after thylakoid intake, and a prolonged passage of nutrients through the intestine. The changed concentrations of CCK, ghrelin and GLP-1 all support a prolonged gastric emptying and intestinal passage after thylakoid intake. The prolonged glucose uptake seen in paper I, III, IV and VI further supports this theory.

In paper I a dose-dependent prolongation of the glucose uptake over the intestine was observed *in vitro*. In the *in vivo* meal-study in paper IV, no difference between the two doses of thylakoids could however be found. There are several phases of digestions and absorption taking place *in vivo*. To find a dose-dependent effect of thylakoids in human subjects, a larger variation in quantity/dose is needed, as was shown in one of the previous studies by Köhnke et al (12).

In paper II, plasma insulin was decreased after an OGTT in the rats fed a high-fat thylakoid diet for 10 days, as compared to rats fed a high-fat control diet. This was explained by increased insulin sensitivity after the 10 days of thylakoid intake. In the OGTT in pigs, paper III, plasma insulin levels were slightly decreased/about the same after 120 minutes, and in the human meal study, paper IV, insulin levels were slightly increased after the thylakoid supplements compared to control. The first day of the three months study, paper VI, insulin levels were slightly decreased in the thylakoid group compared to control, but after three months of daily thylakoid supplementation,

p-insulin levels at 120 minutes were the same for both the thylakoid and control groups. These somewhat conflicting results would hypothetically be explained differently regarding long time versus short time effects, and regarding in what species the study was done. The total doses of thylakoids have been approximately the same for humans, rats and pigs, despite the difference in body size, body weight, food-intake and metabolic rate. By using the food-intake for calculating thylakoid dose/food intake/day, the differences in doses can be a tool for understanding the different results. 5 g thylakoids to a 70 kg human with a daily food-intake of 37.5 g/kg (EFSA) gives the dose of 0.002 g thylakoids/human food-intake/day. In pigs, 0.5 g thylakoids per kg bodyweight, and a body weight of 13 kg with a food-intake of 4% of their body weight/day gives a dose of 0.013 g thylakoids/pig food-intake/day. In rats, 4g thylakoids in a 250 g rat with a daily intake of 30g gives the dose 0.13 g thylakoid/rat food-intake/day. Thus, this could explain why the high dose of thylakoid given to rats resulted in a decreased insulin concentration at 120 minutes, but no effects could be seen in pigs and only either slightly increased insulin concentrations, or no effect, could be seen in humans, as the “effective” doses have a factor 10 between rat and pig, and between pig and human.

Moreover, the pigs were treated to a single dose of thylakoids together with an oral glucose-load, whereas the rats were given thylakoids in the high-fat diet for 10 days before the OGTT. In the rats, the thylakoids together with the increased fat content of the diet, could thereby affect both the metabolism and the mucosal side of the intestine for several days, before the oral glucose-load was administered. As the *in vitro* Ussing study (I) showed a layering of thylakoids in the intestine, we hypothesise that this would affect the uptake of glucose and thereby the secretion of insulin would be less, compared to the control group. The reason for not finding this effect in the long time human study (VI) is probably the dosage difference described above. Also the gut microbiota can influence the glucose and insulin regulation, and as the thylakoids did affect the gut microbiota in the rats, this could also be, at least partly, the reason for the decreased levels of insulin.

The complete glycaemic load is important for what glucose-response a meal generates, and the blood-glucose response is important for the amount of insulin secreted. When comparing the energy distribution between the two meals served in the human studies, the breakfast in the meal study (IV) had a distribution of 71 E% carbohydrates, 11 E% fats and 18 E% proteins. The breakfast given in the three-months study (VI) had a distribution of 60 E% carbohydrates, 28 E% fats and 12 E% proteins. This implicates that the slightly lower carbohydrate load in the VI study, generating a lower glycaemic load, is responsible for the decreased insulin-response at 120 minutes, as compared to the increased insulin levels in the IV study with the higher GI/GL load.

Comparing at the difference between day 1 and day 90 of the VI study, the insulin-response in the control group is much higher the first day compared to day 90. This is the reason for a significantly lower insulin-level in the thylakoid group compared to the control group, seen the first day but not the last day. The thylakoid group generates

about the same concentration of insulin at the time point 120 minutes after food intake, both the first and last day of the study. The reason for a decreased insulin secretion in the control group after the three months study could be explained by the decreased body weight of 3.5 kg (4.4%), which might have generated increased insulin-sensitivity. In comparison, a similar effect in the thylakoid group may have been blunted by the direct thylakoid effect on insulin secretion seen already on the first day.

