Proposed mechanisms of action of serum triglyceride lowering by plant sterols and stanols

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Abstract

Plant sterols and their saturated derivates called stanols (PS) are structurally very similar to cholesterol. Due to this similarity, PS compete with dietary cholesterol for absorption in the small intestine. Consequently, a daily intake of 2 gram PS causes an average reduction of 9% in serum LDL-cholesterol. Recently, it has been recognized that PS also lower fasting serum triglycerides (TG) by 6% on average. Moreover, it was shown that PS lowering of TG is higher in metabolic syndrome patients with high baseline TG levels. However, the mechanism by which PS lower fasting serum TG is currently unknown and in this thesis, a number of potential mechanisms are proposed. To that end, cholesterol and TG homeostasis were reviewed. Given the stronger TG lowering by PS in metabolic syndrome patients, the role of insulin resistance was reviewed as well. TG and cholesterol are assembled and secreted into the circulation by the liver in very-low-density lipoprotein particles (VLDL). It has been suggested that fasting serum TG lowering by PS is associated with reduced numbers of circulating VLDL particles. Hepatic VLDL assembly and secretion depends on hepatic cholesterol and TG content. The latter is determined by free fatty acid (FA) flux, dietary fat intake and *de novo* lipogenesis (DNL). Insulin resistance causes increased VLDL secretion. Here, the following hypotheses on the mechanism of fasting serum TG lowering by PS are proposed: 1) PS reduce intestinal absorption of dietary FA, 2) PS reduce hepatic uptake of chylomicron remnants and/or reduce DNL, 3) Reduced VLDL secretion is an indirect effect of cholesterol lowering by PS, 4) PS reduce levels of proteins involved in assembly and secretion of VLDL, 5) PS have an anti-inflammatory effect on activated Kupffer cells, reducing the expression of TNFα and IL-6 and alleviating IR-induced hypersecretion of VLDL. *In vitro* experiments to test these hypotheses are proposed.

Abbreviations

ABCA1	=	ATP-binding cassette hemi-transporter A1
ABCG1	=	ATP-binding cassette hemi-transporter G1
ABCG5/G8	=	ATP-binding cassette hemi-transporter G5/G8
ACC	=	acyl CoA carboxylase
ACAT	=	acyl CoA cholesterol acyl transferase
apo	=	apolipoprotein
BSEP	=	bile salt export pump
CE	=	cholesterol ester
CETP	=	cholesterol ester transfer protein
ChREBP	=	carbohydrate responsive element binding protein
CM	=	chylomicron
CMR	=	chylomicron remnant
CVD	=	cardiovascular disease
DNL	=	*de novo* lipogenesis
EAS	=	European atherosclerosis society
ELOVL6	=	long chain elongase
ER	=	endoplasmic reticulum
FA	=	fatty acid
FAS	=	fatty acid synthase
FXR	=	farnesoid x receptor
GPAT	=	mitochondrial glycerol 3-phosphate acyltransferase
HDL	=	high density lipoprotein
HL	=	hepatic lipase
HMG-CoA	=	enzyme 3-hydroxy-3-methylglutaryl coenzyme A
IDL	=	intermediate density lipoprotein
IR	=	insulin resistance
IRS	=	insulin receptor substrate
IKK	=	inhibitor of kappa beta kinase
LCAT	=	lecithin-cholesterol acyltransferase
LDL	=	low density lipoprotein
LDL-R	=	LDL receptor
LRP1	=	LDL-R-related protein-1
LPL	=	lipoprotein lipase
LXR	=	liver x receptor
MTP or MTTP	=	microsomal transfer protein
NAFLD	=	non-alcoholic fatty liver disease
NEFA	=	non-essential free fatty acids
NPC1L1	=	Niemann-Pick C1-like 1 protein
PCSK9	=	proprotein convertase subtilisin/kexin type 9
PI3K	=	phosphatidylinositide 3-kinase
PIP3	=	phosphatidylinositide (3,4,5) triphosphate
PL	=	phospholipids
PS	=	plant sterols and stanols and/or their esterified derivatives
SCAP	=	SREBP cleavage-activating protein
SCD1	=	stearoyl-CoA desaturase 1
SRB1	=	scavenger receptor B1
SREBP	=	sterol regulatory response element binding protein
TG	=	triglyceride
TICE	=	trans intestinal cholesterol excretion
TORC1	=	target of rapamycin complex 1
UPR	=	uncoupled protein response
VLDL	=	very low density lipoprotein

Contents

1. Introduction

Plant sterols are well known for their ability to lower serum levels of pro-atherogenic, low-density-lipoprotein (LDL)-cholesterol (Demonty *et al.* 2009). In addition, the potential of plant sterols to lower serum triglyceride (TG) levels, particularly in metabolic syndrome patients, is becoming recognized (Naumann *et al.* 2008, Demonty *et al*. 2013). However the mechanism by which plant sterols lower TG is unknown. In this thesis, the main biological processes underlying cholesterol lowering by plant sterols, cholesterol homeostasis and TG homeostasis in relation to lipoprotein particles were reviewed. From that, a number of hypotheses were generated which propose mechanisms of action of the serum TG lowering effect of plant sterols.

2. Plant sterols and stanols

2.1 Plant sterols and stanols

Analogous to cholesterol in animals, plants contain sterols and their chemically saturated counterparts, plant stanols. Both plant sterols and plant stanols are essential for many aspects of plant physiology like embryonic development, cell growth and proliferation and cellulose synthesis (Schrick 2002 *et al.*, Schrick 2004 *et al.*, Willemsen *et al.* 2003). Plant sterols and stanols are structurally very similar to cholesterol and differences are limited to the short aliphatic side chain, as shown in Figure 1. Plant sterols and stanols are naturally present in vegetable oils, grains, nuts, seeds, fruits and vegetables (Marangoni 2010 *et al*.) and consequently, they are normal components of the human diet. Depending on the population, the average daily intake of plant sterols is 167–437 mg, which is comparable to the intake of cholesterol. Stanol intake is estimated to be about 10% lower (Ostlund *et al.* 2002). The most prominent dietary plant sterols are sitosterol, campesterol and stigmasterol. The main stanols are sitostanol and campestanol (Ostlund *et al.* 2002). Plant sterols and stanols can be esterified to long chain fatty acyl (FA) to form plant sterol or stanol esters. These have superior solubility in hydrophobic food matrices like margarines. For simplicity, unless otherwise stated, free and esterified plant sterols and stanols are collectively denoted PS from here on.

2.2 PS lower serum LDL-cholesterol

Due to their structural similarity, PS compete with cholesterol for integration into intestinal mixed micelles and consequently for absorption by the small intestine (reviewed in Lecerf *et al.* 2011). This mechanism underlies the well established serum cholesterol lowering effect of PS (Demonty *et al.* 2009). Currently, PS are recognized as an efficacious active in cholesterol lowering functional foods. The most recent meta-analysis of 84 human trials studying the effect of PS on serum cholesterol concluded that an average daily PS intake of 2.15 gram reduces serum LDL-cholesterol with 8.8% (-0.34 mmol/L) (Demonty *et al.* 2009). In this analysis, no statistically significant differences in LDL-cholesterol lowering between plant sterols, stanols, or their esterified derivates were identified. In addition, no differences were detected between fat versus non-fat food matrices. Preparations in these studies often contain a mix of (esterified) sitosterol, campesterol and stigmasterol or sitostanol and campestanol. Extensive safety evaluation and post-launch monitoring programmes have demonstrated that PS are safe for human consumption and are used safely (Ayesh *et al.* 1999, Baker *et al*. 1999, Hepburn *et al.* 1999, Waalkens-Berendsen *et al.* 1999, Weststrate *et al.* 1999, Sanders *et al.* 2000, Wolfreys *et al.* 2002, Lea 2004 *et al.*, Lea *et al.* 2006, Willems *et al.* 2013).

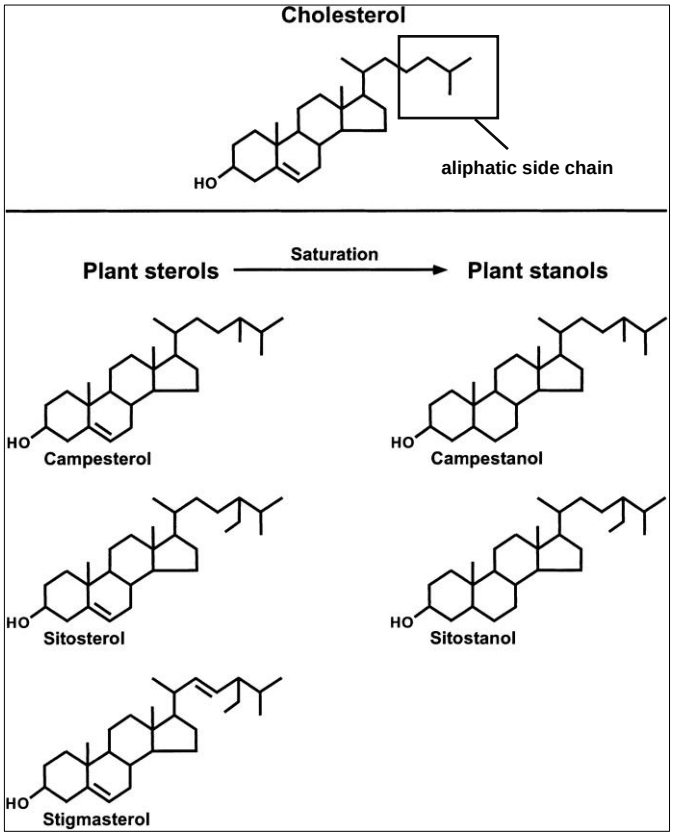

Figure 1 | The molecular structure of cholesterol and the main plant sterols and stanols. Sterols and stanols differ in the structure of their aliphatic side chain. Stanols are saturated derivatives of sterols (adapted from Kerckhoffs *et al*. 2002)

