Novel Lipid Derivatives for Health Benefits

Synthesis, Analysis and Effects in Diet-Induced Obese Mice

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Cover: An apple showing its circumference, reflecting waist circumference as an indirect measurement of obesity.
(photo: Samanthi Madawala)

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Abstract

Obesity is a public health concern and requires effective management with multiple approaches. Dietary supplements of bioactive compounds that can improve fatty acid oxidation and thermogenesis are currently in focus to develop anti-obese food ingredients/nutraceuticals without side-effects. Dietary 1,3-diacylglycerol (DAG) and supplementation of α-lipoic acid (LA) have been shown to reduce fat accumulation and improve lipid profile while phytosterols (PS) are known for reducing blood cholesterol.

In this thesis, novel lipid derivatives of LA with PS and 1,3-DAG were synthesized based on the hypothesis, that they would possess advantages over the individual constituents in controlling obesity due to increased stability, solubility, and bioavailability. A mild esterification method was developed to synthesize conjugates of LA and its reduced form (DHLA), with PS and 1,3-DAG yielding 60-80% of the products. The novel DHLA derivatives showed higher *in vitro* free radical scavenging capacity than the corresponding LA derivatives, and were comparable to pure DHLA.

In a pilot *in vitro* study, LA derivative with 1,3-dilaurin (diLaLA) was completely hydrolyzed by pancreatic lipase, releasing monolipoate, C12:0 and LA. Free LA was detected in plasma 2 h after oral administration of diLaLA to mice. Effects of diLaLA on body weight and lipid metabolism were evaluated in male C57BL/6J diet-induced obese mice on a high fat diet (HFD) and a control treated only with vehicle. After oral administration of diLaLA at 10, 50 and 250 mg/kg body wt. daily for 6 weeks, body weight was lowered ($p<0.05$) in high dose group compared with the control and low dose groups. Treatment with high dose also reduced ($p<0.05$) plasma and hepatic free fatty acid contents, while the concentrations of blood glucose, total cholesterol, and liver triacylglycerol showed a reducing trend compared with the control. Stearoyl-CoA desaturase (SCD)-1 activity, estimated by SCD16 index (C16:1n-7/C16:0), showed lower activity ($p<0.05$) in white adipose tissues while some key genes involved in fatty acid β-oxidation in liver showed upregulation in the high dose group compared with the control. Oral treatment with diLaLA reduced HFD-induced obesity in mice, possibly through enhanced energy expenditure via β-oxidation and suppressing *in vivo* lipogenesis, suggesting its potential as a promising candidate against obesity.

Keywords: Body weight reduction, 1,3-diacylglycerol, dyslipidemia, α-lipoic acid, lipid metabolism, novel lipid derivatives, obesity, β-oxidation, phytosterols, SCD1 activity.

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Dedication

To my late mother and father

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Madawala S.R.P., Andersson R.E., Jastrebova J.A., Almeida M., Dutta P.C. (2012). Phytosterols and α-lipoic acid conjugates: Synthesis, free radical scavenging capacity and RP-LC-MS-APCI analysis. *Polish Journal of Food and Nutrition Sciences* 62(3), 159-169.

II Madawala S.R.P., Andersson R.E., Jastrebova J.A., Almeida M., Dutta P.C. (2011). Novel conjugates of 1,3-diacylglycerol and lipoic acid: Synthesis, DPPH assay and RP-LC-MS-APCI analysis. *Journal of Lipids* doi:10.1155/2011/419809.

III Madawala S.R.P. & Dutta P.C. Pancreatic lipase mediated *in vitro* digestion of a novel hybrid lipid molecule with lauric acid and α-lipoic acid (manuscript).

IV Madawala S.R.P., Gokturk C., Malmlöf K., Dutta P.C. Anti-obesity effects in DIO mice of a novel hybrid lipid with lauric acid and α-lipoic acid - An alternative approach to manage body weight (manuscript).

V Madawala S.R.P., Manubolu M., Asp M., Dutta P.C., Malmlöf K. Effects of a novel hybrid lipid molecule containing lauric acid and α-lipoic acid on lipid metabolism in diet-induced obese mice (manuscript).

Papers I-II are reproduced with the permission of the publishers.

The contribution of Samanthi R. P. Madawala to the papers included in this thesis was as follows:

I Performed synthesis work with support from the co-authors and laboratory analyses (except NMR analysis), evaluation of results and writing the manuscript.

II Performed synthesis work with support from the co-authors, planning and execution of laboratory analyses (except NMR analysis), evaluation of results and writing the manuscript.

III Planning *in vitro* hydrolysis, performed laboratory analyses, evaluation of data, interpretation of results and writing the manuscript.

IV Performed laboratory analyses (except blood glucose), statistical analysis, evaluation and interpretation of the results, and writing the manuscript with co-authors.

V Planning the analytical work and laboratory analyses (except gene expression analysis), statistical analysis, interpretation of results, and writing the manuscript with co-authors.

Abbreviations

ACN	Acetonitrile
AMPK	Adenosine monophosphate-activated protein kinase
APCI	Atmospheric pressure chemical ionization
AT	Adipose tissues
BMI	Body mass index
Cd36	Fatty acid translocase (cluster of differentiation 36)
CFI	Cumulative food intake
CPT	Carnitine palmitoyltransferase
CVD	Cardio vascular diseases
DAG	Diacylglycerol
DHLA	Dihydrolipoic acid
diLaLA	1,3-dilauroyl-2-lipoyl-*sn*-glycerol
DIO	Diet induced obese
DO	Diolein
DOLA	1,3-dioleoyl-2-lipoyl-*sn*-glycerol
DODHLA	1,3-dioleoyl-2-dihydrolipoyl-*sn*-glycerol
DMAP	4-dimethyl amino pyridine
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC	Effective concentration
EDCl	1-ethyl-3-(3-dimethylainopropyl)-carbodiimide hydrochloride
ESI	Electrospray ionization
FA	Fatty acids
FFA	Free fatty acids
GC	Gas chromatography
GSH	Glutathione
HDL-Ch	High density lipoprotein-cholesterol
HFD	High fat diet
HPLC	High performance liquid chromatography

LA	α-Lipoic acid
LDL	Low density lipoprotein
MAG	Monoacylglycerol
MS	Mass spectrometry
MUFA	Mono unsaturated fatty acids
NMR	Nuclear magnetic resonance
Nrf2	Nuclear factor erythroid 2-related factor 2
OECD	Organisation for economic co-operation and development
PPAR	Peroxisome proliferator-activated receptor
PS	Phytosterols
PSLA	Phytosterol lipoate
PSDHLA	Phytosterol dihydrolipoate
ROS	Reactive oxygen species
RP	Reversed phase
RT-qPCR	Real time-quantitative polymerase chain reaction
SCD	Stearoyl-coenzyme A desaturase
SFA	Saturated fatty acids
T-Ch	Total cholesterol
TLC	Thin layer chromatography
T2DM	Type 2 diabetes mellitus
VLDL	Very low density lipoprotein
WHO	World health organisation

1 Introduction

Body weight is regulated by biological factors and remains relatively stable over time in many adults (Fried & Horenstein, 2011). A combination of genetic, environmental and behavioral factors influence accumulation of fat in the body, despite the neurobiological processes controlling the food intake (Haslam & James, 2005; Miller *et al*., 2011). In modern society, sedentary life style with over-consumption of diets rich in energy, promotes extra fat accumulation in the body leading to obesity. Body fat amount is measured indirectly using body mass index (BMI, weight in kg/height in m^2). Individuals with BMI between 25.0 and 29.9 kg/m^2 are considered overweight, while those with BMI above 30 kg/m^2 are categorized as obese (WHO).

Obesity has increased around the world, particularly during the past three decades and it has reached epidemic levels. One out of two adults in OECD countries is overweight or obese (OECD Fact Book, 2013). Obesity is closely associated with dyslipidemia, glucose intolerance and hypertension, collectively known as metabolic syndrome, and is considered a major risk factor for cardiovascular diseases (CVD), type II diabetes mellitus (T2DM) and some types of cancer (Grundy *et al*., 2004; Haslam & James, 2005; Miller *et al*., 2011). In addition to the losses related to productivity and increased health care costs, it is also a concern of the quality of life of the affected individuals.

Thus effective control measures are extremely important to overcome the problems of obesity. Reducing excess body weight, mainly by dietary modifications and exercise, is considered as the basic therapy for reducing fat accumulation. However, long term weight management is a challenging task for many obese individuals, resulting a transient phase of weight loss followed by a return to obesity (Dulloo, 2011; Ara *et al*., 2012). Therefore drug therapy is sometimes recommended, in combination with dietary modifications and exercise, for such individuals. However, long term use of such drugs can be

limited due to their side-effects whereas some individuals may not respond to these drugs (Robinson & Niswender, 2009; Ara *et al*., 2012).

Managing obesity by stimulating energy expenditure through thermogenesis and fatty acid oxidation is currently being examined in the pharmaceutical, nutraceutical and functional food research and industry (Yanagita & Nagao, 2008; Dulloo, 2011). A wide array of natural bioactive food ingredients, such as polyphenols, proteins/amino acids, and lipids/fatty acids, are being investigated for their potential as thermogenic anti-obesity products. They may act on single or multiple targets, increasing energy expenditure and reducing obesity and related health risks. In the Western diet, about 35-40% of total energy intake is from fat (Rudowska *et al*., 2005). Thus lipid based bioactive ingredients can easily be enriched in foods without causing marked changes in eating habits and can be used as part of an integrated approach to reduce obesity.

Some lipid based-bioactive ingredients such as phytosterols (PS) are known for their effect in reducing total cholesterol (T-Ch) and low density lipoprotein cholesterol (LDL-Ch), and have been used as functional foods for many years (AbuMweiss *et al*., 2008). Research has shown that long term substitution of more than 10% of the normal daily triacylglycerol (TAG) intake by 1,3-diacylglycerol (DAG) can significantly reduce body weight and fat accumulation by increasing energy expenditure and thermogenesis, compared with a similar amount of ordinary TAG intake with same fatty acid composition (Rudowska *et al*., 2005; Yanai *et al*., 2007). Supplementation of α-lipoic acid (LA) in doses of 800-1800 mg/d have shown multiple therapeutic properties including weight reducing effects through enhanced energy expenditure (Carbonelli *et al*., 2010; Koh *et al*., 2011).

The bioavailability of a given bioactive compound often depends on its chemical and physical properties, including solubility and stability. Therefore novel derivatives of existing natural bioactive food compounds are being investigated to improve such properties in order to deliver enhanced effect (Meunier, 2007; Koufaki *et al*., 2009). The molecular structure of LA provides an ideal chemical entity that can easily be conjugated with other small bioactive molecules to form multifunctional derivatives (Koufaki *et al*., 2009). Such novel derivatives of LA with other bioactive molecules might provide combined or synergistic effects through their action on single or multiple targets, possibly providing innovative supplements that can increase energy expenditure and suppress fat accumulation, reducing the underlying causes of obesity.

2 Background

2.1 Obesity

Obesity has increased steadily around the world, particularly since the 1980s, and is now becoming a common problem also in developing countries. Today, 53% of adults in OECD countries are overweight and 18% of them are obese (Figure 1). The prevalence of obesity is highest in the USA and Mexico, where it occurs more than 30% of the population. In Denmark, Sweden and Norway, obesity has increased by 40% during the past decade. In Sweden, 13% of the population aged above 15 years was obese in 2010 (OECD Fact Book, 2013). According to predictions, there will be 2.3 billion over weight adults around the world by 2015 and 700 million of them will be categorized as obese (WHO). The OECD has also reported that obesity is responsible for 1-3% of total health expenditure in most OECD countries, whereas it accounts for 5-10% in the USA (OECD Fact Book, 2013). When productivity related losses are added to the health care costs, obesity accounts for over 1% of gross domestic product (GDP) in the USA.

Currently used BMI thresholds to define obesity do not measure the fat content accurately and tend to vary across gender, age and ethnicity. Therefore waist-to-hip ratio and waist-to-height ratio have been suggested as more precise indirect measurements of obesity (Browning *et al*., 2010; Ashwell *et al*., 2012). Recently, dual-energy X-ray absorptiometry (DEXA) emerged as a direct measure of total and regional body fat amount (De Lorenzo *et al*., 2011).

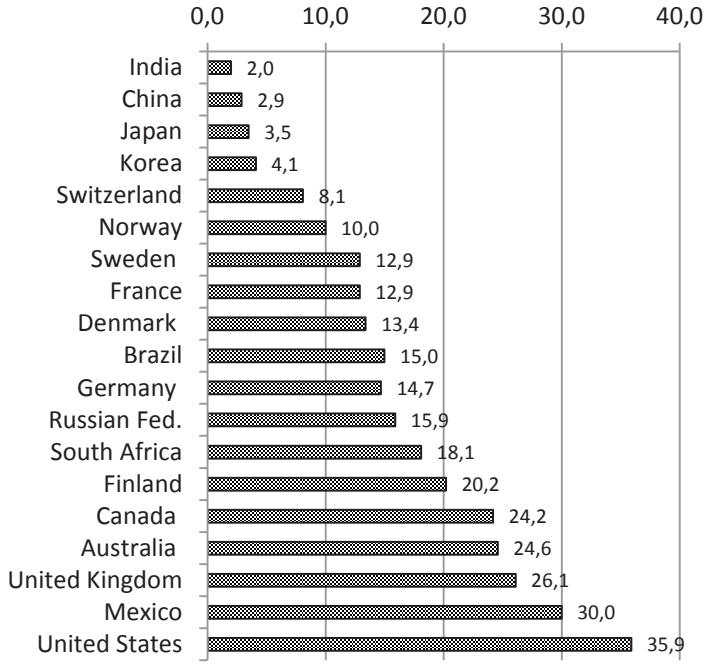

Figure 1. Prevalence of obesity as a % of the population above 15 years age in some selected countries in 2010 (Source: OECD Fact Book, 2013).

Obesity associated risk factor for CVD: Atherosclerosis

CVD is the major cause of death, representing 30% of total death in the world (WHO). Atherosclerosis is the primary underlying cause for CVD. Obesity associated multiple risk factors such as dyslipidemia, hypertension, diabetes and oxidative stress induce atherosclerosis (Rossenfeld, 1989; Grundy *et al*., 2004; Miller *et al*., 2011; WHO). The atherogenic impact of high concentration of blood cholesterol, particularly LDL-Ch, and low concentration of high density lipoprotein-cholesterol (HDL-Ch), is well established (Rossenfeld, 1989; Miller *et al*., 2011; Ference *et al*., 2012). Therefore comprehensive action through integrated approaches are important to reduce above mentioned risk factors. Prolonged exposure to lower LDL-Ch beginning early in life, has been found to reduce the risk of CVD by 55%, compared with a statin treatment started later in the life, highlighting the importance of primary prevention strategies to maintain low LDL-Ch concentration (Ference *et al*., 2012).

2.2 Lipid metabolism

2.2.1 Dietary lipids and metabolism

Lipids are a group of heterogeneous hydrophobic organic molecules. They are mainly fatty acids (FA), their derivatives including mono-, di-, tri-acylglycerols and phospholipids, as well as sterols such as cholesterol. Dietary lipids are essential for growth and development and serve as an energy reserve in the body. Major proportion of dietary lipids consists of TAG (Rudowska *et al.*, 2005).

Digestion of dietary lipids starts in the stomach and continues in the small intestine, where DAG and TAG are hydrolyzed by lipase into monoacylglycerols (MAG) and free fatty acids (FFA) and absorbed into the enterocytes. There they are re-esterified to TAG and incorporated into chylomicrons secreted into blood via lymph. Circulating lipids are associated with proteins and are known as lipoproteins, which contain non-polar lipids in the core facilitating their transport in the body (Harvey & Ferrier, 2011). Lipoproteins contain different amounts of phospholipids, TAG, cholesterol and cholesterol esters, and vary in density (Table 1). Other lipids in the body are mainly compartmentalized in membranes or stored as TAG in adipose tissues (AT).