This brings me into discussing body weight losses in the two longer interventional studies. In the two months study, a restricted diet of 1800 kcal/day was followed, together with a requirement of 60 minutes of low-to-medium intensity physical activity every day. In the three-months study, no restriction apart from a three-meals-per-day regime, and a recommendation of 30 minutes of low-intensity physical exercise per day was followed. The two-months study resulted in an average body weight loss of 4.9 kg (6.4%) in the control group and 5.3 kg (7.3%) in the thylakoid group, respectively. Both groups significantly lost weight, but there were no differences in body weight loss between the groups. The three-months study resulted in a body weight loss of 3.5 kg (4.4%) in the control group and 5.0 kg (6.3%) in the thylakoid group. Both groups significantly lost weight, and there was a significant difference between the groups as the thylakoid group lost more weight than the control group. The reason for a significant body weight loss in the three-month study but not in the two-months study is probably due to the difference in diet and exercise regimes. The restrained diet in the two-month study probably blunt the thylakoid effect on body weight loss, but still the effects in ratings of appetite and urge for palatable food was found. It can thereby be concluded that thylakoids assist in body weight loss by making it easier to follow even a restrained diet regime by inhibiting the urge for energy-dense food. When a less restrained diet is followed, as in the three-months study, the effects on appetite can most probably explain the significantly larger body weight loss in the thylakoid group compared to control.

We conclude that thylakoid supplementation, together with any of the two different diet regimes used in studies V and VI, generates a body weight loss of approximately 7% (average of body weight loss in the studies together). This is highly comparable to the body weight losses obtained with any of the available pharmaceutical drugs for treating obesity today (table 1, page 29). All of the pharmaceutical drugs have side effects, such as steatorrhea, nausea, dizziness, upper respiratory infections, constipation and dry-mouth (81-86). No side effects have been found in any of the thylakoid studies. The faecal fat-content have been analysed both in rats (study II) and human subjects (unpublished data). Both these analyses have shown that thylakoid supplement does not affect the dietary-fat uptake, thus no steatorrhea following thylakoid treatment has been found. In all human studies, the participants have been asked if they have experienced any adverse effects, and from this we can conclude that after a total of three months of daily intake of thylakoids, no side effects have been reported.

The pleasantness and reward of various food items are important for determination of the amount of food ingested (114). To affect appetite and voluntary intake of especially

palatable food, the hormone GLP-1 is of greatest importance. As has been described before, GLP-1 is secreted in response to food intake, and is more sensitive for the fat-content of the meal than for the content of protein and carbohydrates. In the three-months study (VI), secretion of GLP-1 was significantly increased in the thylakoid group compared to control. Moreover, the secretion was prolonged, lasting for two hours with the thylakoid supplement, while the usual secretion of GLP-1 occurs in two phases, one rapid response and a second secretion in the later postprandial phase, as was observed for the control group. The prolonged effect after thylakoid supplementation may be described by a decreased gastric emptying mediated by the rapid secretion of CCK and GLP-1. A decreased gastric emptying would thus promote a prolonged secretion of GLP-1, due to the presence of nutrients in the intestine for a longer time after food intake. Also, as GLP-1 secretion is sensitive for the presence of fat and fatty acids, and thylakoids have been found to prolong the lipolysis, this could further help explain the prolonged secretion of GLP-1. A more pronounced and prolonged GLP-1 concentration may promote the ability to suppress the urge for palatable food in between meals. This is supported by the decreased rating for chocolate and sweets found in study V and VI.

In the other studies in human subjects (IV and V), GLP-1 was not measured. However, we hypothesise that we would have seen increased GLP-1 secretions after thylakoid supplement also in these studies. In the meal-study (IV), increased GLP-1 concentrations could, together with the increased concentration of CCK found, affect the ratings of hunger motivation. Also, a slightly increased concentration of insulin was found in the later time period of this study, which could be explained by an increased concentration of GLP-1, being an incretin hormone. In the two-months study (V) no blood-samples following the breakfast was measured, only the ratings of appetite and urges was measured. The decreased urge for chocolate and carbohydrate rich snacks found in this study (V) could thus definitely be explained by increased GLP-1 and CCK concentrations. Leptin levels are also interesting, as thylakoid supplementation in the two-months study resulted in decreased leptin-levels, but still decreased ratings for palatable food. Normally, as leptin-levels drop following decreased body weight, the sensitivity and urges for palatable food increase in order to help the body restore the lost energy balance.