Cardiovascular disease (CVD) is presently the principal cause of human mortality and the major strategy for CVD is to lower serum LDL-cholesterol via statin therapy (Baigent *et al.* 2010). A substantial number of human studies has demonstrated that LDL-cholesterol lowering by dietary intervention may be an important means to reduce CVD events risk (Robinson *et al.* 2005). Therefore, the LDL-cholesterol lowering activity of PS has resulted in recommendations by the joint European society of cardiology (ESC) and the European atherosclerosis society (EAS) task force to consume PS as part of a healthy diet for the dietary management of dyslipidaemias and cardiovascular disease prevention (Catapano *et al.* 2011, Perk *et al.* 2012). The 2013 EAS consensus panel on PS appraised functional foods containing PS as part of a healthy lifestyle to lower serum LDL-cholesterol and thereby lower CVD risk (Gylling 2013). However, the most recent guidelines on lipid management and lifestyle changes by the American College of Cardiology (ACC) and American Heart Association (AHH) task force did not include PS (Stone *et al.* 2013).

2.3 PS absorption, metabolism and excretion

Intestinal absorption of PS is low, ranging from 4.2 – 13%, depending on the type of PS (Heinemann *et al.* 1993). Much of absorbed PS is directly excreted back into the intestinal lumen by enterocytes via the heterodimeric ATP-binding cassette hemi-transporter G5/G8 (ABCG5/G8) and PS are cleared from the circulation through biliary excretion. Mutations in the ABCG5/G8 gene can cause an inability of enterocytes to pump out PS, leading to highly elevated levels of plasma PS, a pathogenic condition called sitosterolemia (reviewed in Othman *et al.* 2013). In healthy humans, steady state PS plasma concentrations range from 3.8 – 16 μM for sitosterol and from 6.9 – 28 μM for campesterol (Chan *et al.* 2006). For plasma stanols, plasma concentrations range from 0.05 – 0.3 μM (Gylling *et al.* 2010). PS can be incorporated into lipoprotein particles in cells and are transported by lipoproteins in plasma. Therefore, in the following sections cholesterol homeostasis, lipoprotein metabolism, TG homeostasis are reviewed in order to identify potential biological targets for serum TG lowering by PS.

3. Lipoprotein cholesterol metabolism

3.1. Cholesterol homeostasis

3.1.1 Cholesterol

Cholesterol is an essential molecule for physiological homeostasis in animals. It provides rigidity to cell walls and is a chemical precursor for endogenous hormones, vitamin D and bile acids (Lecerf *et al.* 2011). Humans absorb cholesterol from the diet and are able to synthesize it *de novo*. Cholesterol absorption, synthesis and excretion are tightly regulated to control the circulating cholesterol pool. These processes are presented in Figure 2 and will be further discussed in the following sections.

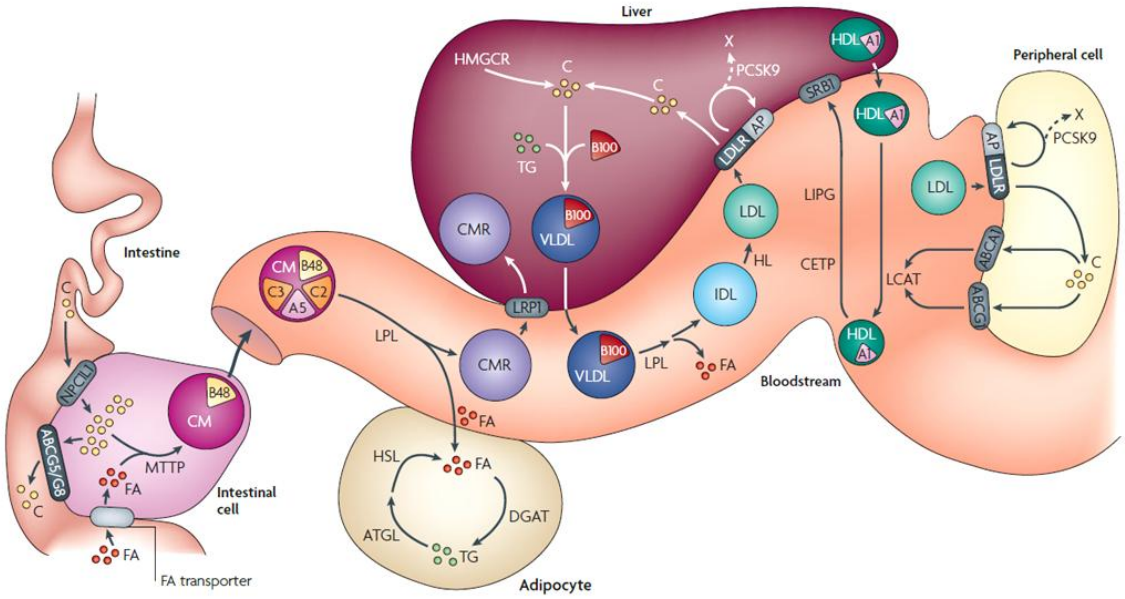

Figure 2 | Lipoprotein metabolism and circulation (from Hegele *et al*. 2009). Details are discussed in the text.

3.1.2. Absorption, synthesis and enterohepatic circulation of cholesterol

Humans ingest 300 mg of dietary cholesterol daily on average (Ostlund *et al.* 2002). The degree of cholesterol absorption depends on genetic and dietary factors as well as on metabolic health. Overall, in healthy persons consuming a moderately low cholesterol diet, the absorption coefficient ranges from 20 – 80% (Sudhop *et al.* 2002, Clarenbach *et al.* 2006). Upon absorption in the small intestine, cholesterol is transported to the liver via lymph and blood in chylomicron lipoproteins which will be discussed in a following section. The majority of the bodies' total cholesterol pool, about 75%, is derived from endogenous synthesis in the liver. Hepatic cholesterol is excreted in very-low-density lipoprotein (VLDL) particles for transport to tissues. Humans cannot degrade cholesterol molecules, but cholesterol is the precursor in bile acid synthesis and excess cholesterol can be cleared via bile acid synthesis. In addition, cholesterol is excreted as a bile constituent via liver-biliary and subsequent faecal excretion (Lecerf *et al.* 2011).

A reduction in exogenous cholesterol intake readily induces changes in absorption and synthesis. When dietary cholesterol intake is high, the relative absorption decreases, but absolute absorption increases (Ostlund *et al.* 1999). Reduced cholesterol absorption causes an increase in endogenous synthesis (Lecerf *et al.* 2011). So, Absorption and synthesis of cholesterol are inversely correlated. In obese individuals, cholesterol synthesis is increased, which causes increased excretion of biliary cholesterol. The larger biliary cholesterol pool competes with exogenous cholesterol and PS for absorption which reduces the cholesterol absorption efficiency (Miettinen *et al.* 2000, Simonen *et al.* 2000).

3.1.3. Key proteins in cholesterol absorption, synthesis and excretion

In the lumen of the small intestine, lipophilic molecules like dietary cholesterol, biliary cholesterol, plant sterols, dietary FA and bile acids associate into mixed micelles. Micellar sterols are taken up by transporter proteins in the brush border of intestinal cells. In humans, Niemann-Pick C1-like 1 protein (NPC1L1) is the major protein involved in absorption of sterols. Plant sterols may inhibit cholesterol absorption via NPC1L1 (Lecerf *et al.* 2011). Endogenous cholesterol synthesis takes place via the mevalonate pathway. In this complex, multistep pathway, the formation of mevalonate by the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is the rate limiting step. Excretion of cholesterol and plant sterols from hepatocytes into the bile occurs via the ABCG5/G8 transporter.

3.1.4. Treatment of elevated serum LDL-cholesterol

In addition to dietary factors, genetic factors affect serum LDL-cholesterol levels and contribute to inter-individual variation. Elevated serum LDL-cholesterol increases CVD risk (reviewed in Stone *et al*. 2013). Hypercholesterolaemic patients can be effectively treated with statins which are inhibitors of HMG-CoA reductase and hence of cholesterol synthesis. In a 2013 Cochrane database systematic review, it was concluded that statins reduce all-cause mortality, major vascular events and revascularisations without excess of adverse events. Moreover, this also applied to individuals for which the risk of a major cardiovascular event was less than 1% (Taylor *et al.* 2013). However, currently, statins are not an over the counter drug, except in the UK, and therefore can often not be used as a non-prescription CVD prophylactic. In addition, in some patients, highest non-toxic dosages of statins lower LDL-cholesterol insufficiently (Nijjar *et al.* 2010). In that case, regular PS intake as part of a healthy diet has been shown an effective additive therapy (Scholle *et al.* 2009, reviewed in Gylling 2013).