The liver functions as the major organ involved in lipid metabolism. In addition to FA from the diet, FAs are also synthesized in hepatocytes via fatty acyl-CoA from carbohydrate metabolism. Stearoyl-coenzyme A desaturase (SCD)-1, catalyzes the rate limiting step in biosynthesis of monounsaturated FA (MUFA), which is a major component in tissue lipids (Dobrzyn *et al.*, 2005; Flowers & Ntambi, 2008). The FA in the liver are re-esterified into TAG and used for the synthesis of very low density lipoproteins (VLDL). Surplus energy supplied from food is reserved as TAG stored in adipose tissues (AT). When FA are required by the body for energy, hormone-sensitive lipase hydrolyzes TAG in adipocytes and FFA are carried by serum albumin to the liver and peripheral tissues, where energy is produced via β-oxidation of FA. In healthy people the plasma FFA concentration remains relatively stable, as removal by peripheral tissues is replaced by lipolysis of subcutaneous AT. In the fasting state, almost all circulating FFA are derived from hydrolysis of subcutaneous AT and transported to peripheral tissues to be oxidized (Fried & Horenstein, 2011).

Table 1. *Lipid composition of lipoprotein classes and some of their physical properties*

Parameter	CM	VLDL	LDL	HDL
Total lipid (%)*	99	91	80	44
Triacylglycerol (%)	85	55	10	6
Cholesterol esters (%)	3	18	50	40
Cholesterol (%)	2	7	11	7
Phospholipids (%)	8	20	29	46
Density (g/mL)	<0.94	0.94-1.006	1.006-1.063	1.063-1.210
Diameter (Å)	6000-2000	600	250	70-120

Abbreviations: CM, chylomicrons; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; *, based on wt./wt. and remaining materials are apoproteins (Source: The AOCS Lipid Library, 2013).

2.2.2 Implications of obesity on lipid metabolism

Obesity is the most common disorder in energy homeostasis, evident as extra fat accumulation (Fried & Horenstein, 2011). During the initial stages of weight gain, the size of adipocytes increases by up to 2-3 times the normal volume, facilitating storage of TAG. With prolonged over-nutrition and excess energy, pre-adipocytes are stimulated and differentiated into mature fat cells, increasing the number of adipocytes and further expanding the fat storage (Fried & Horenstein, 2011).

The adipocyte mass is known as a major endocrine organ and plays a pivotal role in the regulation of body weight and lipid metabolism. In healthy people, one of the adipocyte-derived hormones, leptin, regulates food intake (FI) and body weight. However, changes in molecular signals originating from the increased adipocyte mass in obese individuals are believed to cause some dysfunctions in intracellular lipid metabolism and exceed the normal regulation by leptin (Dobrzyn *et al*., 2005; Fried & Horenstein, 2011). In addition, excess fat mass has been shown to produce extra amounts of pro-inflammatory cytokines. In contrast to other cytokines, the concentration of adiponectin, which stimulate energy expenditure through FFA oxidation and glycemic control in peripheral tissues, has been found to be reduced in obese individuals (Ukkola & Santaniemi, 2002). Moreover, visceral AT shows a high rate of lipolysis, resulting in high influx of FFA to the liver in obese individuals (Ukkola & Santaniemi, 2002; Cornier *et al*., 2008).

In adults, with increasing BMI and total body fat content (TBF%), the concentrations of different plasma lipid components such as T-Ch, LDL-Ch and TAG are increased showing a positive correlation whereas the concentration of HDL-Ch is decreased (Table 2). The contribution of obesity to

serum lipid concentrations is particularly evident when BMI is above 30 or TBF% is very high (Brown *et al*., 2000; Choi *et al*., 2003).

Table 2. *Serum lipid concentrations based on body mass index (BMI) and total body fat (TBF)*

Parameter	No. of cases (*n*)	Serum lipid concentration (mean±SD, mg/dL)			
		T-Ch	TAG	HDL-Ch	LDL-Ch
BMI (kg/m^2)					
Males					
<18.5	49	191±38	99±32	53±11	119±33
18.5-22.9	428	200±36	135±57	46±10	129±31
≥ 23.0	307	211±35	193±96	43±8	124±33
Females					
<18.5	51	183±26	86±37	56±12	108±19
18.5-22.9	403	199±33	114±69	52±11	124±28
≥ 23.0	291	221±42	141±59	47±10	145±28
TBF (%)					
Males					
<12.0	50	173±24	87±38	50±12	108±22
12.0-21.9	481	204±35	142±71	45±11	128±34
≥ 22.0	253	218±37	199±73	41±9	130±37
Females					
<20.0	62	176±19	98±38	53±14	106±19
20.0-30.9	389	199±37	113±56	52±12	124±34
≥ 31.0	294	221±35	139±58	47±9	148±36

Abbreviations: SD, standard deviation; T-Ch, total cholesterol; TAG, triacylglycerol; HDL-Ch, high density lipoprotein cholesterol; LDL-Ch, low density lipoprotein cholesterol (Choi *et al*., 2003).

2.3 Clinical markers of lipid metabolism

2.3.1 Cholesterol

Cholesterol is a steroid alcohol categorized under lipids. About 75-80% of the circulating cholesterol is synthesized in the body, whereas the rest can be of different origin, such as dietary cholesterol (Fried & Horenstein, 2011). Apart from genetic reasons, obesity is the main factor affecting blood cholesterol content. Diets rich in saturated fatty acids (SFA) and *trans*-FA also increase the blood cholesterol (Rossenfeld, 1989; Miller *et al*., 2011).

Age-adjusted serum T-Ch concentration above 200 mg/dL (5.2 mmol/L) is considered as a risk factor for CVD (Table 3), and it has been shown that the relative risk increases progressively with increasing cholesterol concentration

(Miller *et al*., 2011). In general, LDL-Ch represents about 60-75% of T-Ch in serum (Rossenfeld, 1989). Therefore the link between atherogenic effect and serum T-Ch is mainly due to LDL-Ch. In addition, HDL-Ch concentration below 40 mg/dL is specified as a low amount, whereas a concentration above 60 mg/dL is considered highly desirable (ATP III Guidelines, NCEP, 2001). The ratio of T-Ch to HDL-Ch has been suggested as a more robust and precise lipid measurement to estimate the obesity related CVD risk. When, the ratio of T-Ch to HDL-Ch is above 4.5, it is known as dyslipidemia, which clearly increases with increasing BMI (Brown *et al*., 2000).

Table 3. *Classification of serum concentrations of triacylglycerol, total cholesterol and LDL-cholesterol (mg/dL) as risk factors for CVD events*

Designate	Triacylglycerol	Total cholesterol	LDL-Cholesterol
Desirable	<150	<200	<100
Borderline-high	150-199	200-239	130-159*
High	200-499	≥ 240	160-189
Very high	>500		≥ 190

Abbreviations: CVD, cardio vascular disease; LDL, Low density lipoprotein; *, LDL-Ch concentration of 100-129 mg/dL is classified as near optimal/above optimal. Source: ATP III Guidelines, NCEP (2001).

2.3.2 Triacylglycerol

Chylomicrons and VLDL are the main TAG-rich lipoproteins in blood, carrying approx. 85% and 55% (wt./wt.) TAG, respectively (Table 1). High TAG concentrations in blood can be due to increased synthesis and/or decreased catabolism of TAG-rich lipoproteins. According to current designations by NCEP, ATP III Guidelines (2001), a TAG concentration above 150 mg/dL in the blood after 12 h of fasting is considered a risk factor in metabolic syndrome (Table 3). In general, high concentration of TAG is associated with obesity and is considered as a biomarker for visceral adiposity, insulin resistance and T2DM (Miller *et al*., 2011).

Diets rich in fat generally result in elevated postprandial TAG concentration. Incorporation of dietary fatty acids (FA) into chylomicrons during chylomicron synthesis in small intestine depends on chain length and structure (saturation) of FA, and type of TAG or DAG. In contrast, diets rich in carbohydrates, but containing less fat result in lower TAG concentration in the postprandial state, but lead to a higher concentration in the fasting state (Lambert & Parks, 2012). After a carbohydrate-rich meal, *de novo* FA synthesis in the liver is followed by TAG and VLDL synthesis, thus increasing the TAG concentration in the fasting state. Borderline-high and high TAG

concentrations are correlated with atherogenic lipoprotein remnants such as LDL which is a metabolite of VLDL (Miller *et al*., 2011).

2.3.3 Free fatty acids

Expanding visceral fat mass during abdominal obesity releases increasing amounts of FFA, which enter the portal vein and are directed to the liver. At the same time, it has been reported that obesity reduces the concentration of adiponectin derived from AT. As a result, obese individuals have lower clearance of FFA, leading to a chronically elevated circulating FFA concentration (Ukkola & Santaniemi, 2002; Fried & Horenstein, 2011).

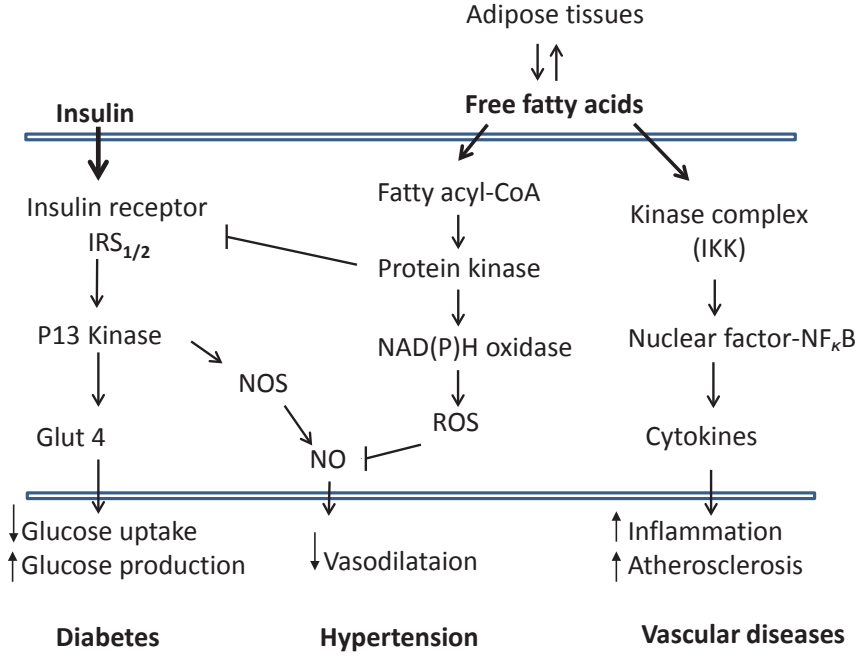

Figure 2. Potential mechanisms induced by enhanced plasma free fatty acid concentration during obesity resulting in diabetes, hypertension and vascular diseases. Abbreviations: Glut, glucose transport protein; NAD(P)H, nicotinamide adenine dinucleotide phosphate; NOS, nitric oxide synthase; NO, nitric oxide; ROS, reactive oxygen species (modified after Boden, 2008).

Once FFA concentration in blood is elevated, it can inhibit the anti-lipolytic action of insulin, further increasing the rate of FFA release. Thus with prolonged obesity, elevated concentrations of FFA reduce insulin-stimulated glucose uptake in skeletal muscles, leading to insulin resistance (Figure 2).

Increased amounts of FFA have been reported to cause accumulation of fatty acyl-CoA and activate serine/threonine kinase, which can interrupt insulin signaling by decreasing tyrosine phosphorylation of insulin receptor substrate (IRS)1/2. Consequently, this interferes with glucose transport protein (Glut 4), reducing glucose uptake in muscles and leading to an increase in hepatic glucose production. Moreover, elevated FFA is believed to induce production of protein kinase which has been reported to activate nicotinamide adenine dinucleotide phosphate (NADP)-oxidase and thereby produce reactive oxygen species (ROS). These ROS can destroy nitric oxide and affect vasodilatation leading to hypertension. In addition, elevated FFA promotes production of inflammatory and pro-atherogenic cytokines, which can impair endothelial function, causing a low grade of chronic inflammatory condition possibly leading to atherosclerosis (Ukkola & Santaniemi, 2002; Boden, 2008).

High influx of portal FFA from abdominal AT is captured in the liver and re-esterified into TAG, which could eventually increase the amount of VLDL synthesized in the liver (Fried & Horenstein, 2011; Karpe *et al*., 2011; Miller *et al*., 2011). Considering its influence in energy metabolism, serum FFA concentration has been suggested as a biomarker for metabolic diseases such as T2DM and CVD (Azzazy *et al*., 2006; Karpe *et al*., 2011). Moreover, elevated plasma FFA concentration can cause an accumulation of TAG, known as ectopic fat deposition, in peripheral tissues other than AT such as muscle and liver tissues, due to insulin resistance (Boden, 2008; Karpe *et al*., 2011).

2.3.4 Oxidative stress

Oxidative stress in biological systems can occur when free radical production exceeds the capacity of cellular antioxidant defense mechanisms, possibly due to diminished antioxidant levels (Packer *et al*., 1995; Biewenga *et al*., 1997; Rochette *et al*., 2013). An inverse correlation between antioxidant levels and central adiposity has been observed in obese individuals. Thus oxidative stress has been suggested as a biomarker for metabolic syndrome. (Higdon & Frei 2003; Keany *et al*., 2003). As mentioned earlier, increasing fat mass in obese individuals has been shown to increase expression of pro-inflammatory cytokines, which may promote oxidative stress during obesity and trigger pro-oxidative and pro-inflammatory processes causing endothelial dysfunction and progression of atherosclerosis, CVD and T2DM (Ukkola & Santaniemi, 2002; Boden, 2008).

LDL-Ch is oxidized by free radicals during oxidative stress conditions causing chemical modifications. Furthermore, the endothelial wall becomes

vulnerable to injuries during oxidative stress conditions and attracts monocytes, which may adhere to the endothelium. When the LDL-Ch concentration increases while oxidative defense mechanism is suppressed, this can trigger the atherosclerosis (Rossenfeld, 1989; Keany *et al*., 2003; Harvey & Ferrier, 2011). Thus chronic oxidative stress has been suggested as one of the mechanistic links between obesity and related chronic progressive pathological conditions (Higdon & Frei 2003; Keany *et al*., 2003; Boden, 2008).

2.4 Management of obesity by body weight reduction

The primary aim in the management of obesity is to reduce excess body weight or fat accumulation in order to improve health. The first approach to achieve this objective is usually through healthy diet and regular physical activity (Ara *et al*., 2012). The effect of weight reduction is positively correlated with serum lipid concentration. According to meta-analyses, reducing body weight by 5-10% can decrease serum TAG by 20%, T-Ch by 5-10% and LDL-Ch by 15%, while it can increase HDL-Ch by 8-10% (Miller *et al*., 2011; Ara *et al*., 2012). A reduction in body weight of 10 kg is reported to reduce T-Ch in humans by 5% (0.23 mmol/L) (Poobalan *et al*., 2004). It has also been shown that a weight loss by approx.10% can reduce the risk of developing T2DM by 30-40%, while obesity related cancer deaths may be reduced by 40-50% (Ara *et al*., 2012; Rodgers & Collin, 2012).

Unlike short-term weight reduction, maintenance of the reduced weight in long term has shown limited success in many obese people. In general, multiple regulatory methods are recommended for a better weight reducing effect through additive, complementary and synergistic effects. This kind of approach is particularly beneficial for individuals who have difficulties in achieving and maintaining satisfactory weight loss through basic strategies such as diet and exercise (Robinson & Niswender, 2009). In such cases a suitable drug therapy is introduced, in combination with modified diets and physical exercise. Though several approved weight reducing drugs are available, long term use of such drugs is limited due to their side-effects. Moreover, some individuals do not respond to such drugs (Ara *et al*., 2012). In severe cases of obesity (BMI above 40), bariatric surgery may be recommended (Ara *et al*., 2012).

2.5 Bioactive compounds against obesity and hypercholesterolemia

Dietary supplements which can improve energy and lipid metabolism could be an important element in an integrated approach for long term control of obesity and associated risk factors. Health promoting compounds isolated from food that are available in medicinal form and have shown to exert some physiological benefits, beyond basic nutritional requirements and reduce the risk of chronic disease are known as nutraceuticals (Moreau, 2004; Palthur *et al*., 2010). Such compounds can be added back to the foods, making them "functional foods" which are similar in appearance to conventional foods. There is growing interest in research and development of thermogenic and "fat-burning" nutraceuticals or functional foods to increase energy expenditure and oxidation of fat (Yanagita & Nagao, 2008; Dulloo, 2011). Being natural components in foods, they might have an advantage over the adverse effects associated with existing weight reducing drugs (Dulloo, 2011). Dietary supplementation and/or substitution of traditional lipids in the diet with functional lipids, can be used as a tool, in combination with other approaches to manage obesity and related health risks (Yanagita & Nagao, 2008).