The lowering effects on blood-lipids seen in both the two-months (V) and three-months (VI) studies can be explained by thylakoids effect on the lipolysis. Also, since thylakoids were shown to affect the gut microbiota in a prebiotic way (study II), the fibre content in thylakoids is highly interesting. The thylakoid fibres might bind bile acids, as beta-glucans from grains have been shown to do (115). The effect would then be an increased bile acid production to compensate for the bile acids lost in the faeces, and thus cholesterol concentrations, and mostly LDL, will be decreased.

# Conclusions

So, does “Thylakoid membranes from green plants affect appetite and promote body weight loss” as the title of this thesis state? Yes, I believe it does! In this thesis I have shown that thylakoids supplemented to the normal diet affect subjective ratings of appetite for chocolate and sweets. To affect how people actually feel is of greatest importance if body weight loss is the goal. The effects of blood-hormones can explain the subjective ratings, as thylakoids have been shown to promote secretion of the satiety promoting hormones CCK and GLP-1, and to decrease the secretion of the hunger hormone ghrelin.

Impaired regulation of glucose and insulin, increased concentration of blood-lipids, increased body weight and BMI, and a negative trend in choice of food intake and amount of daily exercise have all been stated as risk factors for metabolic diseases. The findings in my studies, making up this thesis, show that thylakoids:

- Prolong the glucose uptake both *in vitro* and *in vivo*, in both animals and humans. This is explained by thylakoids creating an extra barrier in the intestine prolonging the absorption, and by thylakoids affecting hormones that in turn promotes a decreased gastric emptying. A repetitive thylakoid supplement for a longer time period also appears to decrease fasting levels of blood-glucose, which would be interesting for patients with impaired glucose and/or insulin regulation.
- Increase the insulin sensitivity, mostly due to the prolonged uptake of glucose over the intestinal wall.
- Affect the appetite regulating hormones CCK, GLP-1, ghrelin and leptin in a favourable way for promoting appetite control and decreasing the urge for, and intake of, palatable energy-dense food.
- Decrease circulating levels of both total cholesterol and LDL. The reason for this is not clear, but might be due to an increased production of bile-acids.
- Promote body weight loss, probably due to the improved regulation of appetite and less urge for snacks in between meals.

In conclusion, the supplementation of thylakoids holds promising effects for better health and for decreasing the incidence of the metabolic diseases. As thylakoids promote body weight loss and decrease the urge for energy-dense food, we propose that thylakoids may be used as an effective tool for novel treatment of overweight, obesity and the metabolic diseases.

# Future perspectives

During my PhD time, I have been able to describe the promising effects that thylakoids exert, but there are still questions left to answer. The underlying mechanisms of how thylakoids exert some of the effects in the body still needs to be investigated, and also longer interventional studies, following test-subjects for more than 3 months, will need to be accomplished. Future studies will aim to answer the following questions:

- Is the energy expenditure affected by the supplementation of thylakoid, or is the found body weight loss only an effect of changed appetite and decreased urge for energy-dense food? How is the body composition changed? We have seen fat-reducing trends in BIA analyses, DEXA scan of rodents (11) and in dissected body-fat stores in rodents (unpublished data), but can thylakoid supplement target body-fat exclusively?
- How do thylakoids affect the intestinal transit-time? We know thylakoids affect hormones controlling gastric emptying, but the gastric emptying and complete transit through the GI tract needs to be physiologically investigated.
- Thylakoids show effects on glucose and insulin homeostasis, but will they be effective in pre-diabetics and patients with impaired regulation of glucose and insulin?
- The effects on ratings of appetite and urge for palatable food – are they enough to promote a more controlled and “healthy” food-intake in “free life”? Does it lead to a decreased body weight and increased health in the long run? Will decreased total cholesterol and LDL also be seen in the long run?
- I have shown that thylakoids affect the gut microbiota in the rat, but can these effects also be seen in humans? If so, can the deviated microbiota explain some of the other effects seen after thylakoid supplement as well? There are several studies today suggesting a more important role of the gut microbiota.





# Acknowledgements

I would like to begin with a BIG *Thank You* to all participants in my interventional meal- and long-time studies, without you this thesis wouldn't have been possible. I have truly enjoyed working in this project, and I hope I have managed to “enlighten” your knowledge about nutrition and in making healthy food choices.