3.1.5. Reverse cholesterol transport

Cholesterol in peripheral tissues can be exported via the ABCA1 and ABCG1 transporters to apolipoprotein A1 (apoA1) to generate nascent high-density lipoprotein (HDL). Cholesterol in HDL is esterified by lecithin-cholesterol acyltransferase (LCAT) which produces cholesterol ester (CE). Cholesterol ester transfer protein (CETP) facilitates the transport of CE from HDL to VLDL and LDL and TG from VLDL and intermediate-density lipoprotein (IDL) to HDL. TG rich HDL is preferentially cleared from the circulation which causes lower HDL plasma concentrations in patients with high plasma TG levels. Clearance of HDL-cholesterol by the liver occurs via scavenger receptor B1 (SRB1). The transport of cholesterol from peripheral tissues to the liver is called reverse cholesterol transport (reviewed in Navab *et al*. 2011).

3.2. Lipoprotein particle assembly and metabolism

3.2.1. Lipoprotein particles

Lipophilic cholesterol and TG molecules are insoluble in lymph and plasma. Therefore, they are packaged in spheroidal multi-molecular complexes called lipoprotein particles, or lipoproteins. These consist of a hydrophobic core, containing CE and TG and a monolayer surface containing free cholesterol, phospholipids (PL) and apolipoproteins. Apolipoproteins (combinations thereof) are lipoprotein specific. They provide a scaffold for lipoprotein assembly. In addition, they function as markers that regulate receptor mediated uptake by the liver or other tissues and regulate the activity of enzymes that metabolize or transfer lipids. An overview of lipoproteins and their composition is given in Table 1. They are classified mainly by their size and density and are referred to as chylomicron (CM) lipoprotein, very-low density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL), which are discussed in a following section. Notably, as depicted in Figure 3, the reduction in density and size from VLDL to HDL is rather a continuous than a stepwise process and many lipoprotein subclasses exist.

Table 1 | Main lipoprotein particles and their composition (from Hegele *et al*. 2009).

Lipoprotein Class	Triglyceride (% by weight)	Phospholipid (% by weight)	Free cholesterol (% by weight)	Esterified cholesterol (% by weight)	Protein (% by weight)	Main apolipoproteins
Chylomicrons	80 - 95	3 - 6	1 – 3	2 - 4	1 - 2	A-I, A-IV, A-V; B-48, C-I, C-II, C-III, E
VLDL	45 - 65	15 - 20	4 – 8	16 - 22	6 - 10	B-100, E, C-I, C-II, C-III
LDL	4 - 8	18 - 24	6 – 8	45 - 50	18 - 22	B-100
HDL	2 - 7	26 - 32	3 – 5	15 - 20	45 - 55	A-I, A-II, E

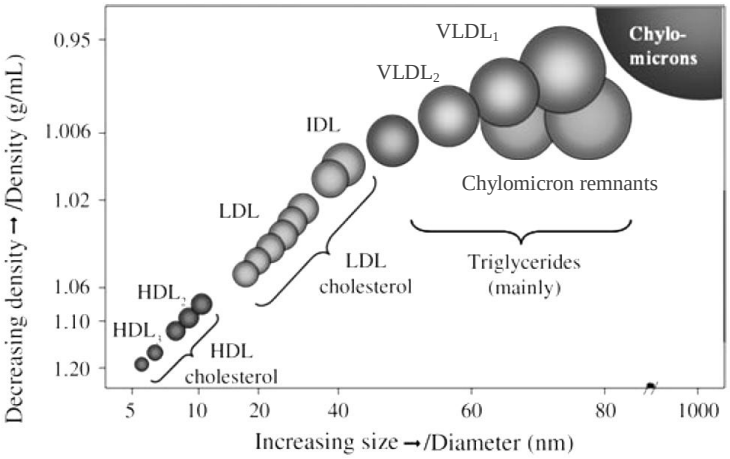

Figure 3 | Size and density of lipoprotein subclasses the reduction in density and size from CM to VLDL to IDL to HDL is rather a continuous than a stepwise process and many lipoprotein subclasses exist (adapted from Frazier-Wood *et al.* 2012).

3.2.2. Lipoprotein assembly and secretion by enterocytes

Cholesterol absorbed by enterocytes is esterified by the enzyme acyl CoA cholesterol acyl transferase (ACAT). Both free cholesterol and esterified cholesterol are incorporated into CM lipoproteins with apolipoprotein B48 (apoB48). Microsomal transfer protein (MTP) transfers TG into CM in the endoplasmic reticulum (ER). TG is synthesized from free FA derived from dietary TG or *de novo* synthesis. Next, CM are excreted into the mesenteric lymph and, via the thoracic duct, enter the bloodstream. There, CM are metabolized by capillary lipoprotein lipase (LPL) which hydrolyses the TG to produce free FA, which are used as an energy source by tissues, or stored in adipose tissue. The remaining CM remnants (CMR) are taken up by the liver via the LDL-R and/or LDL-R-related protein-1 (LRP1). CMR apoB48 and/or apoE are ligands of LDL-R and LRP1 (Lecerf *et al.* 2011).

3.2.3. Lipoprotein assembly and secretion by hepatocytes

Assembly of VLDL takes place in the liver and is initiated by the expression of apoB100 on ribosomes. One single apoB100 protein is integrated into each VLDL lipoprotein and the amount of apoB100 is a measure of VLDL particle number. Novel apoB100 translocates via the secretory pathway into the ER. There, nascent apoB100 acquires membrane phospholipids, TG from lipid vesicles and cholesterol esters via the microsomal transfer protein (MTP) (Choi *et al.* 2011, Sparks *et al.* 2012). Gradually, medium size VLDL-2 particles are constructed which can serve as precursors to large VLDL-1 (Stillemark-Billton *et al.* 2005). Switching of VLDL-2 to VLDL-1 takes place by fusion with larger TG-vesicles that often contain stabilizing apoC3 (Wang *et al.* 1999, Yao *et al.* 2012). Upon maturation, VLDL is secreted into the bloodstream. Hydrolysis of TG in VLDL-1 by LPL releases free FA and thus VLDL1 becomes VLDL-2. Further hydrolysis of VLDL-2 yields IDL which is processed by hepatic lipase (HL) to further reduce TG content and thus generate LDL. LDL is taken up by the liver and peripheral tissues via the LDL-R.

4. Serum triglyceride lowering by PS

4.1.Serum triglycerides

4.1.1. Clinical relevance of serum triglyceride levels

Interventional studies with both dietary and pharmacological (i.e. statins) interventions have shown a direct effect of reduced serum LDL-cholesterol on improvements in CVD end-points (Taylor *et al.* 2013). So far, most data on the effect of serum TG on CVD risk has come from observational studies showing that increased TG are associated with increased CVD risk (reviewed in Miller *et al.* 2011). Nonetheless, in the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study it was shown that TG lowering by the PPARa agonist fibrate causes an additional CVD risk reduction to LDL lowering by statins (Keech *et al.* 2005, Robinson *et al.* 2007). Increased fasting serum TG levels (>1.7 mmol/L) are viewed as an independent risk factor for CVD. The latest recommendations from the American Heart Association (AHH) and the American Association of Clinical Endocrinologists (AACE) are that TG lowering strategies may be applied in individuals at risk (Miller *et al.* 2011, Jellinger *et al.* 2012).

4.1.2. PS lower fasting serum triglycerides

While the serum LDL-cholesterol lowering activity of PS is well established, effects on serum TG levels have long been under scientific debate. Some investigators considered that PS have a neutral effect on plasma TG. They attributed the minor TG lowering effect observed in studies with PS to other (dietary) factors (Derdemezis *et al.* 2010). However, in 2008, a meta-analysis by Naumann *et al*. identified a significant inverse correlation between plant stanol ester intake and fasting serum TG concentrations. TG lowering was most profound in subjects with high baseline TG levels (Naumann *et al.* 2008). Earlier studies on the LDL-cholesterol effect of PS often excluded subjects with high baseline TG levels, e.g. above 1.7 mmol/L (Miller *et al.* 2011). Meta-analyses including these studies therefore resulted in an underestimation of the TG effect of PS. This might explain why the effect had been unnoticed for long (Plat *et al.* 2012).

Accordingly, a pooled analysis of individual subject data from 12 trials concluded that intake of 1.6–2.5 g plant sterols per day lowers fasting serum TG by 6.0% (0.12 mmol/L) on average. A significant correlation between baseline TG values and absolute reductions was found, as presented in Figure 4 (Demonty *et al.* 2013). The similar findings with plant stanols and sterols between the two meta-analyses indicate the robustness of the effect. The above analyses included individuals with relatively high TG baseline concentrations. However, dyslipidaemic metabolic syndrome patients with even higher serum TG were not included. Studies with 2–4 g PS/day in these patients showed profound TG lowering of 19–28% (0.23 to 0.4 mmol/L) (Plat *et al.* 2009, Sialvera *et al.* 2012).

4.1.3. Serum triglyceride lowering by stanol esters is associated with reduced large VLDL-1

A study by Plat *et al.* has provided clues towards the potential mechanism of TG lowering by plant stanol esters. The effect on the distribution of serum lipoprotein subclasses in dyslipidaemic and in normolipidaemic subjects was determined by NMR (Plat *et al.* 2009). In metabolic syndrome patients, plant stanol esters reduced fasting serum TG concentrations by 27.5% (0.23 ± 0.36 mmol/L) compared to the control group. Notably, PS significantly reduced serum levels of TG-rich large VLDL-1 (-57%, -3.8 ± 6.7 nmol/L) and medium size VLDL-2 (-25%, -9.1 ± 17.7 nmol/L). In the normolipidaemic subjects, no significant effects of plant stanols on serum TG were detected. However, a 28% reduction in VLDL-1 (-0.7 ± 3.1 nmol/L) was observed in the plant stanol group. These results suggest that the serum TG lowering effect of PS is associated with reduced serum levels of the largest, TG-rich lipoprotein particle VLDL-1 and, to a lesser extent, medium size VLDL-2. Of note, in this study no effect on CETP activity was found and serum HDL-cholesterol levels were unaltered. Accordingly, in the meta-analysis of Demonty and colleagues, no PS effect on HDL-cholesterol was observed. Therefore, it seems unlikely that TG lowering by PS is caused by unfavourable CETP-mediated remodelling of VLDL (Demonty *et al.* 2013).