2.5.1 Phytosterols

Phytosterols (PS) are closely similar to cholesterol in chemical structure, but are synthesized only in plants (Figure 3). Among a large number of different PS present in foods, sitosterol is the most common (56-79%), followed by campesterol and stigmasterol. Brassicasterol occurs in foods from *Brassicaceae* family such as rapeseed oil. The major structural difference between these PS is based on their side chain and the presence of double bonds in the side chain. Saturated PS are known as phytostanols. In foods, PS mainly occur as free sterols, but they may also occur as sterol esters (Figure 4), glycosides or acylated glycosides. In general PS intake from non-enriched foods may vary from 140-360 mg/d. Cereal products, margarine, vegetable oils, fruits and vegetables are the main sources of dietary PS. Sitostanols are available in trace amounts except in cereals, which contain substantial amounts (Piironen & Lampi, 2004).

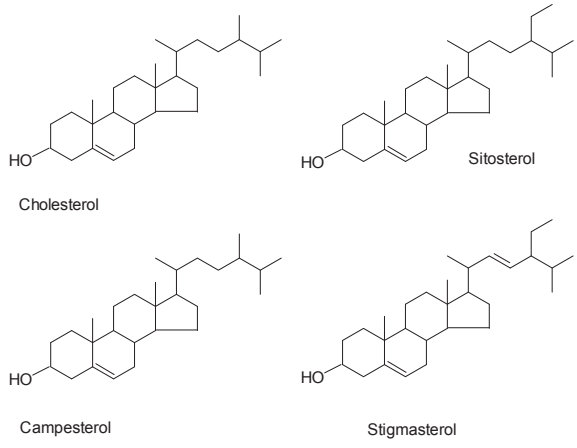

Figure 3. Molecular structure of cholesterol and some common phytosterols, naturally occuring in foods.

Cholesterol-reducing effect of PS

The cholesterol-reducing effect of PS was first reported in the 1950´s (Pollak, 1953). Preparations of solid crystalline sitosterol were used in amounts up to 25 g/d in early studies due to their very low solubility and bioavailability. Esterified PS with FA (Figure 4) was first reported to increase the bioavailability in 1994, with dietary intake of 1g PS/d lowering serum T-Ch by 3.4% and LDL-Ch by 5.9% (Miettinen & Vanhanen, 1994). Consumption of approx. 2 g PS/d (3.3 g PS esters/d) can lower serum T-Ch by 6-10% and LDL-Ch by 8-13% (Plat & Mensink, 2005; AbuMweiss *et al*., 2008; Demonty *et al*., 2009). Nowadays commercial food products enriched with FA esters of PS are common on the market. Margarine products under the names of Benecol® and Becel pro.active® are some of the PS enriched margarines available in Sweden. Derivatives of PS with other bioactive compounds such as ferulic acid or ascorbic acid have been synthesized for potential additive functional properties (Condo *et al*., 2001; Jia *et al*., 2006). The functional properties of added PS are recommended for adults with high serum cholesterol concentrations.

Possible mechanism of action

The exact mechanism behind the cholesterol lowering effect of PS is not fully understood yet, and several mechanisms have been suggested. It is believed

that PS decrease cholesterol absorption in the small intestines (Plat & Mensink, 2005; Harding *et al*., 2010; Davidson, 2011). About 50% of dietary cholesterol is absorbed in free form. Dietary cholesterol esters require prior hydrolysis by cholesterol esterase. In the intestinal lumen, cholesterol molecules are included in micelles with bile salts. PS are more hydrophobic and show higher affinity to micelles compared with cholesterol, thereby reducing incorporation of cholesterol in micelles.

A single dose of PS/d has been shown to be effective, suggesting that the effects of PS remain in the intestines for a while and inducing its effects through some other mechanisms as well (Plat & Mensink, 2005). Several mechanisms by which PS may induce expression of genes involved in transport proteins, increasing the efflux of cholesterol and PS back into the intestinal lumen for excretion, have been suggested (Harding *et al*., 2010; Davidson, 2011).

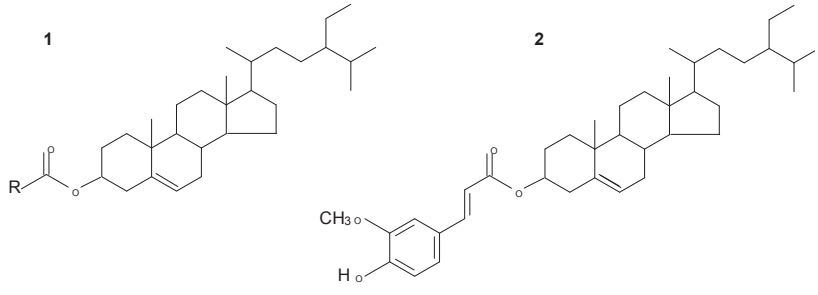

Figure 4. Structures of naturally occuring sitosterol esters; *e.g.* **1**, sitosterol ester of any fatty acid (R-COOH); **2**, sitosterol ester with ferulic acid.

2.5.2 1,3-Diacylglycerols

TAG is the primary component (approx. 95-99%), in ordinary fats and vegetable oils (Yasukawa & Katsuragi, 2008). In the TAG molecule there are three FA esterified at each -*sn* position on the glycerol backbone, whereas in DAG only two -*sn* positions are esterified with FA (Figure 5). As a result, DAG may occur naturally in three different forms depending on the esterified position in the glycerol backbone, *i.e.*, 1,2-DAG, 1,3-DAG or 2,3-DAG. In ordinary vegetable oils, DAG is available in amounts ranging from 1.0-9.5% depending on plant species. During fat digestion by pancreatic lipase, TAG is mainly hydrolyzed into 2-monoacylglycerol (MAG) and corresponding FFA. However, most of the 1,3-DAG is hydrolyzed into the corresponding FFA and *sn-1-* or *sn-3*-MAG due to preferential hydrolysis of pancreatic lipase at the *sn-*

1 and *sn-3 positions*. Nevertheless, the energy value and absorption coefficient of DAG and TAG are similar (Yasukawa & Katsuragi, 2008).

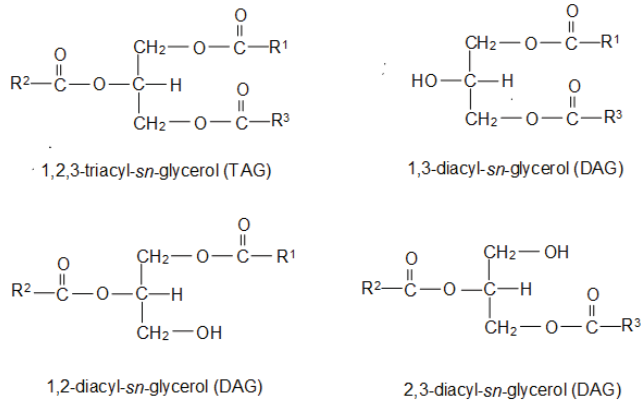

Figure 5. Chemical structure of triacylglycerol and diacylglycerols, where R1, R2 and R3 represent fatty acids of different chain lengths and double bonds, esterified in a glycerol molecule.

Anti-obesity effect of 1,3-diacyglycerol

A number of studies in rodents and humans have collectively revealed that long-term substitution of TAG by 1,3-DAG above 10% of the normal daily TAG intake can significantly reduce body weight and fat accumulation compared with similar intake of TAG with same fatty acid composition (Maki *et al*., 2002; Murase *et al*., 2002; Rudowska *et al*., 2005; Yanai *et al*., 2010). In addition, decrease in postprandial serum TAG has been reported in number of studies with human subjects when more than 50% of the total TAG intake was replaced by 1,3-DAG (Rudowska *et al*., 2005; Tada, 2008). Toxicological studies on rats have not indicated any deleterious effects specific to DAG intake up to 2500 mg/kg body wt./d doses, while humans were reported to well tolerate doses high as 45 g/kg body wt./d (Borzelleca *et al*., 2008). Dietary 1,3-DAG is considered under generally recognized as safe (GRAS) food substance in the USA, since 1,3-DAG is present in measurable quantities in common foods (Empie, 2008).

Possible mechanism of action

According to studies cited above, 1,3-DAG mediated effects can be due to metabolic differences related to its molecular structure compared with that of the TAG molecule (Rudowska *et al*., 2005; Yanai *et al*., 2007; Yasukawa & Katsuragi, 2008). Although the exact mechanism is not fully known yet,

several possible pathways have been suggested. One suggestion is that digestion products of 1,3-DAG *i.e.*, *sn-1-* or *sn-3*-MAG are poorly re-esterified into TAG after absorption into enterocytes and slow down subsequent synthesis of chylomicrons compared with 2-MAG from TAG digestion (Figure 6). The MAG- and DAG-acyltransferase enzymes primarily involved in re-synthesis of TAG, use 2-MAG as their substrate during TAG synthesis. This is known as the 2-MAG pathway. Since 1- or 3-MAG cannot be used as a substrate by 2-MAG acyltransferase, they are suggested to be re-esterified via the less active glycerol-3-phosphate pathway, which requires further break down of 1- or 3-MAG into glycerol and FFA (Yanai *et al*., 2007). This extra step may demand more energy and it was evidenced by enhanced expression of uncoupling protein (UCP)-2 and thermogenesis in the small intestines of 1,3-DAG fed C57 BL/6J mice (Murase *et al*., 2002; Rudowska *et al*., 2005).

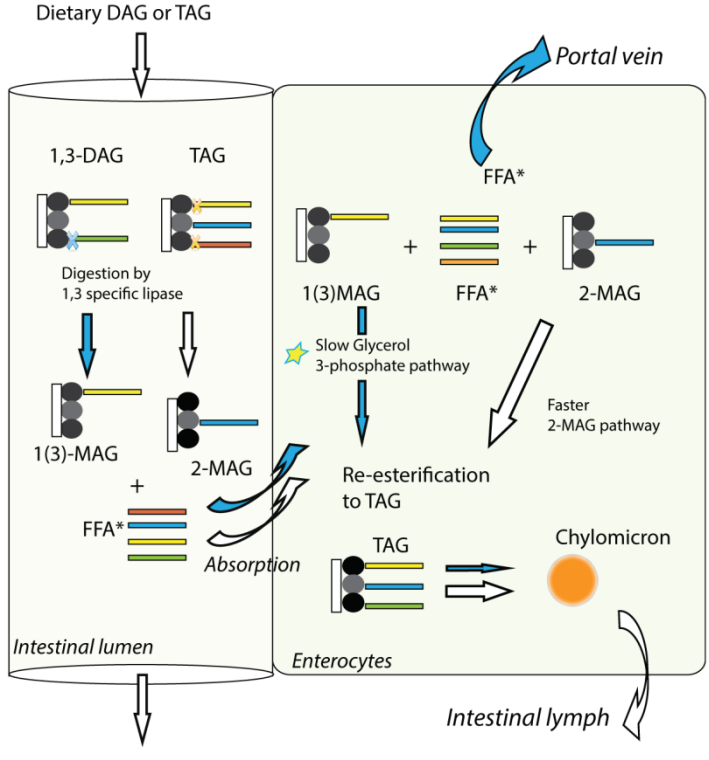

Figure 6. Pathways showing the suggested differences between 1,3-diacylglycerol (DAG) and triacylglycerol (TAG) during digestion, absorption and re-esterification into TAG in the small intestine; Abbreviations: FFA, free fatty acids (modified after Yanai *et al*., 2007).

In addition, less efficient chylomicron synthesis may promote transport of more FFA to the liver, facilitating enhanced β-oxidation and energy expenditure rather than accumulation in adipose tissues (Murase *et al*., 2002; Rudowska, 2005; Hibi *et al*., 2008). The C57 BL/6J mice fed with 4% 1,3-DAG in a 14% fat containing diet, showed up regulation of enzymes involved in β-oxidation such as acyl-CoA oxidase and medium-chain acyl-CoA dehydrogenase, in small intestine (Murase *et al*., 2002). A recent study using Caco-2 cells revealed that 1-MAG, the final digestion product of 1,3-TAG, might induce serotonin receptors and increase plasma serotonin concentration resulting in upregulation of genes involved in FA β-oxidation, FA metabolism and thermogenesis (Yanai *et al*., 2010). A higher respiratory quotient was observed in healthy individuals fed with 50% dietary 1,3-DAG replacing TAG compared with the control on 100% TAG (Hibi *et al*., 2008).

2.5.3 α-Lipoic acid

Naturally occurring α-LA is a bioactive compound also known as 1,2-dithiolane-3-pentanoic acid or 6,8-thiotic acid (Figure 7). It is synthesized in mitochondria, where lipoamide is used as an essential cofactor in α-ketoacid dehydrogenase complex, catalyzing oxidative decarboxylation during energy metabolism (Packer *et al*., 1995). In dietary sources, LA occurs as a lysine residue known as lipoyllysine. The most abundant sources of LA are spinach, broccoli, kidney, heart and liver which contain 0.9-3.2 μg/g dry weight (Biewenga *et al*., 1997; Rochette *et al*., 2013). LA was isolated and chemically identified for the first time by Lester Reed and co-workers in 1951 (Packer *et al*., 1995). Chemically, it is derived from octanoic acid (C8:0) and consists of a terminal carboxylic acid and a dithiolane ring where the C6 and C8 carbons are connected with a disulfide bond.

Due to its chiral center at C6, LA can exist as isomers of R-(+) or S-(-) forms. Therefore, a racemic mixture of synthetic LA contains both isomers in equal proportions. It has been reported that, 20-40% of a racemic LA mixture can be absorbed in humans, whereas R-(+)-LA has shown 40-50% higher plasma concentration compared with the S-(-) form (Teichert & Preiss, 2008; Packer & Cadenas, 2011). In addition, after absorption S-(-)-LA has shown 28 fold slower reducing capacity into DHLA, compared with R-(+)-LA. Therefore the naturally available R-(+) form is considered more appropriate in oral supplements. Pure R-(+)-LA is less stable than racemic LA and tend to polymerize (Carlson *et al*., 2007).

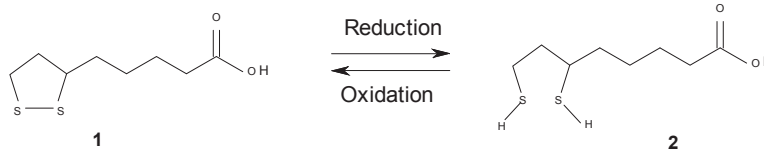

Figure 7. Chemical structures, showing reduction of disulfide bond into thiol groups in α-lipoic acid. 1) α-lipoic acid; 2) dihydrolipoic acid.

Health benefits of α-lipoic acid

In addition to its well established use as a pharmacotherapy in diabetes, today LA has become a common supplement or nutraceutical (Shay *et al*., 2009). Supplementation with free, exogenous LA in doses higher than obtained naturally from foods (200-600 mg/d) has been shown to have multiple therapeutic properties against diabetes, chronic inflammatory diseases, hypertension, vascular diseases and oxidative stress (Shay *et al*., 2009; Rochette *et al*., 2013), whereas doses of 800-1800 mg/d have demonstrated a significant weight reduction in human studies (Carbonelli *et al*, 2010; Koh *et al*., 2011). Oral supplementation with LA 800 mg/d to pre-obese and obese individuals for 4 months resulted in an 8% reduction in body weight in the pre-obese group and a 9% reduction in the obese group (Carbonelli *et al*., 2010).

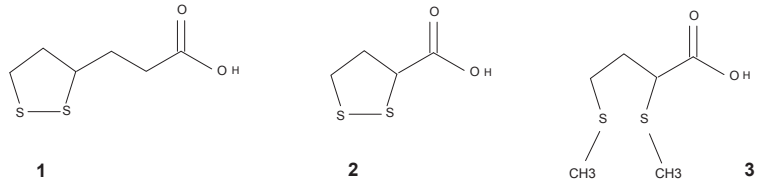

Figure 8. Metabolites of α-lipoic acid formed via β-oxidation and S-methylation in the liver. The compounds shown are: **1)**, bisnorlipoic acid; **2)**, tetranor lipoic acid; **3)**, 2,4-bis(methylthio)butanoic acid observed in human plasma and urine (modified after Teichert & Preiss, 2008).