I am truly thankful to my main supervisor **Charlotte Erlanson-Albertsson** for introducing me to this *most interesting field of research!* Thank you for always supporting me, and allowing me to develop into an independent researcher under your supervision. To be a part of the research-path of thylakoids, from the beginning to the now already available nutritional product, has been amazing and I wish and hope for many more years of collaborations.

My co-supervisor **Björn Weström** - thank you for believing in me, and encouraging me ever since I was a bachelor student back in 2005. We have many more interesting studies to carry out!

My family, and first of all my father **Lars** – you was the one introducing me to science and you always encourage me to reach beyond my current knowledge, thank you for your never-ending love and support. **Elisabeth**, thank you for your encouragement and always interesting scientific discussions. My grandmother **Marianne**, aunt **Aja** with family, my family in law; **Anki, Tommy, John** and **Åsa** with family and my uncles with families – thank you for never-ending support! **Åsa** – thank you for all the laughter, holidays, dinners and good times, both in the past and to come! Also, **Emily** and **Elin**, thank you for always being on my side!

The loves of my life, my husband **Ola**, precious son **William** and my sister and soul mate **Cecilia**. Ola, your love and encouragement has helped me going despite long nights in front of the computer or in the lab, and thank you for always supporting my chosen career. Cecilia, thanks for your energy, love, support and also your active participation and assistance in my studies! William – I love you more than words can say!

My amazing colleagues since many years now, **Karolina Östbring** and **Eva-Lena Stenblom** – what should I have done without you guys! We've had so much fun and produced so many interesting research data (and so many thylakoid-shots and food-products!). I am truly grateful for getting to know you and hope for life-long friendships! **Per-Åke Albertsson** and **Sinan Cem Emek**, thank you for answering so

many biochemical questions over the years, and always being so positive and supporting me! Also, big thanks to previous colleagues; **Rickard Köhnke**, **Nadia Osman** and **Emma Gårdsmark**. My newest colleague **Emil Eggecioglu** - thank you so much for your help during my maternal leave! We have many interesting studies ahead! **Magnus Hillman** – thank you for your never ending support and all the time you have spent for scientific (and non scientific) discussions. You are like a beaker (of whiskey maybe ☺) filled with wisdom and knowledge, and I am truly grateful for all your help! My roomie **Birgitte Ekholm**, I have very much enjoyed all our discussions and I am so thankful for all technical and laboratory support you have given me! **Jonathan Dereke**, thank you for your statistical support and for fruitful discussions. All members of the “breakfast club”, **Ulrikke Voss**, **Elin Sand**, **Eva Ekblad** and **Anna Themner-Persson** – thank you for your support and for all those “all hearty” breakfasts over the years!

All students; **Daniel Erlandsson** (special thanks to you Daniel – you have done wonderful work!), **Egzona Vitija**, **Ida-Maria Wahlström**, **Line Skarping**, **Maria Fransson**, **Maria Håkansson**, **Sofia Nilsson** and **Ulrika Toremalm** – thank you so much for your work and I wish you all good luck in your future careers!

I am so thankful for all colleagues and technical personnel assisting me in different parts of my work; **Mona Landin-Olsson**, **Stefan Pierzynowski**, **Jens F Rehfeld**, **Camilla Björklöv**, **Margit Bergström**, **Anna K Pålsson**, **Bertil Nilsson**, **Ann-Marie Räihä**, **Liudmyła Lozinska**, **Katarzyna Szwiec**, **Marek Kardas**, **Else Åkerberg Krook**, **Eva Assarsson**, **Ulla Andersson**, **Siv Ahrné**, **Göran Molin**, **Caroline Linninge**, **Ingegerd Sjöholm**, **Marilyn Rayner**, **Krzysztof Podgorski** and **Rita Wallén**. I am also so proud of the products *Appethyl* and *Aptiless*, two thylakoid products now available on the market. Many thanks to *Green Leaf Medicals* **Dan Edwall** and **Anders Struksnes** for believing in our product and making thylakoid supplement available for everyone!

Last but not least, all other wonderful people working at B11 at BMC and at the Biology center, and other people I might have forgotten to mention – thank you!

Finally, my biggest gratitude to FORMAS, VINNOVA, The Royal Physiographic Society in Lund, Färs och Frosta Foundation, Håkanssons Foundation and Vetenskapsrådet for funding my PhD-time and my studies!

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