Together, studies indicate that PS can offer a combined benefit of lowering serum LDL-cholesterol and TG. Although the TG lowering by PS in normolipidaemic individuals is modest, it appears to be more substantial in metabolic syndrome patients with high baseline TG levels. Thus, the effect seems strongest in subjects that would presumably benefit the most. Since VLDL is the main lipoprotein transporting TG from the liver to the tissues, it makes good sense that a reduction in serum TG is reflected by a reduction in VLDL. Production and secretion of VLDL depends on hepatic TG content and on assembly via apoB100, MTP and apoC3. The following section gives an overview of these factors, particularly in the context of insulin resistance (IR), a characteristic of the metabolic syndrome which is associated with hepatic inflammation.

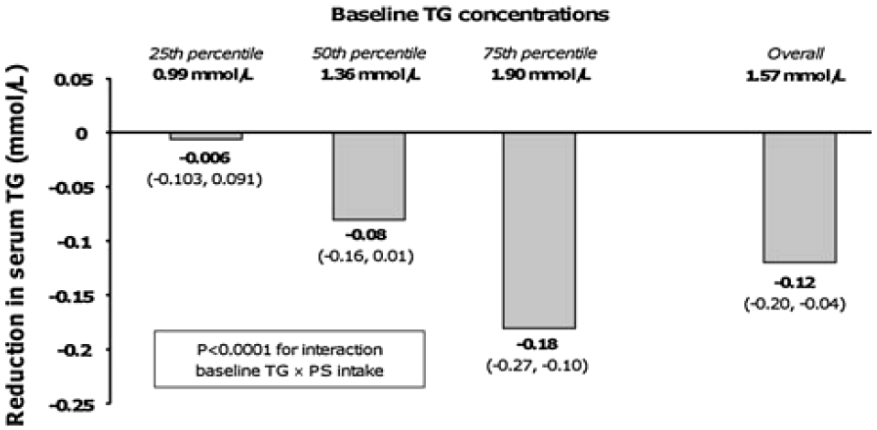

Figure 4 | Absolute reductions in serum TG by PS correlate positively with TG baseline levels (from Demonty *et al.* 2013).

4.2. Hepatic triglyceride content

4.2.1. Hepatic triglyceride in health and in insulin resistance

VLDL is assembled and secreted by the liver for delivery to peripheral tissues and hepatic TG levels are the major determinant of hepatic VLDL secretion (Fabbrini *et al.* 2009). The hepatic TG content depends on circulating levels of free FA, dietary fat intake and *de novo* lipogenesis (DNL). Liver TG metabolism is regulated by insulin. In the healthy state, insulin suppresses VLDL secretion. One of the symptoms of IR, characteristic of metabolic syndrome and type 2 diabetes mellitus, is impaired insulin suppression of VLDL secretion, resulting in increased serum TG levels and lower HDL levels. Notably, Sørensen *et al.* have shown that a rise in serum VLDL levels goes ahead of defective regulation of glucose levels in normoglycemic, obsese men indicating the onset of IR (Sørensen *et al.* 2011). Thus, in IR, the suppressive action of insulin on VLDL secretion is lost first. This is often associated with the development of non-alcoholic fatty liver disease (NAFLD) (reviewed in Smith *et al.* 2011). In a study that employed stable isotope labelling, it was found that in obese metabolic syndrome patients with NAFLD, fasting hepatic TG was derived from 59% non-essential (adipose tissue derived) free FA (NEFA), 26% DNL and from 15% dietary FA. This distribution also applied to TG in VLDL. Liver TG synthesis inversely employed NEFA and dietary FA (Donnelly *et al.* 2005). A similar distribution was found in a study with healthy individuals in the fasting state, but with a smaller contribution of DNL and dietary FA (Timlin *et al.* 2005). Below, these contributors to hepatic TG are further discussed with a focus of the effect of IR. The determinants of hepatic TG content and related VLDL production and secretion in health and in IR are depicted in Figure 5.

4.2.2. Free fatty acids

Free FA circulate in the blood bound to albumin. They are released by lipase hydrolysis of TG in CM and VLDL lipoproteins and of TG in adipose tissue. The latter is referred to as free FA flux (Nielsen *et al.* 2012). Free FA function mainly as an energy source for tissues. Free FA are taken up by the liver via passive transport and receptor mediated transport (Abumrad *et al.* 2012). Hepatic FA is a substrate for TG synthesis and free FA flux contributes to liver VLDL-TG secretion (Nielsen *et al.* 2012). In IR, impaired insulin suppression of adipose tissue lipolysis is thought to contribute to increased FA flux (Jensen *et al.* 2007). Notably, studies have shown that in IR patients, increased FA uptake can result in increased apoB100 secretion without elevated TG secretion (Zhang *et al.* 2004). This most likely results in smaller, more atherogenic lipoprotein. To date, no effects of PS on adipose tissue lipolysis have been reported.

4.2.3. Hepatic uptake of triglyceride rich lipoprotein remnants

Postprandial TG-rich CM remnants and VLDL remnants are a major source of hepatic TG. They are taken up by the liver via the LDL-R, or LRP1 receptor. Comparable to IR reduced suppression of hepatic VLDL production, IR reduces suppression of CM production in the small intestine similarly (Duez *et al.* 2006). So, in IR, diet-derived TG from CMs is increased. Moreover, IR is associated with reduced lipoprotein lipase activity (LPL), partly via increased secretion of apoC3, an inhibitor of LPL and hepatic lipase (HL). In combination, these effects cause reuptake of increased amounts and larger TG-rich CM and VLDL remnants by the liver. Rideout *et al*. showed that in a study with C57BL/6J mice, PS reduced intestinal FA absorption,

which could be an indication of impaired CM production (Rideout *et al.* 2010). In line with this suggestion, in a study with male Golden Syrian hamsters, β-sitosterol and stigmasterol were shown to reduce MTP mRNA levels (Liang *et al.* 2011).

4.2.4. Hepatic de novo *lipogenesis*

Hepatic *de novo* lipogenesis (DNL) is synthesis of FA in the liver via glucose-derived acetyl-CoA. Lipogenesis contributes substantially to hepatic TG content and hence to hepatic VLDL secretion and is increased in obesity with IR (Diraison *et al.* 2003, Donnelly *et al.* 2005). Main genes involved in lipogenesis are acyl CoA carboxylase (ACC), long chain elongase (ELOVL6), fatty acid synthase (FAS), mitochondrial glycerol 3-phosphate acyltransferase (GPAT) and stearoyl-CoA desaturase 1(SCD1). These genes are under the control of the transcription factors sterol regulatory binding element protein-1c (SREBP-1c) (Postic *et al.* 2008, Choi *et al.* 2011) and the carbohydrate responsive element binding protein (ChREBP) (Dentin *et al.* 2006). Nuclear transport of ChREBP is positively regulated via increased hepatic glucose flux through generation of the signaling molecule xylulose 5-phosphate (Uyeda *et al.* 2006). In addition, expression of both SREBP-1c and ChREBP is activated by the liver X receptor (LXR) through binding to the LXR-response element in their promoter sequences (Schultz *et al.* 2000, Cha *et al.* 2007, Choi *et al.* 2011). LXR is a direct transcription activator of the FAS gene as well (Joseph *et al.* 2002). Insulin induces LXR expression and thereby activating the expression of SREBP-1c, ChREBP and FAS. So, in the postprandial state, DNL is upregulated.

In addition to regulation on a transcriptional level, active SREBP-1c is formed from an inactive precursor, which is regulated by ChREBP, insulin, SREBP cleavage-activating protein (SCAP) and by SREBP-1c itself. Recently, target of rapamycin complex 1 (TORC1) has been identified as a novel regulator of SREBP-1c nuclear transcriptional activity. Phosphorylation by of the protein lipin 1 by TORC1, prevents lipin 1 nuclear translocation and inhibition of SREBP-1c (Peterson *et al.* 2011, Bakan *et al.* 2012). Moreover, cytosolic lipin 1 has phosphohydrolyase activity, facilitating TG synthesis and VLDL catabolism (Bou Khalil *et al.* 2009).

Thus, in metabolic syndrome patients with IR, continued insulin-driven LXR activation, elevated hepatic glucose flux and constitutively higher TORC1 activity increase lipogenesis, providing high levels of TG substrate. Combined with elevated apoB levels, this favors VLDL assembly and secretion (Schultz *et al.* 2000, Chen *et al.* 2004, Cha *et al.* 2007, Postic *et al.* 2008, Choi *et al.* 2011). Notably, several studies suggest that increases in DNL lead to increased VLDL TG content and therefore VLDL size, but not to an increase in VLDL number (Melish *et al.* 1980, Horton *et al.* 1999, Grefhorst *et al.* 2002). Interestingly, PS were shown to reduce hepatic SREPB1c transcription in the apoE3-/- metabolic syndrome mouse model (Calpe-Berdiel *et al.* 2005).