Orally supplemented free LA is absorbed showing approx. 30 min. of plasma half-life. Then it is extensively metabolized by β-oxidation in the liver, resulting in bisnor LA, tetranor LA and 2,4-bis(methylthio)butanoic acid (Figure 8), which are excreted rapidly through urine (Teichert & Preiss, 2008). LA given to Sprague Dawley rats in doses of 180 mg/kg body wt./d for 2 years did not show any adverse effects (Cremer *et al*., 2006). In humans, supplementation of 1800 mg/d did not cause any adverse effects, but a few

individuals reported an itching sensation (Koh *et al.*, 2011). There is no agreement on the upper limit or recommended dose of LA for human use.

Possible mechanism of action

The exact mechanism behind the multiple biological effects of LA supplementation is not clearly understood yet. After absorption in the body, LA is reduced to dihydrolipoic acid (DHLA) by lipoamide dehydrogenase (Figure 7). The LA and DHLA redox couple can function very efficiently as a biological antioxidant due to their high reduction potential (-320 mV). *In vitro* studies have shown that LA and DHLA can scavenge peroxinitrite, various ROS such as hydroxyl radicals, hypochlorus acid and hypochlorite. LA can also terminate singlet oxygen and chelate metal ions. In addition, DHLA can regenerate vitamin C and E, endogenous thiols such as intracellular glutathione (GSH), and cysteine involved in physiological redox systems (Biewenga *et al*., 1997). Furthermore, recent research has shown that R-(+)-LA can increase GSH concentration by inducing GSH synthesis through antioxidant response element (ARE) mediated gene transcription regulated by nuclear factor Nrf2 levels, which respond to oxidative stress (Shay *et al*., 2009; Packer & Cadenas, 2011).

Alhough LA is metabolized and rapidly cleared from the body, perceived effects observed during the post-administration period suggest that LA and DHLA stimulate neuro-hormonal functions in target cells and indirectly influence multiple cell signaling pathways in peripheral tissues (Shay *et al*., 2009). Dietary supplementation with LA 0.1-0.5% (wt./wt.) has been found to improve blood lipid profiles by reducing FFA, TAG, T-Ch and LDL-Ch concentrations, whereas it increased HDL-Ch concentration in plasma and liver in rodents (Kim *et al*., 2004; Yang *et al*., 2008; Park *et al*., 2008). LA has been reported to downregulate expression of genes involved in the biosynthesis of cholesterol (Yang *et al*., 2008).

LA can induce expression of genes such as acyl-CoA dehydrogenase, acyl-CoA oxidase, hydroxyacyl-CoA dehydrogenase and carnitine palmitoyltransferase (CPT)-1α involved in oxidation of FA in the liver. Oral supplementation with LA enhanced adenosine monophosphate dependent protein kinase (AMPK) activity and increased glucose uptake and FA oxidation in peripheral tissues whereas it suppressed hypothalamic AMPK and reduced the food intake in rodents (Kim *et al*., 2004; Koh *et al*., 2008; Wang *et al*., 2010). It has been shown that up regulation of AMPK induced the phosphorylation of insulin receptor protein (IRS). Moreover, R-(+)-LA has

been reported to increase the abundance of glucose transport proteins $GLUT_1$ and $GLUT_4$, promoting glucose uptake (Shay *et al*., 2009; Wang *et al*., 2010; Packer & Cadenas, 2011).

2.5.4 Derivatives of α-lipoic acid

The molecular structure of LA is considered an ideal chemical entity which can be covalently bound with other bioactive compounds (Koufaki *et al*., 2009). LA conjugated with another small molecule is known as a hybrid molecule when the two molecules represent two chemical entities and possess different biological functions. Thus the two molecules may interact with a single target or two or more independent/related targets (Meunier, 2007).

Hybrid molecules of LA and trolox (a water soluble form of vitamin E) showed higher antioxidant activity against lipid peroxidation on rat liver microsomal membranes compared to pure LA or trolox (Koufaki *et al*., 2009). A phenolic compound, hydroxytyrasol esterified with LA, was found to exert antiproliferative effect on human colon cancer HT-29 cells (Bernini *et al*., 2011). A derivative of PS linked with LA via $POCl_3$ has been described by Milanova *et al*. (2001). According to our knowledge, there is no published data on any *in vitro* or *in vivo* study to evaluate potential biological effects of this PS derivative. Derivatives of LA with different amides have been synthesized for multiple aspects such as neuroprotective and anti-inflammatory properties and have shown higher activity when tested using *in vitro* models (Sen *et al*., 1998; Koufaki *et al*., 2009; Melagraki *et al*., 2009). Innovative novel supplements which can deliver a higher biological effect by using existing natural bioactive food components may be useful in an integrated approach to manage obesity and associated health risks.

3 Objectives

The overall aims of this thesis were to, *i*) synthesize novel lipid derivatives of α-lipoic acid (LA) based on the hypothesis that these conjugates would possess advantages over the individual compounds due to their increased solubility, stability and bioavailability, *ii*) investigate the potential effects of one LA derivative with 1,3-diacylglycerol (1,3-dilauroyl-2-lipoyl-*sn*-glycerol; diLaLA) against high fat diet-induced obesity. Specific objectives were to;

1) Synthesize two series of novel LA derivatives with phytosterol (PS) and 1,3-diacylglcerol (DAG).

2) Characterize novel lipid conjugates by RP-HPLC-MS analysis and evaluate their *in vitro* free radical scavenging capacity.

3) Study pancreatic lipase mediated *in vitro* digestibility of diLaLA and trace free LA in plasma samples after oral administration of diLaLA to mice in a pilot study.

4) Evaluate the effects of diLaLA on body weight, food intake, and some clinical markers of lipid metabolism, in plasma and liver in diet induced obese (DIO) mice fed a high fat diet (HFD).

5) Investigate the effects of diLaLA on estimated stearoyl-CoA (SCD)-1 activity in plasma, liver, and white adipose tissues and targeted genes involved in β-oxidation of fatty acids in the liver.

4 Materials and Methods

This section briefly describes the materials and methods used in this thesis. The basic outline of the whole study and major analytical steps involved, are shown in Figure 9. In the first half of the study, two series of novel derivatives of LA with PS and 1,3-DAG were synthesized in our laboratory. In the second half of the study, similarly synthesized LA derivative with 1,3-dilaurin (1,3-dilauroyl-2-lipoyl-*sn*-glycerol; diLaLA) was used for *in vitro* hydrolysis test (Paper III) and to evaluate anti-obesity effects by *in vivo* study (Papers IV-V). Since the *in vivo* study required pure novel derivative (diLaLA) in relatively large amount, diLaLA was obtained from a contract supplier, Onco Targeting AB (Uppsala, Sweden). Derivatives of LA with PS synthesized for their potential cholesterol reducing effects (Paper I), were not further studied for *in vivo* effects. Details of all the methods can be found in Papers I-V.

4.1 Synthesis of novel derivatives (Papers I-II)

Synthesis of LA derivatives with PS (Paper I)

Two series of novel LA derivatives with PS and 1,3-DAG were synthesized according to a modified method, described by Chiu *et al*. (1996) and Uyeda *et al*. (2005). First, LA derivatives were synthesized with PS (phytosterol lipoate; PSLA), and phytosterol dihydrolipoate (PSDHLA) was obtained by reducing PSLA (Paper I). The synthesis reaction of LA derivatives with PS is shown in Figure 10.

A mixture of PS containing β-sito-, campe-, stigma- and brassica- sterol was used for the synthesis of PSLA (Paper I). In brief, 1g PS, dissolved in 9 mL CH_2Cl_2, was mixed with 64 mg 4-dimethyl amino pyridine (DMAP), 580 mg LA, and 468 mg 1-ethyl-3-(3-dimethylainopropyl)-carbodiimide

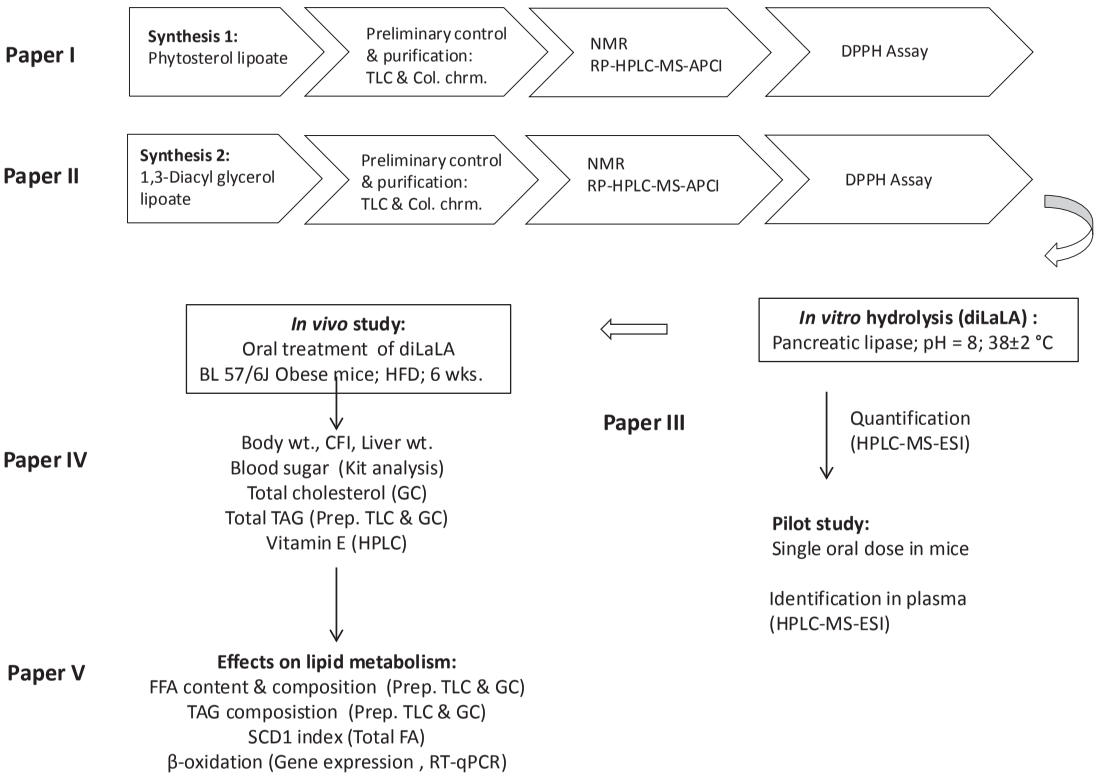

Figure 9. Overview of the studies and analytical procedures used in Papers I-V of this thesis

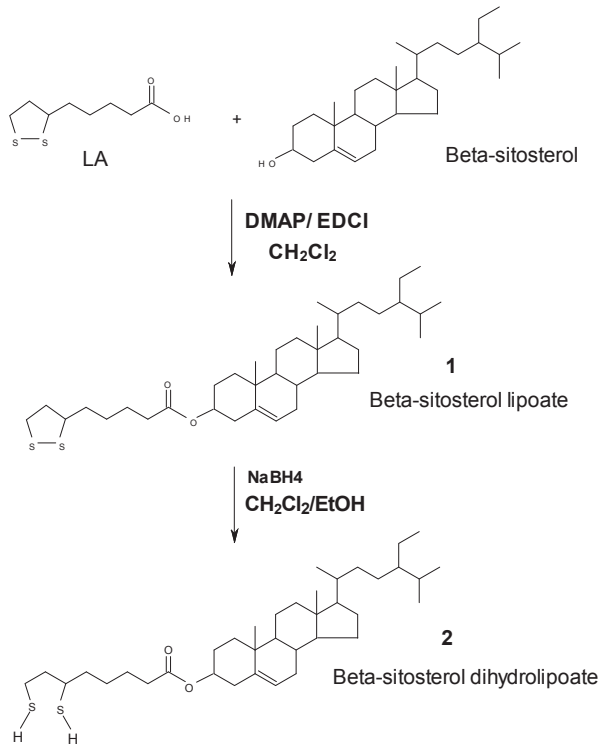

Figure 10. Synthesis of phytosterol lipoate (PSLA) and phytosterol dihydrolipoate (PSDHLA), showing beta-sitosterol as the example yielding: **1**) beta-sitosterol lipoate, and **2**) beta-sitosterol diyhydrolipoate.

hydrochloride (EDCI) under N_2 at 0 °C. After an overnight stirring at room temperature, the mixture was acidified with 1 M HCL and washed with aqueous saturated NaCl. The product was extracted twice with CH_2Cl_2, collected through dry Na_2SO_4. The synthesis reaction and purity of the product was primarily controlled by thin layer chromatography (TLC; Si gel 60, 10x20 cm, 0.25 mm thickness), developed in hexane:diethyl ether:acetic acid (75:25:0.85, v/v/v). Compounds were visualized by spraying 10% phosphomolybdic acid solution in ethanol and dried at 120 °C for 15 min. Crude extract from synthesis was purified by solid phase extraction (SPE; 1 g silica) using hexane.

The reducing reaction to obtain PSDHLA was performed according to Chittiboyina *et al*. (2006) with some modifications (Figure 10). Purified PSLA dissolved in CH_2Cl_2: EtOH (1:5, v/v) was mixed with $NaBH_4$ in portions while

stirring under N_2 at room temperature. After 2 h, the mixture was acidified with 1 M HCl, washed with aqueous NaCl and extracted twice with CH_2Cl_2 passed through dry Na_2SO_4. After evaporating solvent, purification was performed by preparative TLC (Si gel 60, 10x20 cm, 0.50 mm thickness plates) using the solvent system mentioned above for PSLA.

Synthesis of LA derivatives with 1,3-DAG (Paper II)

In the second study, LA was esterified with 1,3-diolein (DO) to synthesize 1,3-dioleoyl-2-lipoyl-*sn*-glycerol (DOLA), and 1,3-dioleoyl-2-dihydrolipoyl-*sn*-glycerol (DODHLA) was obtained by chemical reduction (Figure 11). The esterification procedure was similar to the synthesis of PSLA as described above, except that the preliminary TLC control was performed using hexane:diethyl ether:ethyl acetate:acetic acid (75:20:5:1, v/v/v/v) solvent system. Purification of DOLA was performed by column chromatography (Si gel 60 Å, 200-400 mesh) and fractions were collected with hexane:diethyl ether:acetic acid (100:4:1, v/v/v).

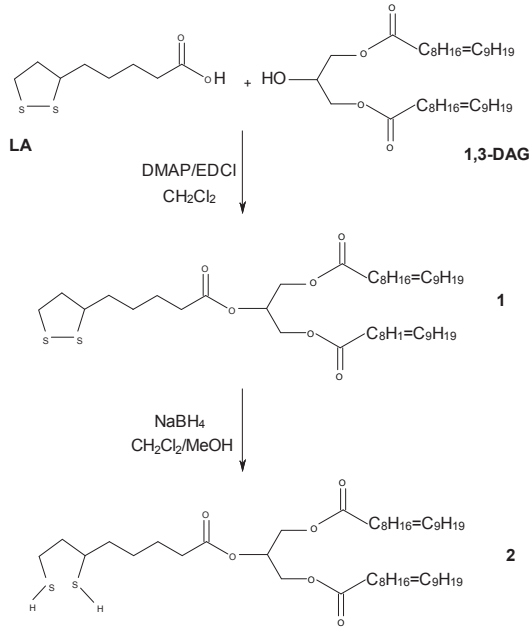

Figure 11. Synthesis of **1**) 1,3-dioleoyl-2-lipoyl-*sn*-glycerol (DOLA), and **2**) 1,3-dioleoyl-2-dihydrolipoyl-*sn*-glycerol (DODHLA).