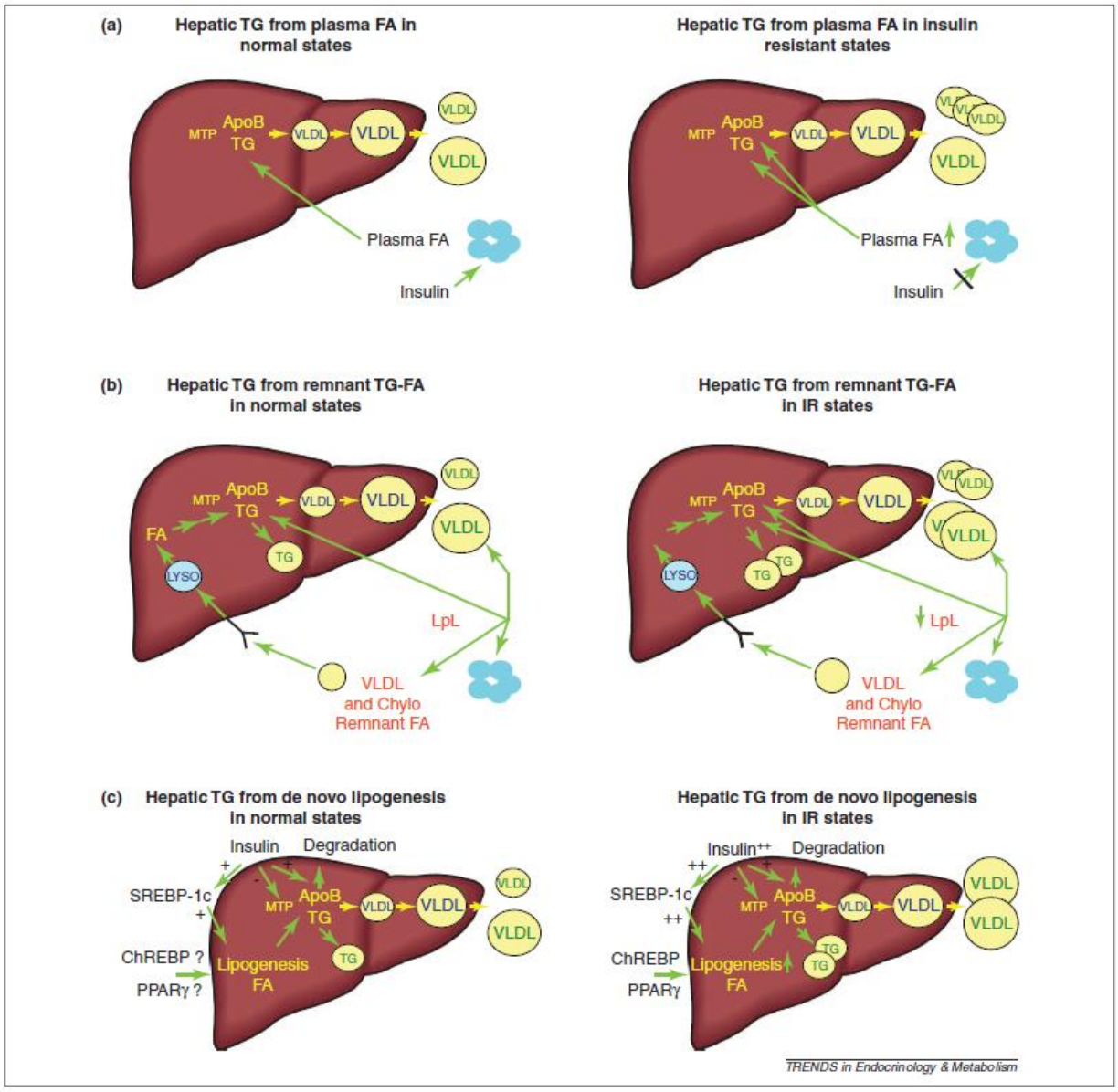

Figure 5 | Determinants of hepatic TG content and related VLDL production and secretion in health and in insulin resistance (from Choi *et al*. 2011).

4.3. Factors regulating VLDL assembly and secretion

VLDL assembly and secretion primarily depends on apoB100. Insulin is the main apoB100 regulator and causes apoB100 degradation. In addition, MTP is required for VLDL assembly and apoC3 may affect the TG content of VLDL. In the metabolic syndrome, hepatic inflammation contributes to IR which leads to dysregulation of suppression of apoB100 by insulin. The TG lowering effect of PS might depend on a direct effect on apoB100, MTP, or apoC3. Alternatively, PS may indirectly affect these protein(s) via insulin, either by direct interaction with insulin signaling, or by alleviating IR via suppression of hepatic inflammation.

4.3.1. apolipoprotein B100

Insulin reduces apoB100 protein levels. ApoB levels are controlled by protein degradation, not by inhibition of expression (Sparks *et al.* 2012). Binding of insulin to the insulin receptor results in the insulin receptor substrate (IRS) mediated activation of phosphatidylinositide (PI)3-kinase (PI3K) which results in formation of phosphatidylinositide (3,4,5) triphosphate (PIP3). PIP3 marks apoB100 protein, either lipid associated or not, for degradation by the autophagy-lysosomal pathway, or the proteosomal pathway, leading to reduced VLDL assembly and secretion (Sparks *et al.* 1996, Phung *et al.* 1997, Fisher *et al.* 1997). In the absence of insulin receptor activation, PTP-1B and PTEN inhibit PIP3 activation, maintaining default VLDL production (Taghibiglou *et al.* 2002, Delibegovic *et al.* 2009). Notably, a number of human studies showed a reduction in total serum apoB by PS (Temme *et al.* 2002, Ntanios *et al.* 2002, Homma *et al.* 2003, Sialvera *et al.* 2012)

4.3.2. Microsomal transfer protein

MTP levels have a direct effect on VLDL assembly and secretion (Sparks *et al.* 2012). A study by Kamagate *et al*. showed that insulin suppresses hepatic MTP expression via phosphorylation of transcription factor for MTP, FoxO1, which leads to its exclusion from the nucleus. In IR, insulin-induced FoxO1 phosphorylation is impaired, resulting in reduced MTP suppression, providing one rationale for increased VLDL assembly (Kamagate *et al.* 2008). However, studies with several mouse models of IR have provided confounding results on the relationship between insulin, MTP expression and VLDL secretion (Bartels *et al.* 2002, Biddinger *et al.* 2008). Sparks *et al.* demonstrated that in transfected primary rat hepatocytes MTP induced increase in apoB100 secretion is not affected upon insulin treatment (Sparks *et al.* 2011). Irrespective of the effect of insulin, nevertheless, MTP is positively correlated with VLDL assembly and secretion. To date, no studies on the effect of PS on MTP have been reported.

4.3.3. apolipoprotein C3

While hepatic lipid droplets have traditionally been viewed as rather passive bystanders, they are currently gaining recognition active players in the balance between hepatic levels of FA and TG, and VLDL production and secretion. Their regulatory functions were shown to be associated with several lipid droplet coat proteins. Qin *et al.* demonstrated that in humans, a missense mutation in the C-terminal domain of apoC3 strongly diminished VLDL secretion and formation of large, TG-rich VLDL. The investigators hypothesized that fusion of hepatic lipid droplets with VLDL-precursors to form VLDL-1 is mediated by apoC3 (Qin *et al.* 2011). However, it has been shown by Nagashima and colleagues that lower expression levels of wild-type apoC3, induced by the drug pioglitazone, did not reduce hepatic apoB100 and TG secretion in humans (Nagashima *et al.* 2005). To date, no studies on lipid droplet functioning in IR have been reported.

4.3.4. Hepatic inflammation

Steatosis and IR are associated with low grade inflammation in the adipose tissue and the liver. The inflammatory status of the liver is driven by the release of pro-inflammatory cytokines (TNFα, IL-6) by specialized liver resident macrophages called Kupffer cells. It has been suggested that Kupffer cells release these cytokines in response to activation of their innate immune receptor TLR4 by FA-ligands (Shi *et al.* 2006). Notably, mice depleted of Kupffer cells do not develop IR in response to a high fat or high sugar diet (Huang *et al.* 2010, Lanthier *et al.* 2011). It was demonstrated that this process requires TNFα (Huang *et al.* 2010). In support of these findings, Qin *et al*. showed that in hamsters, TNFα injection caused VLDL overproduction via inhibition of insulin-mediated VLDL suppression (Qin *et al.* 2008).

Kupffer cell derived TNFα is an activator of the signalling cascade that leads to nuclear translocation of the pro-inflammatory transcription factor NF-κB. During this cascade the inhibitor of kappa beta kinase (IKK) becomes activated, which phosphorylates a serine residue on a component of the insulin signalling cascade, insulin receptor substrate 1 (IRS1), inhibiting insulin signalling (Rondinone *et al.* 1997, Zierath *et al.* 1998). In addition, NF-κB itself contributes to increased expression of hepatic SOCS3. Pro-inflammatory IL-6 further promotes SOCS3 expression in hepatocytes via serine phosphorylation induced activation of the transcription factor STAT3 by mTOR (Kim *et al.* 2008). SOCS3 inhibits insulin signalling by binding to the insulin receptor, preventing activation by tyrosine-phosphorylation of IRS1. Furthermore, SOCS3 promotes proteosomal degradation of IRS1, adding to the insulin inhibitory effect. Thus, in a high fat environment, Kupffer cells secrete pro-inflammatory TNFα and IL-6 that differentially inhibit insulin signalling, promoting VLDL secretion. Suppressive effects of PS on inflammation have been demonstrated reported, but more research is needed (Lanthier *et al.* 2011, Kurano *et al.* 2011, Plat *et al.* 2013 unpublished data).

5. Hypotheses on serum triglyceride lowering by PS

From the previous sections it was hypothesized that the PS mediated lowering of fasting serum TG could be via 1) reducing hepatic TG content (via intestinal FA absorption, hepatic CM uptake, or DNL), 2) inhibiting hepatic VLDL assembly and secretion, 3) improving low-grade inflammation related IR, or 4) targeting all these biological targets. Thereupon, 5 hypotheses were defined, which are presented and discussed in the following sections. In Figure 6, these hypotheses of the potential mechanisms of action of the serum TG lowering by PS are depicted with the putative biological targets.

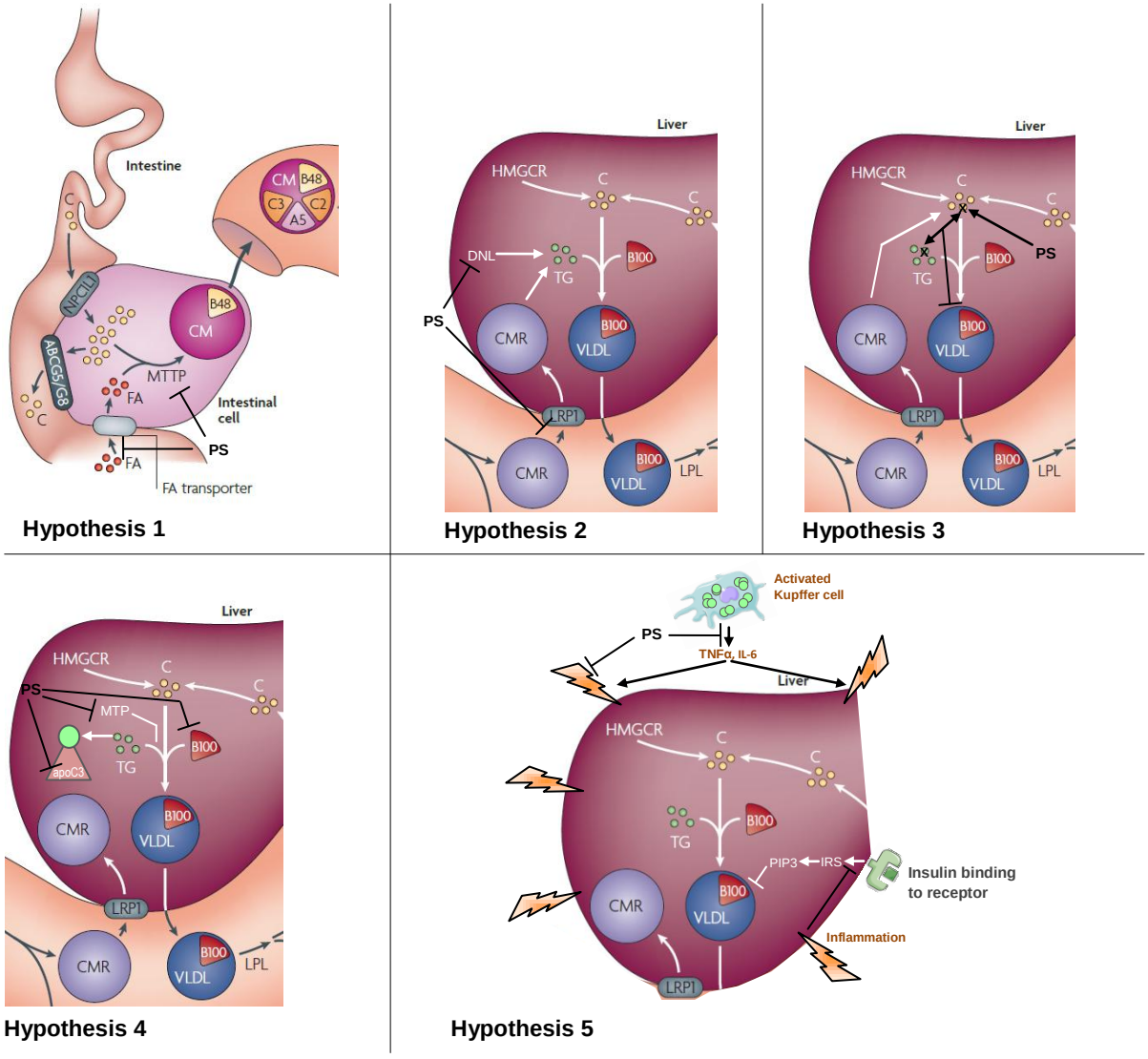

Figure 6 | Proposed mechanisms of action of serum TG lowering by PS. 1) PS reduce intestinal absorption of dietary FA, 2) PS reduce hepatic uptake of TG-rich CM remnants and/or DNL, 3) Reduced VLDL secretion by PS results from their LDL-cholesterol lowering, 4) PS reduce levels of proteins involved in assembly and secretion of VLDL-TG, 5) PS have an anti-inflammatory effect on activated Kupffer cells, reducing the expression of TNFα and IL-6 and alleviating IR-induced hypersecretion of VLDL (Adapted from Hegele *et al*. 2009).

5.1. Reducing hepatic triglycerides

5.1.1. Hypothesis 1
PS reduce intestinal absorption of dietary FA

PS could affect intestinal absorption of dietary FA by impairing FA solubility. There are no indications that PS influence receptor mediated FA transport, but it is possible that passive FA absorption is reduced via impaired solubility in the intestinal lumen, which would lead to increased faecal excretion. PS could directly suppress FA integration in mixed micelles, or reduce FA solubility by downregulation of bile acid secretion. Reduced FA absorption would decrease hepatic TG levels and lower VLDL-TG secretion. Finally, PS could affect integration of TG in CMs. Studies have provided supporting and conflicting evidence related to these issues, which is discussed below.

In a study by Rideout *et al.*, C57BL/6J mice were given a 'Western type' diet supplemented with 2% PS for 6 weeks (Rideout *et al.* 2010). Comparable to earlier studies with C57BL/6J mice, plasma LDL-cholesterol was not decreased (Calpe-Berdiel *et al.* 2005, Plosch *et al.* 2006, Weingartner *et al.* 2008), but hepatic total cholesterol showed a 5 fold reduction compared to control mice. It has been proposed that the absence of a PS mediated cholesterol lowering effect in normolipidaemic C57BL/6J mice is caused by the low baseline cholesterol levels in these mice and that further reduction is compensated for by *de novo* synthesis. In mice fed the PS diet, a significant reduction in plasma TG of 25% and in hepatic TG of 26% was found and, interestingly, faecal excretion of the saturated fatty acids palmitate (C16) and stearate (C18) doubled. The investigators assume this was due to reduced intestinal FA absorption (Rideout *et al.* 2010). No changes in the mRNA levels of the long chain fatty acid uptake protein CD36 were identified in the study by Rideout *et al.*, but protein expression levels were not analysed. In a recent review by Abumrad *et al*., CD36 was concluded to be more a FA transport regulator than transporter and its net contribution to FA absorption was stated minimal, particularly in high fat diets (Abumrad *et al.* 2012). Consequently, the observed reduced intestinal absorption of FA was most likely not related to CD36.

Additional *in vivo* studies with different animal models have provided conflicting results regarding the effect of PS on faecal lipid excretion. In a study by Brufau and colleagues, guinea pigs were given a high saturated fat diet with 2.45% PS for 4 weeks (Brufau *et al.* 2007). Contrary to the study by Rideout *et al*., no changes were observed in the excretion of the medium chain FAs palmitate (C16) and stearate (C18), but excretion of the medium-short chain FAs laurate (C12) and myristate (C14) decreased, while an increase in the long chain arachidonate (C20) and behenonate (C22) excretions was detected. Although total saturated fatty acid faecal excretions did not change in the PS supplemented group compared to the control group, the shift towards greater excretion of longer chain, more hydrophobic saturated fatty acids, does suggest a reduction in intestinal lipid solubilizing potential. The authors propose a direct role for PS in preferred integration of the smaller FAs laurate (C12) and myristate (C14) into mixed micelles promoting their absorption, but the possibility of reduced bile acid secretion partly impairing the intestinal solubility of the most hydrophobic long chain arachidonate (C20) and behenonate (C22) cannot be excluded.

One study with a fermented PS including campestenone did cause increased lipid excretion in mice (Suzuki *et al.* 2007), but studies in in apoE-/- mice given 2% mixed PS (Calpe-Berdiel *et al.* 2005), or in male rats given 0.5% campestanone (Ikeda *et al.* 2006) did not report altered intestinal lipid absorption. In humans, only one study investigated the effect of plant sterol and stanol esters on fat digestion in ileostomy subjects. At a typical dose of 2.5g PS, no effect on faecal lipid excretion was detected (Normén *et al.* 2006).