Table 4. Summary of RP-HPLC-MS analysis (Papers I- III)

Study	Analyte	Column	HPLC conditions	Ionization	MS conditions
Paper I	LA, DHLA, PS PSLA, PSDHLA	C8, Zorbax SB, 150 x 4,6 mm, i.d., 5µm, column T at 50 ˚C	Isocratic elution: ACN, 17 mmol acetic acid (92.5:7.5, v/v), flow rate at 1.0 mL/min	APCI	Vaporizer T 350 ˚C, drying gas T 350 ˚C, nebulizer pressure 60 psi, flow at 9 L/min capillary voltage 3000 V
Paper II	LA, DHLA 1,3-DO, DOLA, DODHLA	C18, Thermo Hypersil GOLD 150 x 4,6 mm , i.d., 3 µm, column T at 20 ˚C	Gradient elution: ACN, 2-propanol, 5 mmol acetic acid (65:30:5, v/v/v) changed to (25:70:5, v/v/v), flow rate at 0.6 mL/min	APCI	Vaporizer T 300 ˚C, drying gas T 350 ˚C, nebulizer pressure 60 psi, flow at 9 L/min capillary voltage 3000 V
Paper III	LA, C12:0, MAG, Plasma lipids*	C18, Sunniest column, 100 x 2.0 mm, i.d., 5 µm, column T at 40 ˚C	Gradient elution: ACN, 20 mm acetic acid + 7% ACN (80:20, v/v) changed to (95:5, v/v), flow rate at 0.1 mL/min	ESI	Drying gas T 350 ˚C, nebulizer pressure 40 psi, flow at 12 L/min, capillary voltage 4000 V

RP, reversed phase; LA, α-lipoic acid; DHLA, dihydrolipoic acid; PS, phytosterols; PSLA, phytosterol lipoate; PSDHLA, phytosterol dihydrolipoate; DO, diolein; DOLA, 1,3-dioleoyl-2-lipoyl-*sn*-glycerol; DODHLA, 1,3-dioleoyl-2-dihydrolipoyl-*sn*-glycerol; C12:0, lauric acid; MAG, monoacylglycerol; i.d., internal diameter; T, temperature; ACN, acetonitrile; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; * lipids extracted from pooled plasma samples of mice using, the method by Folch *et al*. (1957).

Purified DOLA dissolved in CH_2Cl_2:MeOH (1:5, v/v) was reduced to obtain DODHLA as mentioned above for PSDHLA. Purification of DODHLA was performed by column chromatography (Si gel 60 Å, 200-400 mesh) and fractions were collected with hexane:diethyl ether:acetic acid (100:1:0.1, v/v/v). More details of the methods can be found in Paper II.

NMR analysis

All novel conjugates synthesized above were confirmed by ^{1}H-, ^{13}C-NMR and 2-D experiments (COSY, TOCSY and HSQC-dept), as explained in Paper I and II.

4.2 RP-HPLC-MS analysis (Papers I-II)

Novel LA and DHLA derivatives and initial compounds used in synthesis (PS, 1,3-DO, LA and DHLA) were analyzed by RP-HPLC-MS using atmospheric pressure chemical ionization (APCI). The chromatographic and MS conditions are summarized in Table 4. LA, DHLA, PSLA and PSDHLA were analysed in negative mode, whereas PS, PSLA, 1,3-DO, DOLA and DODHLA were analyzed in positive ion mode. The compounds were identified by their respective mass spectra. The fragmentation pattern of the novel derivatives was studied and fragment ions were identified with reference to the ions observed in the initial compounds of each derivative.

4.3 DPPH assay (Papers I-II)

Free radical scavenging capacity of the novel derivatives was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical according to Brand-Williams *et al*. (1995). First 0.06 mmol/L DPPH in toluene was prepared. A series of concentrations for each test compound was prepared separately, using toluene as the solvent (Wettasinghe & Shahidi, 2000). Concentrations of LA and derivatives of LA were prepared to obtain final molar ratio (MR) of 1-8 or 1-12 between test compound and DPPH in the final mixture. Concentrations of DHLA and DHLA derivatives were prepared between 0.025-1.0 MR. Each concentration of all compounds was analyzed in triplicates using 0.1 mL test compound mixed with 3.9 mL DPPH in toluene. Absorbance was measured at 515 nm, using toluene as the blank sample, every 15 min until steady state. The mean % of remaining DPPH free radical ($DPPH^{\cdot}$) at steady state was plotted to study the reaction kinetics of novel derivatives. Free radical scavenging

capacity defined as efficient concentration (EC_{50}) for DHLA and DHLA derivatives was calculated using the respective kinetic curves.

4.4 *In vitro* hydrolysis of novel derivative - diLaLA (Paper III)

As mentioned earlier, pure R-(+)-LA conjugated with 1,3-dilaurin (1,3-dilauroyl-2-lipoyl-*sn*-glycerol; diLaLA) was studied for *in vitro* hydrolysis, in a pilot study prior to an *in vivo* study. The *in vitro* hydrolysis was performed using porcine pancreatic lipase (type II), according to a combined method described previously (Narayan *et al*., 1993; Sahin *et al.,* 2005). In brief, porcine lipase (0.3% of reaction medium; wt./wt.) dissolved in 7 mL Tris buffer at pH 8 was mixed with 25 mg diLaLA dissolved in 100 µL hexane. Then 0.25 mL 0.05% bile salt and 2.2% calcium chloride per mL buffer were added and incubated at 38±2 ˚C with continuous agitation. *In vitro* hydrolysis was tested in triplicate at 5, 15, 30, 60 and 120 min incubation periods to monitor the formation of products by hydrolysis. The hydrolysis pattern was primarily monitored by analytical TLC developed in hexane:diethyl ether:acetic acid (50:50:1, v/v/v), and visualized by exposing the plate to iodine vapor for 10 min. At the end of the incubation time, the mixture was acidified to pH 2, after the TLC control. The digestion products were extracted with diethyl ether and analyzed by RP-HPLC-MS. Chromatographic and MS conditions used, are summarized in Table 4.

In addition, a preliminary test was performed to trace free LA in blood samples after a single oral treatment of 250 mg diLaLA/kg body wt. in lean C57/BL6J mice (details of the animals and sample collection is given under *in vivo* test below & Table 5). Blood samples collected after 2 h and 18 h, pooled separately, lipids were extracted (Folch method, 1957) and analyzed for free LA and C12:0 using RP-HPLC-MS-ESI (Table 4).

4.5 Study design: *In vivo* trial (Papers IV-V)

Novel molecule diLaLA was evaluated for potential biological effects against HFD induced obesity using obese C57BL/6J male mice (Taconic, USA), orally treated with diLaLA dissolved in rapeseed oil (used as vehicle). The study was approved by the animal experimental ethical committee (Dnr: C189/11) and conducted at Contract In Vivo Design (Uppsala, Sweden). In addition to DIO mice, an extra group of age matched lean male mice (Lean) was included as a parallel study and their blood samples were used in Paper III. Details of treatment groups, research diets and dose levels of the test compound are given

in Table 5 and Papers IV-V. The mice were around 16 weeks old and grouped based on their initial body weight.

Mice were housed as 2-3 mice/cage and had free access to food and water. During a 10 day acclimatization period, DIO mice were fed a HFD (product no. D 12451; 45% of total calories from fat; 4.73 kcal/g) purchased from Research Diet Inc. (New Brunswick, NJ, USA) and lean group was fed an ordinary rodent chow diet (3 kcal/g) obtained from Lantmännen (Kimstad, Sweden). The treatment was conducted for 6 weeks, and DIO mice were fed the same HFD, throughout the treatment period. The lean group was introduced to above mentioned HFD, at the start of the experiment. Body weight and cumulative food intake (CFI) were recorded 2-3 times/week, during the treatment period.

Animals were observed for general behavior, clinical signs of toxicity, motor activity and fur quality. On the last day of the treatment, lean mice were treated with 250 mg/diLaLA/kg body wt. and blood samples were collected at regular intervals during 0.5-18 h after administration. These plasma samples were used in Paper III. At sacrifice macroscopy was performed, the liver was inspected for steatosis and liver weight was recorded. Plasma, liver and white adipose tissue (AT) samples were frozen immediately and stored at -80 ˚C until further analysis. Further details of clinical observations, termination of the experiment and sample collection are given in Papers IV-V. A deviation report on mice excluded from the study is attached as a supplement report in the appendix to Paper IV.

Table 5. Allocation of test animals, diets and dose levels among different treatment groups of BL57/6J male mice (Papers III-V)

Parameter	Diet induced obese (DIO) (Papers IV-V)				Lean (Paper III)
Treatment group	DIO-V	DIO-10	DIO-50	DIO-250	Lean
Type of diet	HFD	HFD	HFD	HFD	HFD
No. of animals/group (*n*)	7	7	7	7	7
Initial body wt. (g)	36.7±2.2	35.8±2.9	36.3±1.8	35.9±3.7	26.1±1.9
Dose of diLaLA (mg/kg body wt./d) dissolved in vehicle (V)	0+V	10+V	50+V	250+V	50+V

Abbreviations: HFD, high fat diet containing 45% of total calories from fat; *n*, number of male mice allocated per each treatment group at the beginning of the study; diLaLA, test compound 1,3-dilauroyl-2-lipoyl-*sn*-glycerol; V, vehicle (rapeseed oil) used to dissolve diLaLA. The test compound and/V was orally administered by gavage, daily for 6 weeks.

4.5.1 Biochemical analysis

DIO mice fed with HFD, were used to evaluate effects on obesity in this thesis. Blood samples from non-fasting animals were analyzed for glucose levels using Accu-Chek® Aviva from Roche (Mannheim, Germany) according to the manufacturer's instructions. After studying body weight, CFI and blood glucose concentration data, it was decided to analyze lipids in plasma, liver and AT samples only from DIO-V and DIO-250 groups, for practical reasons. Detailed methods of all biochemical analysis are given in Papers IV-V.

Concentrations of TAG and FFA were quantified using heptadecanoic acid (C17:0) and triheptadecanoin as internal standards (IS), added to plasma. Lipids were extracted using a slightly modified version of the method by Folch *et al*. (1957), and FFA and TAG fractions were separated by preparative TLC using hexane:diethyl ether:acetic acid, (85:15:1, v/v/v) solvent system. Fatty acid methyl esters (FAME) of FFA and TAG were prepared by in-situ methylation and analyzed by GC (Glaser *et al*., 2010). A recovery test was performed similarly, using standard oleic acid and triolein spiked in pooled plasma samples (n = 3), and recovery % was calculated based on non-spiked controls (n = 3).

Total cholesterol (T-Ch) and total fatty acid composition (TFA) in plasma and liver samples were quantified using 5α-cholestane and C17:0, respectively, as IS. Samples were saponified directly according to a combined method (Rule *et al*., 2002; Ubhayasekera *et al*., 2010). The non-saponifiable fraction was extracted, silylated and analyzed for T-Ch by GC. Remaining saponifiable fraction with IS C17:0 was extracted and methylated to analyze TFA. Similarly, white AT samples were directly saponified and analyzed for TFA (Lepage & Roy, 1984). Stearoyl-CoA desaturase (SCD)-1 activity was estimated by SCD16 index (palmitoleic acid, C16:1n-7/palmitic acid, C16:0) and SCD18 index (oleic acid, C18:1n-9/stearic acid, C18:0) using TFA data from plasma, liver and white AT samples (Warensjö *et al*., 2009). Vitamin E (α-tocopherol) content in plasma and liver samples was quantified by HPLC using an external standard method (Azardmard-Damirichi & Dutta, 2008) after extracting lipids, according to the method of Hara & Radin (1978).

4.5.2 Gene expression analysis

Liver samples from the DIO-250 (n = 3) and DIO-V (n = 2) groups were analysed for target genes involved in β-oxidation and energy metabolism using RT-qPCR (Qiagen Gmbh, Hilden, Germany). Each sample was analysed in duplicate and average expression level was calculated separately for all the genes analysed. Relative expression levels for each gene in liver samples of

DIO-250 were expressed as fold change, compared with DIO-V. All data were calculated using on line RT^2 Profiler^{TM} PCR Array Data Analysis version 3.5 Qiagen (Qiagen Gmbh, Hilden, Germany) and statistical comparison between two treatment groups was not performed due to limited number of replicates in DIO-V. Details of the method are found in paper V.

4.5.3 Statistical analysis

Results from *in vitro* tests are expressed as mean±standard deviation (SD), (Papers I-II) and results from animal experiment and biochemical analysis as mean±standard error of mean (SEM). Data on body weight, CFI, blood glucose and liver weight in DIO groups were analyzed using one way analysis of variance (ANOVA) and treatment groups were compared using Bonferroni´s or Dunnet´s multiple comparision test. Results from biochemical analysis of plasma, liver and white AT samples of the DIO-250 and control groups were compared using a two sample t-test assuming equal variance. Differences were considered significant at $p<0.05$. All statistical analysis were performed using the software, GraphPad Prism (GraphPad Software Inc., California, USA) and Minitab version 16.0 (Minitab Inc., State College, PA, USA).

5 Results and Discussion

5.1 Synthesis of novel derivatives

Two series of LA and DHLA derivatives with PS and 1,3-DAG were prepared by a simple two step procedure (Figure 10 & 11), performed under mild conditions. In the first step, LA with PS or 1,3-DAG was esterified, in the presence of DMAP and EDCl. In the second step, the disulfide bond was reduced using $NaBH_4$ to obtain DHLA derivatives of either PS or 1,3-DO. Among different solvent systems tested for TLC control, hexane:diethyl ether:acetic acid (75:25:0.85, v/v/v) showed satisfactory separation of the non-polar derivatives PSLA and PSDHLA, as well as the initial polar compounds PS and LA (results are not shown).

Synthesis of DOLA and DODHLA was performed similarly, except that TLC control (Figure 12) was performed using the solvent system hexane:diethyl ether:ethyl acetate:acetic acid (75:20:5:1, v/v/v/v). Purified novel derivatives PSLA, PSDHLA, DOLA and DODHLA were characterized by NMR analysis (Papers I & II). Novel derivatives PSLA and DOLA showed yield of approx. 60% and 80%, respectively, after purification by chromatographic methods. Both of the products remained as light yellow colored oils, in the room temperature in contrast to LA and PS which occur as powder and are poorly soluble in oil or water.

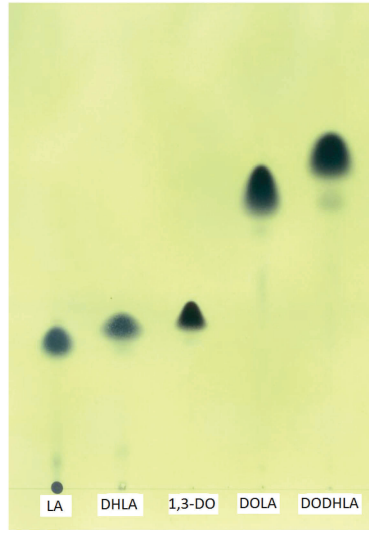

Figure 12. Thin layer chromatogram (TLC) showing the separation of initial compounds and novel derivatives (Paper II). TLC was developed in hexane:diethyl ether:ethyl acetate:acetic acid (75:20:5:1, v/v/v/v) and compounds were visualized by spraying phosphomolybdic acid to develop color at 120 ˚C for 15 min. Abbreviations: LA, α-lipoic acid; DHLA, dihydrolipoic acid; 1,3-DO, 1,3-dioleoyl-*sn*-glycerol; DOLA, 1,3-dioleoyl-2-lipoyl-*sn*-glycerol, and DODHLA, 1,3-dioleoyl-2-dihydrolipoyl-*sn*-glycerol.

5.2 RP-HPLC-MS-APCI analysis

Derivatives of LA with phytosterols (Paper I)

As expected, the polar initial compounds LA and DHLA showed relatively higher signal in ESI mode, whereas 1,3-DO, PS and non-polar conjugates after esterification showed higher signal in APCI mode. LA and DHLA were analyzed in negative ion mode, due to their acidic properties. However, they were co-eluted due to the high proportion of organic solvent used in the mobile phase. Since our main focus was to study the novel derivatives, high organic proportion in the mobile phase and APCI mode were selected for further analysis.

In the case of PS derivatives, a satisfactory separation of different PS (β-sito-, campe-, stigma- and brassica- sterols) and their LA derivatives could be achieved with C8 column at 50 ˚C column temperature (Figure 13) compared with the C18 columns tested (details in Paper I). Among the PS, brassicasterol was eluted first, followed by campesterol and stigmasterol whereas sitosterol was eluted last. However, a baseline separation between campesterol and stigmasterol could not be achieved even with C8 column and the method may

need further optimization. Corresponding LA and DHLA derivatives of PS eluted later, but followed the same order. A mixture of PS was used in the synthesis of PSLA, since this was commercially more feasible compared to expensive pure PS. The composition of this initial PS mixture was pre-analyzed by GC in our laboratory and found to contain 53, 29, 15 and 1% of β-sito-, campe-, stigma- and brassica- sterol, respectively. Similar proportions of corresponding PSLA derivatives were observed after the esterification reaction when analyzed by RP-HPLC-MS.