As mentioned in the previous paragraph, a possible mechanism for reduced intestinal FA absorption could be decreased duodenal bile acid excretion, affecting intestinal lipid solubilization. In support of these findings, a water-soluble derivative of stigmasterol, stigmasterol-acetate, inhibited CDAC induced transcription the bile salt export pump (BSEP) via farnesoid x receptor (FXR) antagonism in HepG2 cells and in primary hepatocytes of FXR +/+ mice, but not of FXR -/- mice (Carter *et al.* 2007). Notably, in this study, stigmasterol itself was only tested for its FXR antagonizing potential, not for the effect on endogenous BSEP expression. Stigmasterol did exhibit modest FXR antagonism, yet, contrary to stigmasterol-acetate, not statistically significant. However, it was not taken forward in subsequent experiments. This is remarkable, especially in view of the profound effects of small molecular differences in sterol biology, e.g. cholesterol versus plant sterols. Finally, β-sitosterol did not show any FXR antagonising activity in this study. To the contrary, in a recent study with the mouse breast cancer cell line TM2H, β-sitosterol showed FXR activation (van den Heuvel *et al.* 2012). These findings provide a plausible mechanism for a PS mediated reduction in bile acid excretion. Support for this hypothesis *in vivo* is provided by a study by El Kasmi that suggested that in C57BL/6J mice, parenteral nutrition that contained 1 mg/100 mL stigmasterol caused an increase in plasma bile acid levels, associated with suppressed BSEP transcription via antagonism of FXR (El Kasmi *et al.* 2013). However, in a study by Calpe-Berdiel and co-workers, supplementation of a 'Western' type diet with 2% PS given to apoE-/- mice for 4 weeks, no changes in faecal bile acid excretion, or lipid excretion, were detected (Calpe-Berdiel *et al.* 2005). Moreover, in studies in humans that were given 3 g of sitostanol esters per day (Gylling *et al.* 1996), 2 g mixed stanols per day (Miettinen *et al.* 2000), or 8,6 g plant sterols per day, no changes of faecal bile acid excretion were found (Weststrate *et al.* 1999).

In summary, a collection of *in vitro* and *in vivo* studies have provided conflicting results regarding the effect of PS on faecal FA and bile acid excretion. This is not surprising given the large variation in animal model species, differences in the type(s) and dosages of PS used and in the background diet. Nonetheless, studies in human subjects failed to show any effect of PS on neither faecal FA excretion, nor bile acid excretion, even if the daily intake was about 4-fold higher than the typical intake. Thus, based on current evidence, increased faecal FA excretion and concomitant reduced intestinal FA absorption by PS in humans seems unlikely. Further research is needed comparing different PS types and dosages in appropriate metabolic syndrome model systems, both *in vitro* and *in vivo*.

5.1.2. Hypothesis 2

PS reduce hepatic uptake of TG-rich CM remnants and/or hepatic DNL

Postprandial uptake of TG-rich CM remnants is a major contributor to hepatic TG content and related VLDL production. Therefore, it cannot be excluded that PS lowering of serum TG results from suppressed hepatic CM remnant uptake. This could be a consequence of affected intestinal CM production by PS, which would partly overlap with Hypothesis 1. One study in male Syrian hamsters has shown a reduction in intestinal MTP mRNA. This could be associated with reduced CM production, but no experiments were performed to investigate this (Liang *et al.* 2011). Reduced hepatic CM remnant uptake could also be a consequence of a decrease in expression of the CM receptors LDL-R and LRP1 in the liver, or increased expression in other tissues. In that view, in a study by Plat and co-workers, an interesting observation was made that daily consumption of 4 g plant stanol esters increased monocyte and T lymphocyte surface expression of LDL-R by 37% and 25% respectively in humans (Plat *et al.* 2002), albeit in a fasting state. Possibly, PS increase the capacity of the PBMC compartment to capture apoB lipoproteins.

A few studies have analyzed the effect of PS on liver TG. In a study with apoE-/- mice fed 'Western type' diet with 2% PS for 4 weeks a non-significant 17% reduction in liver TG was detected (Calpe-Berdiel *et al.* 2005). Serum TG were not analyzed in this mouse study, but given the small PS-induced average reduction in serum TG in humans of 6% (Demonty *et al.* 2013), changes in hepatic TG are expectedly small. The study by Calpe-Berdiel might have been underpowered to analyse the effect on hepatic TG. Interestingly, in the same study, changes in hepatic gene expression in response to PS were analyzed and a significant reduction in SREBF1 transcription was detected. The SREBF1 gene encodes SREPB1c, the major transcription factor of genes involved in DNL. Nonetheless, in a yet unpublished study by Plat *et al*., LDL-R-/- mice given a high fat diet with 2% PS for 3 weeks, showed a marked significant decrease of 40% at minimum in plasma TG, without a concomitant significant decrease in hepatic TG (Plat *et al.* 2013 unpublished data). However, a non-significant decrease of 20% at maximum in hepatic TG was observed and, as in the Calpe-Berdiel study, relatively high variation combined with a small effect might have rendered the study underpowered to identify significant changes in hepatic TG. Notwithstanding, as will be discussed in Hypothesis 5, this study showed a profound reduction of hepatic inflammation by PS which could underlie plasma TG lowering.

Contrary to the results obtained in the transgenic apoE-/- and LDL-R-/- metabolic syndrome mouse models, hepatic TG lowering by PS has been observed in wild type C57BL/6J mice. In the aforementioned study by Rideout *et al*., C57BL/6J mice on a 'Western type' diet supplemented with 2% PS for 6 weeks showed a reduction in hepatic TG of 26% (Rideout *et al.* 2010). Paradoxically, a significant increase of 23% in DNL was found, reflected by a strong upregulation of hepatic FAS mRNA and a trend towards an increase in mRNA of SREBP1c. Possibly, DNL was induced as a (partial) compensatory measure, similarly to what has been proposed for plasma cholesterol in response to PS in C57BL/6J mice (Rideout *et al.* 2010).

In vivo studies of the effect of PS on hepatic TG content and lipogenic gene expression are limited. Available studies have used different mouse models. Results suggest that PS can reduce hepatic TG levels, but the identified effects on genes involved in DNL are conflicting. Some studies suggest that reduced DNL might be the underlying cause of reduced hepatic TG. Other studies suggest that DNL is employed as a compensatory mechanism to restore hepatic TG reduction. It can be speculated that this might depend on to what degree the model represents physiology in the metabolic syndrome. Sufficiently powered studies in the appropriate models are needed to investigate the effect of PS on hepatic DNL in more depth. The selection of lipogenic genes analysed in those studies could be expanded by employing a (phospho)proteomics approach. Proposed experimental work will follow in the next chapter.

5.2. Hepatic VLDL assembly and secretion

5.2.1. Hypothesis 3

Reduced VLDL-TG secretion is an indirect effect of cholesterol lowering by PS

The suppressive effect of PS on VLDL-TG secretion could be an indirect effect from LDL-cholesterol lowering. In a number of animal models of various species, reduced plasma LDL-cholesterol by PS was associated with reduced hepatic cholesterol *in vivo* (Uchida *et al.* 1983, Ntanios 1998, Hayes 2002, Connor 2005, Kobayashi 2008). Perfusion experiments with isolated livers from rats that had been fed high cholesterol diets showed that VLDL production was correlated with cholesterol concentration. Increased cholesterol caused an increase in the number of VLDL secreted (Fungwe *et al.* 1992). Additional animal studies strongly suggested that liver cholesterol content directly regulates the number of VLDL particles and that TG synthesis is regulated accordingly to enable assembly of VLDL with proper ratios of its constituents (Khan *et al.* 1990, Thompson *et al.* 1996, Kang *et al.* 2000). In humans, cholesterol synthesis was demonstrated to be directly positively associated with hepatic VLDL secretion in normolipaedemic subjects (Riches 1997), in obese subjects (Cummings *et al.* 1995 - 1) and in non–insulin-dependent diabetic patients (Cummings *et al.* 1995 - 2). Coordinated synthesis of cholesterol and TG and associated assembly of VLDL-TG is demonstrated to be regulated by SREBPs. These sense hepatic cholesterol levels and induce lipogenesis accordingly (Horton *et al.* 2002). In view of the above, a very recent report suggested that VLDL secretion by isolated hamster primary hepatocytes *in vitro* is directly regulated by administered LDL without recapitulation of the intracellular cholesterol pool (Sniderman *et al.* 2013).An additional indirect manner by which cholesterol lowering could cause serum TG lowering could be via increased expression of LDL-R and LRP1, causing increased uptake of TG-rich apoB lipoprotein remnants. This would explain why TG lowering by PS is highest in metabolic syndrome patients with high baseline TG levels from high concentrations of circulating remnants. Similarly, statins have been shown to lower the expression of hepatic LDL-R and LRP1 *in vitro* and *in vivo* (Moon *et al.* 2011, Imagawa *et al.* 2012).

5.2.2. Hypothesis 4

PS reduces levels of proteins involved in hepatic assembly and secretion of VLDL-TG

Assembly of TG into mature VLDL-1 particles involves many proteins. ApoB provides the scaffold, MTP transfers phospholipids and TG to apoB, apoC3 stabilizes large TG-droplets and facilitates fusion with VLDL precursors to form VLDL-1. A decrease in hepatic levels of apoB, MTP, or apoC3 would generally result in decreased assembly and secretion of VLDL-1. For example, acute suppression of VLDL assembly and secretion by insulin is mainly due to its inhibitory effect on apoB production (Sparks *et al.* 2012).