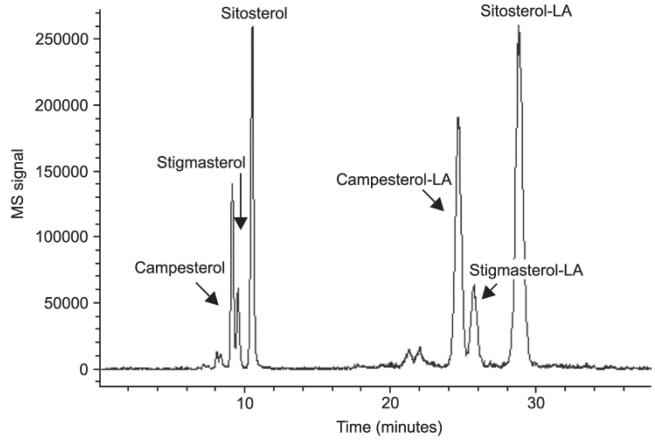

Figure 13. Total ion chromatogram recorded in positive ion mode in RP-HPLC-MS-APCI, showing separation of β-sito-, stigma-, and campe- sterols in the initial PS mixture, and their derivatives with LA.

Different PS and their PSLA derivatives provided relatively higher signal in positive mode, whereas PSDHLA showed higher signal in the negative mode. The dominant and characteristic ions observed for the novel derivatives demonstrated their fragmentation pattern (Paper I). PS showed typical dehydrated ion, $[M\text{-}H_2O+H]^+$, along with $[M\text{-}2H+H]^+$ and $[M\text{-}4H+H]^+$ ions, as described previously (Rozenberg *et al*., 2003; Bedner *et al*., 2004). In positive mode, the protonated molecular ion $[M+H]^+$ was characteristic in all PSLA molecules showing ions at *m/z* 603, 601, 589 and 587 for β-sito-, stigma-, campe-, and brassica- sterol lipoate, respectively. At the same time, fragment ions at *m/z* 397-, 395-, 383-, and 381- confirmed their corresponding characteristic PS moiety from β-sito-, stigma-, campe-, and brassica- sterol, respectively. The fragmentation at the esterified position was further illustrated by the dominant fragment ion at *m/z* 205 from LA moiety observed in the

negative ion mode analysis of PSLA. Dominant $[M-H]^-$ was observed for PSDHLA in the negative mode.

Derivatives of LA with 1,3-DAG (Paper II)

Among different columns tested, C18 column was proved suitable for separation of LA, DO, DOLA and DODHLA (Figure 14). When retention times of LA derivatives were compared with their corresponding DHLA derivatives, DHLA derivatives eluted before their corresponding LA derivatives. LA derivatives with the closed dithiolane ring showed a more hydrophobic nature compared to their reduced counterparts which possess an open ring with two thiol groups.

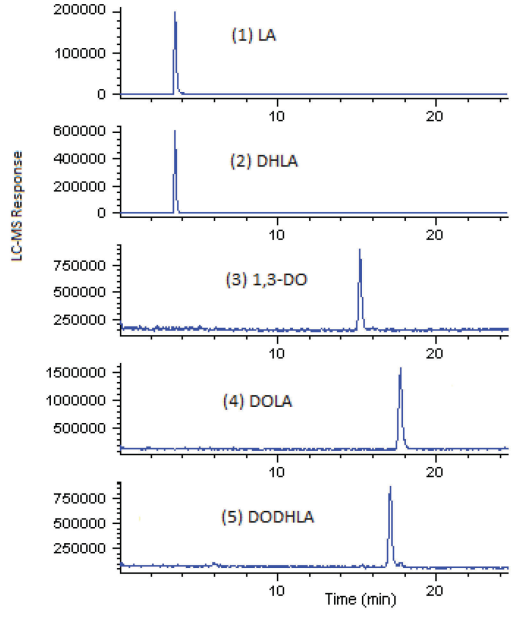

Figure 14. Total ion chromatogram recorded in RP-HPLC-MS-APCI showing retention times of the initial compounds and their derivatives. Abbreviations: 1) LA, α-lipoic acid; 2) DHLA, dihydrolipoic acid; 3) 1,3-DO, 1,3-dioleoyl-*sn*-glycerol; 4) DOLA, 1,3-dioleoyl-2-lipoyl-*sn*-glycerol, and 5) DODHLA, 1,3-dioleoyl-2-dihydrolipoyl-*sn*-glycerol.

In the case of DO, DOLA and DODHLA, relatively higher signal was observed in positive ion mode. Due to its hydroxyl group at the *–sn2* position, DO produced the dominant $[M-H_2O+H]+$ ion at *m/z* 603. In addition, ion at *m/z* 339 showed dissociation of one FA from DO, resulting in a protonated MAG $[M-RCOOH+H]^+$ while molecular ion at *m/z* 621 was less abundant. In

contrast, DOLA was relatively more stable and showed the dominant molecular ion at *m/z* 809 and fewer fragment ions. However, fragment ions at *m/z* 527 and 339 reflected some formation of $[M\text{-}R_1COOH+H]^+$ and $[M\text{-}R_1COOH\text{-}R_2COOH+H_2O+H]^+$ ions, respectively. DODHLA showed a similar fragmentation pattern, but the dominant ion was *m/z* 529 fragment ion $[M\text{-}R_1COOH+H]^+$ with less abundant molecular ion $[M+H]^+$.

5.3 Free radical scavenging capacity

The results from the DPPH assay showed that the *in vitro* free radical scavenging capacity of PSLA and DOLA was very low and comparable to the free radical scavenging capacity and kinetics observed for LA. In contrast, PSDHLA and DODHLA showed high interaction with DPPH free radical and were comparable with DHLA (Figure15). Reaction kinetics was plotted using a series of concentrations for each test compound, as described by Brand-Williams *et al*. (1995). At MR = 1, the remaining DPPH% was only 18% in PSDHLA, whereas it was 99% in PSLA. Similarly, DOLA and LA also showed more than 99% remaining $DPPH^{\cdot}$, whereas DHLA and DODHLA showed 14% and 6% remaining $DPPH^{\cdot}$, respectively, when MR = 1. The relatively longer time taken to achieve steady state in DHLA, PSDHLA and DODHLA compared to LA and LA derivatives suggests continuous interaction of novel derivatives with $DPPH^{\cdot}$.

The antioxidant properties of LA is believed to be due to the intra-molecular disulphide bond in the dithiolane ring of LA, which possesses high electron density due to its special position of the two sulfur atoms (Packer *et al*., 1995; Biewenga *et al*., 1997). In biological systems LA is enzymatically reduced to DHLA, which is considered more potent antioxidant due to its two thiol groups (Teichert & Preiss, 2008). High antioxidant capacity of DHLA compared with LA is well documented (Packer *et al*., 1995; Biewenga *et al*., 1997; Shay *et al*., 2009). However, LA did not show sufficient interaction with $DPPH^{\cdot}$ free radical in present study. In this thesis, *in vitro* free radical scavenging capacity of novel LA and DHLA derivatives was analyzed and compared with that of pure LA and DHLA to determine, whether conjugation has any effect on the potential antioxidant properties. The results observed showed that conjugation did not affect the free radical scavenging capacity of novel derivatives.

Stable $DPPH^{\cdot}$ free radical is considered suitable for analysis with lipophilic compounds, performed at micro-molar levels (Brand-Williams *et al*., 1995). Due to their nonpolar nature, PSLA and DOLA are not completely soluble in methanol or ethanol, which are considered typical solvents for the DPPH test.

Therefore toluene was used as the solvent in DPPH assay, according to Wettasinghe & Shahidi (2000). Since, there were no previously published data on the *in vitro* free radical scavenging capacity of LA and DHLA using toluene as solvent, they were also evaluated under similar conditions, in order to compare with novel conjugates. The effective concentration EC_{50} is known as a comparative measurement of antioxidant capacity between different antioxidant compounds (Brand-Williams *et al*., 1995). The EC_{50} values obtained from the kinetic curves for DHLA, PSDHLA and DODHLA were 0.39±0.04, 0.43±0.05 and 0.22±0.03, respectively. The variation between different compounds can be due to the molecular structure and differences in the solubility of test compounds. In addition to the reducing capacity of the test compound, the interaction between DPPH˙ may also vary between different compounds due to steric hindrance depending on molecular structure and differences in solubility (Brand-Williams *et al*., 1995).

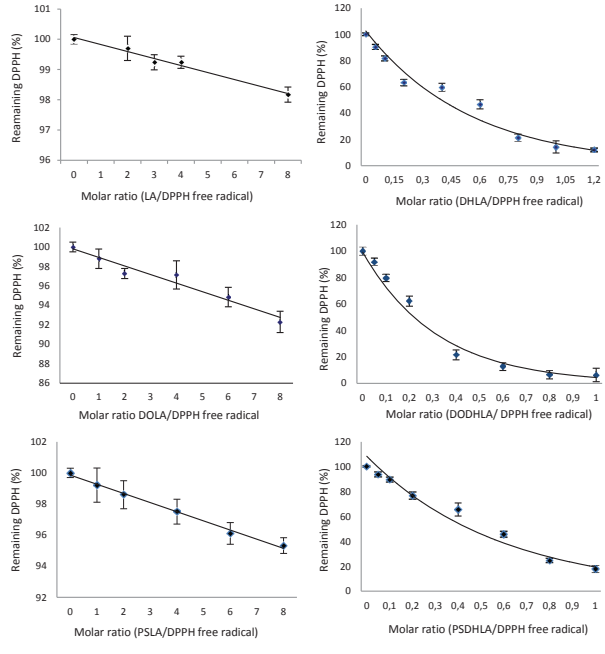

Figure 15. Free radical scavenging capacity of novel derivatives measured by DPPH assay. Results are expressed as remaining DPPH free radical (%) as a function of molar ratio between testing compound and DPPH free radical. Abbreviations: LA, α-lipoic acid; DHLA, dihydrolipoic acid; DOLA, 1,3-dioleoyl-2-lipoyl-*sn*-glycerol, DODHLA, 1,3-dioleoyl-2-dihydrolipoyl-*sn*-glycerol; PSLA, phytosterol lipoate; PSDHLA, phytosterol dihydrolipoate.

5.4 *In vitro* digestion of diLaLA

In vitro hydrolysis of diLaLA was performed by simulating conditions similar to the small intestine, but with excess pancreatic lipase to ensure that the reaction was not substrate-limited. Intermediate product 1(3)-lauryl-2-lipoyl-*sn*-glycerol was observed in incompletely hydrolyzed mixtures, during optimization of the reaction and when diLaLA was not initially dissolved in 100 µL hexane as described by Narayan *et al*. (1993). After optimization, major products from the hydrolysis at the initial stage were lauric acid (C12:0) and MAG (Figure 16). The amount of free LA increased gradually from approx. 3% at 5 min up to 20% by 60 min of the incubation time (Figure 17). The concentration of free LA increased until 60 min while the MAG concentration decreased, showing further hydrolysis of monolipoate. Under the test conditions, pancreatic lipase showed hydrolysis of LA, esterified at the *sn-2* position in the diLaLA molecule, in addition to its well documented hydrolysis at the –*sn*1 and the –*sn*3 positions. It has been found that pancreatic lipase can hydrolyze FA at the –*sn*2 position in TAG depending on the incubation time, enzyme concentration, temperature and type of lipase enzyme (Jennings & Koh, 1999).

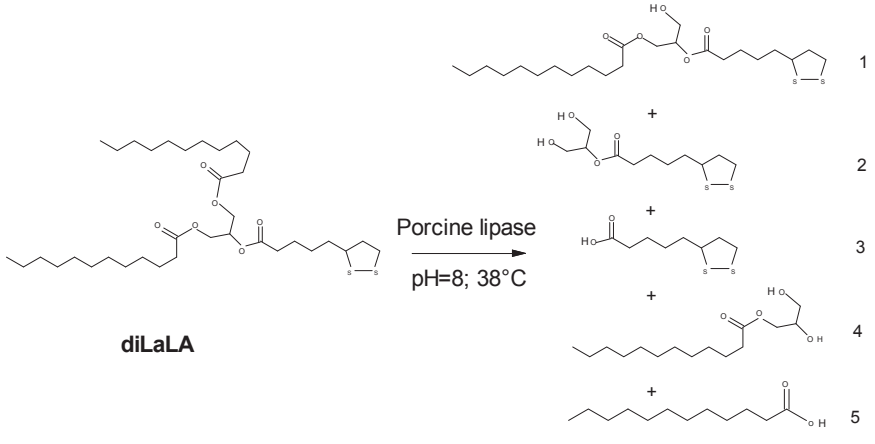

Figure 16. Chemical structure of the products formed in *in vitro* hydrolysis of novel derivative 1,3-dilauroyl-2-lipoyl-*sn*-glycerol (diLaLA), mediated by porcine pancreatic lipase (Type II). Products identified using RP-HPLC-MS-ESI: **1**) 1(3)-lauroyl-2-lipoyl-*sn*-glycerol; **2**) 2-lipoyl-*sn*-glycerol (monolipoate); **3**) α-lipoic acid (LA); **4**) 1(3)-lauroyl-*sn*-glycerol (monolaurate), and **5**) lauric acid (C12:0).

The concentration of C12:0 was slightly reduced until 60 min, probably due to acyl migration, that might have occured between partial glycerides during pancreatic hydrolysis (Jennings & Koh, 1999; Christophe, 2008). The MAG

fraction observed at 15 min was isolated by preparatory TLC and analyzed by RP-HPLC-MS showed that 78% of it comprised monolipoate, whereas 14% was monolaurate. Since pure standard was not available, formation of monolipoate during incubation was followed by monitoring the area recorded by SIM chromatogram for selected characteristic ions at *m/z* 263 and *m/z* 298. The TLC control and RP-HPLC-MS analysis showed that *in vitro* hydrolysis of diLaLA was completed within 15 min, releasing monolipoate, monolaurate, free C12:0 and LA under the test conditions.

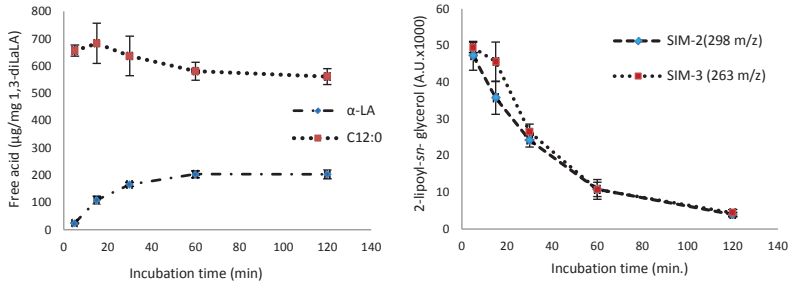

Figure 17. Formation of digestion products during *in vitro* hydrolysis of novel TAG molecule (diLaLA) using porcine pancreatic lipase (Type II) at 8 pH and 38±2 °C. Each point is mean±SEM of triplicates analyzed by RP-HPLC-MS-ESI.

Identification of free LA and C12:0 in mice plasma (Paper III)

For the first time, free LA and C12:0 were observed in the lipid extract of a pooled plasma sample collected after 2 h oral administration of 250 mg diLaLA/kg body wt. in lean mice (Figure18). The peaks were identified by comparing them with the respective mass spectra and retention times of standard LA and C12:0 (Paper III). Despite co-eluting unknown compounds, negative deprotonated ion $[M\text{-}H]^-$ at *m/z* 205 and characteristic fragment ion $[M\text{-}H_2S\text{-}H]^-$ at *m/z* 171 provided enough evidence to identify free LA in plasma sample as observed in previous studies Papers I-II, and previous literature (Montero *et al*., 2012). Neither LA nor C12:0 was observed in plasma samples collected 18 h after oral administration of diLaLA. According to the literature, orally ingested pure LA is absorbed directly, metabolized rapidly and excreted via urine (Schupke *et al*., 2001; Teichert & Preiss, 2008).

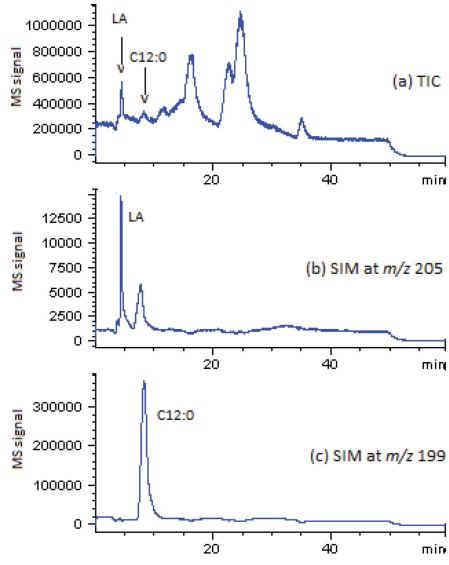

Figure 18. Free α-lipoic acid (LA) and lauric acid (C12:0) detected in lipids, extracted from a pooled plasma sample 2 h after oral treatment with 250 mg diLaLA/kg body wt. in lean mice. Lipid extract was analyzed using RP-HPLC-MS-ESI in negative ion mode. Abbreviations: a) TIC, total ion chromatogram of plasma lipid extract; b) SIM at *m/z* 205, selective ion monitoring chromatogram at *m/z* 205 for LA, and c) SIM at *m/z* 199, selective ion monitoring chromatogram at *m/z* 199 for C12:0.

5.5 Anti-obesity effects of diLaLA

Clinical observations(Papers IV-V)

During the study, three mice in the DIO-10 group were excluded, one mouse due to failure in oral gavage and two due to physical injuries that affected food intake (FI) and weight gain. During clinical observations, no signs of substance-related toxicity, was observed. Furthermore, there were no toxic- or pathological- symptoms in relation to administration of vehicle or test compound. At sacrifice, steatosis was observed in one animal from each group. Weight of the liver among different groups did not show any significant difference (Table 6). The C57BL/6J mice model is widely considered a reliable and robust experimental model to evaluate the effect of novel therapeutic interventions related to diet induced obesity (Winzel & Ahrén, 2004).

5.5.1 Effects on body weight and food intake

The body weight of all DIO mice on HFD was increased in all treatment groups during the 6 week treatment period, except in DIO-250 group (Figure 19) which showed significant difference ($p<0.05$) compared with the control group treated vehicle alone (DIO-V) or the low dose (DIO-10 and DIO-50) groups. At the end of the 6 weeks treatment, only the high dose group, DIO-250 showed a significant effect on body weight change demonstrating a loss in body weight compared with other groups ($p<0.05$) who clearly gained weight over time (Figure 20).

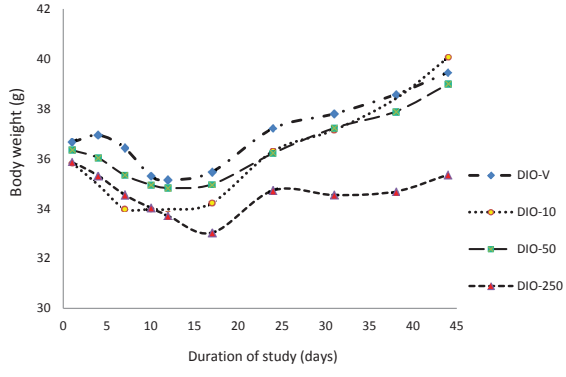

Figure 19. Changing pattern of the body wt. in different treatment groups of diet induced obese (DIO) male mice fed a high fat diet (HFD) during 6 weeks treatment with 1,3-dilauroyl-2-lipoyl-*sn*-glycerol (diLaLA). Mice in treatment groups DIO-V, DIO-10, DIO-50 and DIO-250 were orally treated with diLaLA, dissolved in vehicle (rapeseed oil) at dose levels 0, 10, 50 and 250 mg/kg body wt., respectively.

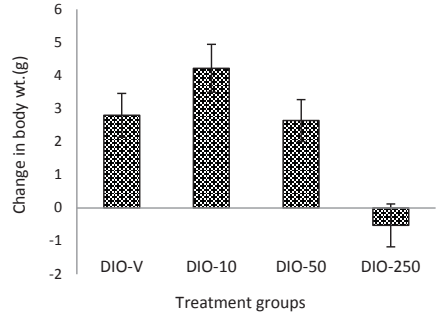

Figure 20. Body weight change observed in different treatment groups at the end of 6 weeks treatment with diLaLA. Abbreviations and dose levels as described in Figure 19.

Doses at 10 or 50 mg/kg body wt. showed non-significant changes in body weight gain at the end, compared with the control suggesting that tested low oral doses of diLaLA were not sufficient to deliver any weight reduction. Thus oral administration of diLaLA at 250 mg/kg body wt. daily, for 6 weeks was effective in reversing HFD induced body weight gain in DIO mice, compared with the control.

A dip in body weight gain observed during the second week of the treatment, was not related to diLaLA since it was also observed in the control group. This could be due to some physical strain caused by gavage feeding. The animals were housed in groups of two or three per cage, for practical reasons and cumulative food intake (CFI) was calculated for each group based on the FI data on cage basis. A marginally lower CFI was observed in all diLaLA treated mice compared with the control, indicating some reduction in CFI, but there were no significant difference between treatment groups (Table 6).

Since this was the first study on the novel derivative diLaLA, there were no previously published data on weight-reducing effects to compare the results. Previously published data on oral supplementation with pure LA have shown a significant reduction in body weight in rodents (Kim *et al*., 2004; Cremer *et al*., 2006; Park *et al*., 2008; Prieto-Hontoria *et al*., 2009; Wang *et al.*, 2010). However, a concomitant reduction in FI in the rodents tested, were observed in all those studies except that by Ide *et al*. (2013), who did not observe any significant reduction in FI, similar to present data. Therefore the results support, the idea that there is another mechanism underlying the body weight-reducing effect mediated by diLaLA. Wang *et al*. (2010) found that the respiratory quotient (volume CO_2/volume O_2) in LA treated C57BL/6J mice was higher than that in the control, suggesting higher energy expenditure induced by LA. Moreover, dietary supplementation of LA (0.5% wt./wt.) in rats was reported to up regulate expression of uncoupling protein (UCP)1 in brown AT. UCP1 is located in the inner mitochondrial membrane and dissipate proton electrochemical energy as heat, regulating energy expenditure in rodents (Kim *et al*., 2004).

The weight-reducing effect mediated by consumption of 1,3-DAG replacing TAG in the diet, was also explained by enhanced energy expenditure through FA oxidation and thermogenesis, in rodents as well as humans (Maki *et al*., 2002; Murase *et al*., 2002; Rudowska *et al*., 2005; Hibi *et al*., 2008). In addition, a serotonin mediated increase in β-oxidation of FA has also been suggested for 1,3-DAG induced weight reduction (Yanai *et al*., 2010).

Table 6. *Effect of orally administered 1,3-dilauroyl-2lipoyl-sn-glycerol (diLaLA) in DIO mice on high fat diet at the end of the 6 weeks treatment period (Paper IV)*

Parameter	DIO-V	DIO-10	DIO-50	DIO-250
CFI (kcal/kg body wt.)	1618±90	1515±82	1429±38	1493±42
Liver weight (g)	1.4±0.1	1.3±0.1	1.4±0.1	1.3±0.1
Blood glucose con. (mmol/L)	9.6±0.5	9.8±0.9	9.8±0.2	8.6±0.4
TAG con. in plasma (mg/dL)	45.7±7.2	NA	NA	59.7±3.6
TAG con. in liver (mg/g)	62.0±13.2	NA	NA	51.3±12.3
Vitamin E con. in plasma (µg/dL)	NA	NA	343.9±62.4	438.1±51.7
Vitamin E con. in liver (µg/g)	47.1±7.6	NA	NA	48.1±7.5

Abbreviations: CFI, cumulative food intake; TAG, triacylglycerol; DIO, diet induced obese mice; V, control treated only with vehicle; -10, -50, -250, treated with diLaLA at -10, -20 and -250 mg doses/kg body wt. daily for 6 weeks, respectively; con., concentration in samples from non-fasting animals; NA, not analyzed. All values are mean±standard error of mean, no. of animals (*n)/group* = 7, except for DIO-10 where $n = 4$.

Effects on blood glucose concentration

The blood glucose concentration in DIO-250 was the lowest among all groups, while that in DIO-V, DIO-10 and DIO-50 was rather uniform (Table 6). Although difference between the DIO-250 and the DIO-V was not significant, this might indicate a trend for reducing glucose concentration. This was probably due to improved insulin sensitivity. According to previous literature dietary supplementation of LA increase glucose uptake and insulin sensitivity in rodents as well as humans (Kim *et al*., 2004; Shay *et al*., 2009; Wang *et al*., 2010; Packer & Cadenas, 2011). This is further supported by significantly low FFA concentration observed in DIO-250 mice compared with the control, which will be discussed later in this thesis.

5.5.2 Effects on lipids in plasma and liver

Effects on total cholesterol concentration

The concentrations of T-Ch in both plasma and liver samples from DIO-250 mice were relatively low compared with the control DIO-V, although significant differences were not observed (Figure 21). Supplementation of pure LA was reported to reduce total cholesterol in serum and liver in rodents (Yang *et al*., 2008; Ide *et al*., 2013). A previous study with C57BL/6J mice supplemented with LA (0.1% wt./wt.) in HFD reported decrease in plasma T-Ch and LDL-Ch, mediated by down regulation of genes involved in the cholesterol synthesis pathway (Yang *et al*., 2008). Moreover, there might be a parallel effect of body weight reduction, since plasma cholesterol shows a

positive correlation with body weight (Poobalan *et al*., 2004; Miller *et al*., 2011).

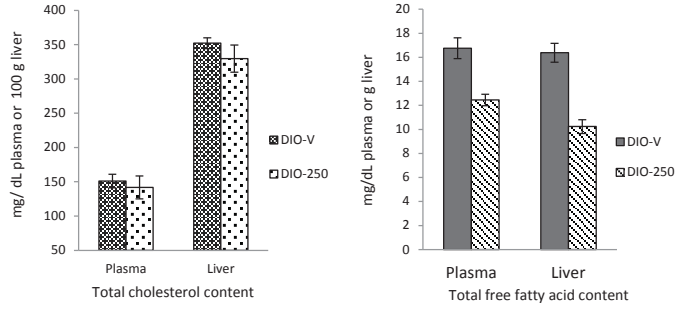

Figure 21. Concentration of total cholesterol and free fatty acids in plasma and liver samples of diet induced obese (DIO) mice, after 6 weeks treatment with 1,3-dilauroyl-2-lipoyl-*sn*-glycerol (diLaLA). Abbreviations: DIO-250, DIO mice treated with diLaLA 250 mg/kg body wt. daily; DIO-V, control treated only with vehicle. Values are mean±standard error of mean (n = 7).

Effects on TAG concentration

The results from the recovery test showed 91±4% recovery for TAG analyzed, by preparative TLC method used in this thesis. The total plasma and hepatic TAG concentrations between DIO-250 and the control DIO-V did not differ significantly (Table 6), probably due to the high variation observed among animals and the small group size (n = 7). In contrast to other results observed in DIO-250 mice, plasma TAG concentration was higher compared with the control. This unexpected result was probably due to time of sampling in relation to food intake. The mice in the present study had free access to food and were non-fasting when sacrificed.

On the other hand, total hepatic TAG concentration observed in DIO-250 mice was 17% lower compared with the control and was in agreement with other results observed in the study. According to the literature, liver TAG generally reflects relatively long term effects compared with the plasma TAG and only 9-13% of dietary TAG are recycled through the liver daily (Lambert & Parks, 2012). The reduced TAG concentration observed in the liver of DIO-250 mice is in agreement with previously published data in rats supplemented with LA (Park *et al*., 2008). The concentration of individual FA observed in TAG fraction was proportionately lower in DIO-250 mice, compared with the control (Figure 22). However, the relative % of individual FA remained rather uniform between DIO-250 and control. The concentrations were reduced in the major FA, C18:1(n-9), C16:0 and C18:2(n-6) which comprised approx. 38%, 25% and 21% of the total amount, respectively.

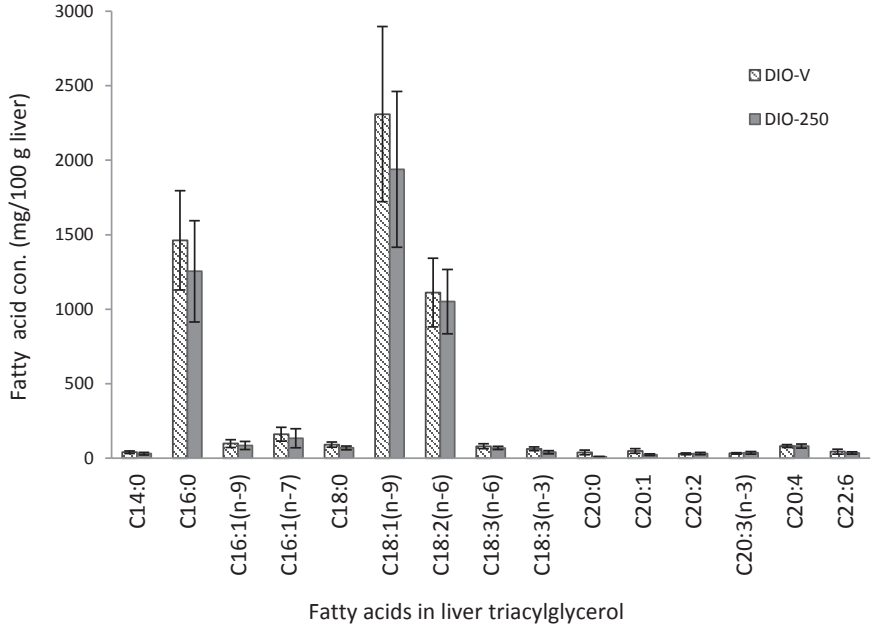

Figure 22. Concentration of individual fatty acids in the triacylglycerol fraction in liver samples of diet induced obese (DIO) mice, after 6 weeks treatment with 1,3-dilauroyl-2-lipoyl-*sn*-glycerol (diLaLA). Abbreviations: DIO-250, DIO mice treated with diLaLA 250 mg/kg body wt. daily; DIO-V, control treated only with vehicle. Values are mean±standard error of mean (n = 7).

Effects on FFA concentration

Oral administration of diLaLA at 250 mg/kg body wt. resulted in significant decrease in total FFA concentration in both plasma ($p<0.05$) and liver ($p<0.01$) compared with the control group (Figure 21). In DIO-250 mice, plasma FFA concentration was reduced by 26%, while liver FFA concentration was reduced by 38%. The recovery test showed 99±23% recovery, for FFA analyzed by preparative TLC method used in this thesis. Among individual FFA observed, concentrations of C16:0, C16:1n-9, C16:1n-7, C18:1n-9, C18:2n-6, C20:4 and C22:6 were significantly lower ($p<0.05$) in plasma (results are not shown). In liver samples, all identified individual FFA, except C12:0, C14:0, C16:1n-9 and C16:1n-7 were significantly lower ($p<0.05$) in DIO-250 mice, compared with the control (Figure 23). However, the relative % of individual plasma FFA between DIO-250 and the control remained constant. The major FFA in both plasma and liver were C16:0, C18:1(n-9) and C18:2(n-6).

These findings are in agreement with previous studies on rats supplemented with LA in the diet (Kim *et al*., 2004; Park *et al*., 2008). Increased FFA concentration in plasma is commonly associated with obesity and has been

identified as an important factor behind insulin resistance (Boden, 2008; Cedernaes *et al*., 2013). Extra FFA provided by visceral AT to the liver, are re-esterified to TAG, resulting in increased concentrations of VLDL which is believed to generate atherogenic LDL-Ch (Miller *et al*., 2011). Therefore the diLaLA-induced decrease, in plasma and liver FFA concentrations in diet induced obese mice in this thesis, highlights the potential positive health effects of this novel derivative against obesity associated risk factors.

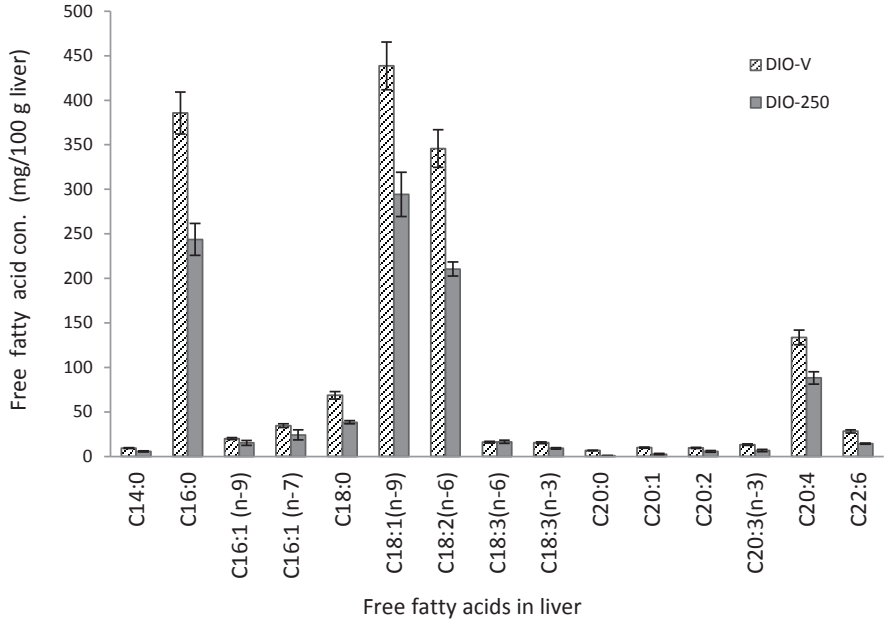

Figure 23. Concentration of individual fatty acids in the free fatty acid fraction in liver samples of diet induced obese (DIO) mice after 6 weeks treatment with 1,3-dilauroyl-2-lipoyl-*sn*-glycerol (diLaLA). Abbreviations: DIO-250, DIO mice treated with diLaLA 250 mg/kg body wt. daily; DIO-V, control treated only with vehicle. Values are mean±standard error of mean (n = 7).

5.5.3 Effects on estimated SCD1 activity

The results in this thesis show that estimated SCD16 index (16:1n-7/16:0) was significantly reduced (p<0.05) in white AT of DIO-250 mice compared with the control, while a similar trend was found in liver samples (Figure 24). However, no difference in SCD16 index was observed in plasma. This could be due to the relatively short duration of the experiment, because AT are known to reflect earlier changes in obesity compared with liver and plasma samples (Cedernaes *et al*., 2013). Lipogenic enzyme SCD1 catalyzes the biosynthesis of major FA in tissue lipids by introducing a double bond to

palmitic acid (C16:0) and stearic acid (C18:0), converting them into palmitoleic acid (C16:1) and oleic acid (C18:1), respectively. In general, the C16:1 content in the diet is relatively low and most of the C16:1 is derived from endogenous synthesis catalyzed by SCD1. Therefore FA index can be used as a surrogate measurement of SCD1 activity and considered as a biomarker for *de novo* lipogenesis in humans as well as rodents (Ntambi, 1999; Warensjö *et al*., 2009).

In general, SCD18 index (18:1n-9/18:0) reflects FA chain elongation and subsequent desaturation (Warensjö *et al*., 2009; Cedernaes *et al*., 2013). In this thesis, non-significant differences were observed in SCD18 index in DIO-250 mice compared with the control. Slightly lower activity was observed in liver samples of the DIO-250 group, compared with the control. In contrast, opposing changes were observed in AT, probably due to high C18:1 content in the diet. Despite alterations in the endogenous FA pool through different physiological processes, the overall FA composition of the diet is also reflected by FA composition in AT in rats (Weber *et al*., 2002; Oosterveer *et al*., 2009).

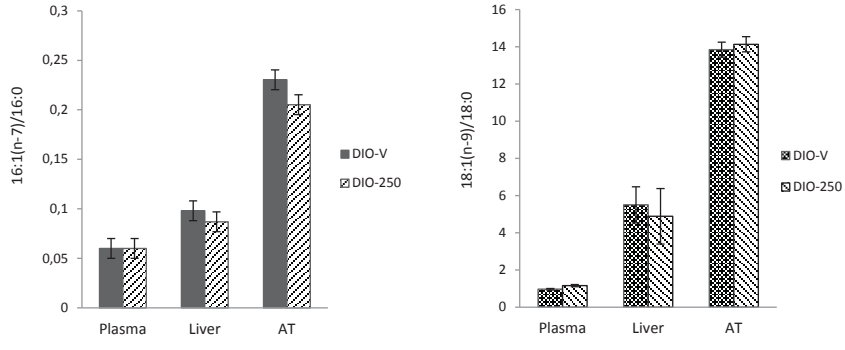

Figure 24. Stearoyl-CoA desaturase (SCD)-16 index (C16:1n-7/C16:0) and SCD-18 index (C18:1n-9/C18:0) as a measurement of SCD1 activity in plasma, liver and white adipose tissue samples of diet induced obese mice (DIO), after 6 weeks treatment with 1,3-dilauroyl-2-lipoyl-*sn*-glycerol (diLaLA). Abbreviations: DIO-250, DIO mice treated diLaLA 250 mg/kg body wt. daily; DIO-V, control treated only with vehicle. Values are mean±standard error of mean (n = 7).

SCD1 activity influences fat storage and body weight through its activity in lipogenesis. Increased SCD1 activity is associated with obesity and estimated SCD1 activity is considered a biomarker for increased adiposity (Flowers & Ntambi, 2008; Jeyakumar *et al*., 2009; Warensjö *et al*., 2009). Downregulation of SCD1 activity has been reported in relation to management of obesity and improvement of insulin sensitivity, and thus estimated SCD1 activity can be

used to evaluate the efficacy of anti-obesity treatments (Jeyakumar *et al*., 2009).

5.5.4 Effects on β-oxidation

The results from RT-qPCR analysis provide preliminary insights how treatment with diLaLA induced a weight reducing effect through upregulation of key genes involved in β-oxidation of FA in the liver in DIO mice on HFD (Figure 25). Although the results from gene expression analysis were not tested for statistically significant effect due to limited number of replicates, the results measured as fold change clearly showed upregulation of peroxisome proliferator activated receptor (PPAR)-α, FA translocase Cd 36, and carnitine palmitoyltransferase (CPT)-1 in DIO-250 mice, compared with the control. Upregulation of PPAR-α can trigger target genes involved in rate limiting steps in mitochondrial FA uptake for the FA β-oxidation pathway, such as Cd 36 and CPT1 (Burri *et al*., 2010).

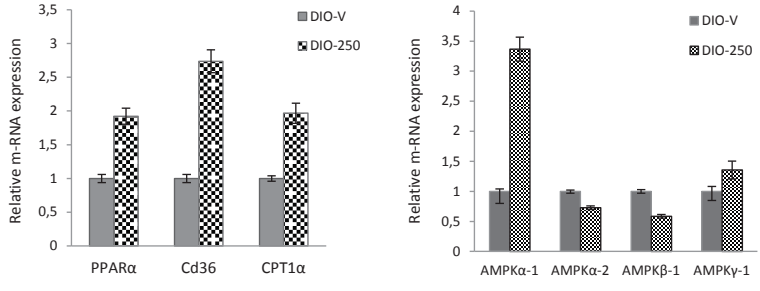

Figure 25. Relative expression of genes analyzed by RT-qPCR in liver samples of DIO-250 (n = 3; r = 2) presented as fold change, compared with the control DIO-V (n = 2; r = 2). Abbreviations: DIO-250, DIO mice treated with 1,3-dilauroyl-2-lipoyl-*sn*-glycerol (diLaLA) 250 mg/kg body wt. daily for 6 weeks; DIO-V, control treated only with vehicle; PPARα, peroxisome proliferator-activated receptor alpha; Cd36, fatty acid translocase (cluster of differentiation 36); CPT1α, carnitine palmitoyltransferase 1 alpha, and different isomers of AMPK, adenosine monophosphate activated protein kinase.

At the same time, isoforms of Adenosine monophosphate-activated protein kinase (AMPK), -α1 and -γ1 were also upregulated whereas -α2 and -β1 were down regulated in liver samples of DIO-250 mice compared with the control (Figure 25). AMPK is known as a cellular energy sensor and a key regulator in hepatic energy metabolism. Hepatic AMPK can limit anabolic pathways and facilitates catabolic pathways to increase ATP production. Activation of AMPK increase FA oxidation, reduces hepatic lipogenesis and modulates

transcription of genes involved in lipogenesis and mitochondrial biogenesis (Viollet *et al*., 2009).

The low levels of hepatic FFA and TAG along with reduced SCD1 activity observed in diLaLA treated mice showed an overall lipogenic suppression effect induced by diLaLA in the liver. According to Dobrzyn *et al*. (2005) this lipogenic suppression via reduced SCD1 activity is supported by enhanced expression of AMPK. Their study demonstrated that metabolic changes in SCD1-deficient mice (SCD-/-) were due to higher energy expenditure induced by AMPK activation (Dobrzyn *et al*., 2005). The authors further explained that activation of AMPK decreases malonyl-CoA levels in the liver, consequently stimulating CPT1 activity and increasing FA transport to mitochondria for β-oxidation. The results in this thesis also show upregulation of AMPK isoform -α1 and -γ1 and CPT1 in DIO-250 mice compared with the control. However, AMPK isoforms α2 and β1 were downregulated in DIO-250 mice compared with the control. It has been shown that AMPK-α2 is associated with FA oxidation mediated by leptin through nervous stimulation via the brain (Dobrzyn *et al*., 2005). According to Kim *et al*. (2004), the LA mediated anti-obese effect observed in rodents was due to enhanced expression of AMPK in peripheral tissues which was mediated by leptin independent manner. It should be noted that, pure LA can reduce plasma FFA and TAG via AMPK dependent and independent manner (Park *et al*., 2008). Moreover, replacing dietary TAG by 1,3-DAG in mice also resulted in upregulation of enzymes involved in β-oxidation, such as acyl-CoA oxidase and medium-chain acyl-CoA dehydrogenase (Murase *et al*., 2002).

5.6 Final remarks

This thesis demonstrates that the novel designed TAG molecule (diLaLA), a conjugate of two naturally existing molecules 1, 3-dilaurin and R-(+)-LA significantly reduced body weight and FFA concentration in both plasma and liver in DIO mice. These novel derivatives were completely soluble in vegetable oil such as rapeseed oil, in room temperature showing relatively higher solubility than the initial compounds, which occur as a powder. A previous study, which tested 0.3% (wt./wt.) racemic LA mixed with medium chain TAG oil in a diet (10% fat) given to hamsters for 4 weeks did not observe any effect on body weight (Wollin *et al*., 2004). The authors suggested that their results might be due to low solubility of LA powder in TAG oil, probably leading to poor bioavailability. Therefore improved solubility in oils is important in potential applications as functional lipids or supplement

because it facilitates uniform blending, consistent composition and effectiveness.

The solubility of novel TAG molecule may also depend on the fatty acids esterified in the glycerol backbone. It should be noted that the novel derivative, diLaLA investigated for anti-obesity effects in this thesis, constituted of C12:0. Naturally 1,3-DAG may constitute of any common fatty acid such as C18:1, as it was used to synthesize for the first time, in the beginning of this study (Paper II). According to previous research, fatty acid composition of DAG molecule would not affect the weight reducing properties associated with 1,3-DAG (Murase *et al*., 2001; Murase *et al*., 2002). However, fatty acid composition may affect the stability of the novel derivative. In addition to the advantage of stability, lower cost of production prompted us to continue the study of this thesis (Papers III-V), using diLaLA containing, C12:0.

To our knowledge, there is no naturally occurring TAG molecule which has one or more LA molecule/s esterified in its glycerol backbone. The *in vitro* hydrolysis of diLaLA tested as a preliminary screening of intestinal digestion mediated by pancreatic lipase showed it can be completely digested, releasing monolipoate, C12:0 and LA. However, it should be noted that the conditions used in this single-step *in vitro* experiment were far from actual *in vivo* conditions in the gastrointestinal tract of mice. In live conditions, diLaLA might be subjected to acidic pH and other biochemical conditions in the stomach, which might result in some prior hydrolysis of the novel TAG molecule before digestion by lipase. Considering these factors, the observed effects of diLaLA can be due to both 1,3-DAG and LA.

It should be noted that according to previous studies, 1,3-DAG is required in relatively higher amounts (g amounts), replacing dietary TAG to show a significant *in vivo* effect on energy expenditure and reduce fat accumulation, compared with pure LA supplemented in relatively small amounts (mg amounts). However, which initial constituent in diLaLA molecule caused this weight reducing effect at molecular level, is difficult to speculate at this stage since we don´t know how this novel TAG molecule is digested in the gastrointestinal tract in live animals. Nevertheless, the results from previous studies conducted with pure 1,3-DAG and LA separately provide some idea of how the observed outcomes could have been mediated by diLaLA.

Nevertheless, the results presented in this thesis are consistent with previous studies, which showed similar effects by dietary supplementation of pure LA in rodents (Kim *et al*., 2004; Park *et al*., 2008; Prieto-Hontoria *et al*., 2009; Wang *et al.*, 2010). It is of interest to note that Kim *et al*. (2004) tested dietary supplementation of LA at 0.25, 0.5 and 1.0% (wt./wt.) levels corresponding to approx. 150, 300 and 600 mg pure LA/kg body wt., respectively. A similar

high dose of LA *i.e*. 500 mg/kg, was used in C57BL/6J mice treated for four weeks by Wang *et al*. (2010). In contrast, mice studied in this thesis were treated with oral doses of diLaLA, which contained only 32% LA based on the molecular weight of diLaLA. Thus the doeses at 10, 50 and 250 mg diLaLA/kg body wt. tested in this thesis, may correspond to 3, 16 and 80 mg of LA/kg body wt., respectively. The results in this thesis, show that diLaLA is effective in mediating a significant reduction in body weight and FFA concentration in HFD diet induced obese mice. This effect was found at the dose level 250 mg diLaLA/kg body wt. which could be probably a relatively low dose level of LA, compared with previous studies in rodents treated with pure LA, indicating possible synergistic effect of this novel derivative. Probably this indication of enhanced effect might be due to 1,3-DAG in addition to LA. It has been shown that TAG in the diet replaced by 1,3-DAG, enhanced energy expenditure, reduced fatty acid synthesis in liver, fat accumulation and body weight as mentioned previously in this thesis (Maki *et al*., 2002; Murase *et al*., 2001; Murase *et al*., 2002; Rudowska *et al*., 2005; Yanai *et al*., 2010). Further, it could also be due to increased solubility and stability of the novel conjugate which might have improved bioavailability, as discussed previously.

6 Main findings

- A mild esterification method to synthesize PS and 1,3-DAG derivatives with LA, resulting in approx. 60 and 80% of final products with a higher solubility in vegetable oils, than initial compounds.

- The *in vitro* free radical scavenging capacity of novel DHLA derivatives of PS and 1,3-DAG were comparable to that of pure DHLA, as evaluated by DPPH method under test conditions.

- Pancreatic lipase mediated *in vitro* hydrolysis of diLaLA resulted in complete digestion, releasing monolipoate, free C12:0, and LA under tested conditions.

- Free LA was detectable in plasma samples of mice 2 h after oral treatment with diLaLA.

- Oral treatment with 250 mg diLaLA/kg body weight daily for 6 weeks, significantly reduced body weight gain, and plasma and hepatic FFA concentrations in DIO mice on HFD.

- Oral treatment with 250 mg diLaLA/kg body weight daily for 6 weeks also lowered the SCD1 activity, estimated by SCD16 index in white adipose tissues, while inducing expression of some key genes involved in β-oxidation of fatty acids in the liver of DIO mice.

- The novel derivative, diLaLA reduced body weight in DIO mice possibly through enhanced energy expenditure via β-oxidation and suppressing *in vivo* lipogenesis, suggesting its potential in alleviating some risk factors associated with obesity.

7 Future research

- Phytosterol lipoates need to be investigated for their potential cholesterol lowering effects in animals.

- An *in vivo* study is important to monitor the pharmacokinetic and pharmacodynamic parameters and evaluate the relative bioavailability of orally administered diLaLA.

- The effects of diLaLA on expression of genes involved in FA β-oxidation and lipogenic enzymes such as SCD1 needs to be further investigated.

- A long term study to evaluate whether diLaLA mediated anti-obesity effects are maintained long term.

- A toxicological study to investigate possible adverse effects of long term oral treatment with diLaLA.

- Identification of possible digestion products of diLaLA in *in vivo* conditions and their metabolites in plasma and urine after oral administration would be of interest.

- Anti-obesity effect of similar LA derivatives with 1,3-DAG, containing fatty acids other than C12:0 would also be of interest.

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