A number of human studies have shown a reduction in total serum apoB by PS ranging from 5.6–8% (Temme *et al.* 2002, Ntanios *et al.* 2002, Homma *et al.* 2003, Sialvera *et al.* 2012). It is not clear to what extend these changes are reflected in hepatic apoB. In these studies no distinction between VLDL-apoB and LDL-apoB was made. However, LDL is produced by VLDL TG hydrolysis without affecting apoB. Nevertheless, apoB containing VLDL remnants could be taken up by the liver before being hydrolyzed to LDL. So, the PS reduction on serum apoB could result from reduced apoB secretion, but increased hepatic VLDL remnant uptake cannot be excluded. So far, no studies investigating the effect on hepatic MTP have been reported. One study investigated the effect of plant stanol esters on serum apoC3, but no changes were detected (Plat *et al.* 2009).

Importantly, inhibition of the VLDL assembly and secretion machinery without concomitant reduction in hepatic TG could be considered undesirable. Accumulation of TG could lead to NAFLD. Yet, a novel antisense apoB mRNA drug, Mipomersen, which is currently in phase 3 clinical tests, has been shown to reduce serum apoB levels by 20 – 50% in humans. Although investigators consider the development of steatosis a potential risk that should be closely monitored, Mipomersen is currently viewed safe for use in hypercholesterolaemic patients (reviewed in Ricotta *et al.* 2012 and Gelsinger *et al.* 2012). Therefore, the maximal serum apoB reduction of 8% by PS is most likely an acceptable risk (Temme *et al.* 2002). Importantly, it is likely that the observed apoB reduction is the consequence, not the cause, of PS-driven reduction of VLDL production via the mechanisms proposed in the other hypotheses.

5.3. Inflammation related insulin resistance

5.3.1. Hypothesis 5

PS have an anti-inflammatory effect on activated Kupffer cells, reducing the expression of TNFα and IL-6 and alleviating their induction of IR

Pro-inflammatory activation of specialized liver resident macrophages denoted Kupffer cells is essential for the development of IR via TNFα and IL-6 (Huang *et al.* 2010, Lanthier *et al.* 2011). Some human studies have suggested that PS have an anti-inflammatory effect based on significant reductions in circulating hs-CRP, TNFα, or IL-6, although many studies failed to demonstrate this effect, or the effect was dependent on the co-administration of anti-inflammatory poly unsaturated fatty acids like docosahexaenoic acid (DHA) (reviewed in Rosa *et al.* 2012).

However, it is questionable whether potential PS-induced small decreases in hepatic TNFα and IL-6, would be detectable systemically. Nevertheless, Kupffer cell depletion did not improve metabolic and inflammation parameters in C57BL/6J mice fed a high fat died for 4, or 16 weeks (Lanthier *et al.* 2011). The investigators concluded that although Kupffer cells are crucial for the onset of metabolic syndrome, they are probably not a suitable therapeutic target once symptoms are established. One *in vitro* study with mouse primary peritoneal macrophages showed that sitosterol and campesterol augmented LPS induced expression of TNFα and IL-6, albeit to a lesser extent than cholesterol (Kurano *et al.* 2011). Moreover, a recent study by Plat and colleagues found that in LDL-R-/- mice, supplementation of a high fat diet with 2% PS abolished hepatic inflammation as shown by a reduction in infiltrated macrophages and neutrophils, and by a reduction in the expression of the inflammatory markers IL-1β, TNFα, MCP-1 and ICAM. No results on IL-6 were reported. Furthermore, PS restored the amount of hepatic foamy (CD68+) Kupffer cells to the same level as in mice receiving a normal diet (Plat *et al.* 2013 unpublished data).

In summary, the combined findings of the aforementioned studies are in support of the hypothesis that PS lower hepatic TNFα and IL-6 production by Kupffer cells, which could reduce IR. It is interesting that in one mouse study, Kupffer cell depletion did not improve inflammation parameters, while in another mouse study, PS abolished hepatic inflammation and restored inflammatory Kupffer cells to normal numbers. From these findings, it could be speculated that the presence of Kupffer cells of a tolerogenic phenotype, similar to type 2 macrophages, is essential to maintain a metabolically healthy, non-inflamed state and that PS promote differentiation of Kupffer cells towards that phenotype. In line with hypothesis 3, this could be an indirect effect of cholesterol lowering, in the same way as lower serum LDL-cholesterol reduces inflammatory macrophage foam cell formation (reviewed in McLaren *et al.* 2011).

6. Proposed research

To test the hypotheses on the potential mechanism of action of serum TG lowering by PS, a series of experiments are proposed here. As these will be performed to the interest of Unilever R&D, and given the strict Unilever R&D policy against animal testing, all proposed experiments are *in vitro*. Results from these experiments however, could eventually be validated in human studies.

It is proposed to develop cell culture models of intestinal cells and of hepatic cells to test the effect of defined PS on:

-Integration of saturated FA (C12-C22) into mixed micelles,
-absorption of FA by intestinal cells
-production and composition of CM by intestinal cells
-total hepatocytic TG in relation to hepatocyte uptake of CM and DNL
-total hepatocytic cholesterol, free and esterified
-hepatocyte bile acid secretion
-hepatocyte lipoprotein production and secretion, in particular VLDL-TG

Although all aspects are interrelated and are expected to eventually affect VLDL-TG production, initial experiments could focus on the effect of PS on hepatocyte TG metabolism and VLDL production in the presence of varying doses of specific PS or mixes thereof. Importantly, contrary to many of the reported *in vitro* studies, PS should be tested at physiologically relevant concentrations. FAs could then be administered with albumin as carrier. Alternatively synthetic CM-like particles are offered, or in a more advanced stage, CM produced in the intestinal cell experiments are isolated and administered to the hepatocytes. Methods for the production of mixed micelles, employing free cholesterol, FAs, PS and bile acids have been applied before at the Unilever laboratory.

Well established intestinal and hepatic cell lines, Caco-2 and HepG2 respectively, are available at the Unilever laboratory. As these immortal cell lines provide an unlimited supply and have been extensively used by other researchers, they are useful tools for the development and validation of the culture systems described above. However, being cancer cell lines their biology is altered and results obtained might be less physiologically relevant. Therefore, it is proposed to use human primary intestinal cells and primary hepatocytes instead, which are currently commercially available. By continuous condition culturing of cells in energy-rich media the development of a metabolic syndrome phenotype might be possible.

Best analysis methods for FA absorption, CM production and composition, and VLDL-TG production need to be discussed. Initially, combined HPLC and apoB48 (CM), apoB100 (VLDL) ELISAs could be performed together with intracellular total TG staining. This could be extended LC-MS-MS for tracing of FA stable isotopes, or even NMR for full lipoprotein subclass analysis.

If PS suppressive effects on hepatocyte VLDL-TG production (cell content) and secretion (supernatant content) are detected, it is proposed to perform a comprehensive analysis on the transcription (qPCR) and expression levels (Western blot, Luminex multiplex analysis) and, if applicable, phosphorylation status of the proteins referred to in the previous chapters. These are related to:

-Bile acid secretion: FXR/RXR, BSEP, CYP7A1
-Hepatic uptake of CM and VLDL remnants: LDL-R, LRP1
-DNL: LXR, SREBP-1c, ChREBP, ACC, ELOVL6, FAS, GPAT, SCD1, SCAP, TORC1, Lipin 1
-VLDL-1 assembly and secretion: apoB, MTP, apoC3
-IR: IRS, PI3K, PIP3
-Inflammation: TNFα, IL-6, IL-1β

Results from these experiments should provide insight into which mechanism(s) are likely involved in the TG lowering effect of PS, either directly, or via LDL-cholesterol lowering.

In an advanced stage of this research project, monocyte-derived macrophages as a model for Kupffer cells could be incorporated. First in isolation to test the effect of specific PS on their inflammatory status. Later in co-culture with hepatocytes. This would enable to address hypothesis 5. Furthermore, the integration of this final cell type crucial for the development of IR would provide a comprehensive model system that can be of great use to answer future research questions in the areas of enterohepatic lipid physiology and glucose control in the context of the metabolic syndrome.

7. Conclusion & Discussion

The serum LDL-cholesterol lowering effect of plant sterols and stanols and their esterified derivatives is well recognized. A daily intake of about 2g of PS lowers serum LDL-cholesterol by about 9% (Demonty *et al.* 2009). It is generally accepted that the PS mechanism of action of LDL-cholesterol lowering is competition with cholesterol for integration into mixed micelles, which partially inhibits intestinal cholesterol absorption. An additional recently recognized LDL-cholesterol lowering mechanism by PS involves LXR-induced trans intestinal cholesterol excretion (TICE) (van der Veen *et al*. 2009).

In addition to LDL-cholesterol lowering, PS modestly, but significantly lower fasting serum TG. A 2013 meta-analysis showed that PS intake of 1.6 – 2.5 g/day lowers fasting serum TG by 6.0% (0.12 mmol/L) on average in humans (Demonty *et al.* 2013). Furthermore, the absolute reduction in serum TG was shown to increase with high baseline TG levels, as in metabolic syndrome patients. Plat and colleagues confirmed the strong effect in metabolic syndrome patients and showed that TG lowering was associated with a significant reduction in the number of large TG-rich VLDL lipoprotein particles, mainly subclass VLDL-1, but also medium sized VLDL-2 (Plat *et al*. 2009).

VLDL is assembled in and secreted by the liver. VLDL formation requires apoB100. The number of VLDL formed is directly dependent on the amount of apoB protein available. Furthermore, VLDL-1 assembly requires MTP for the addition of PL and TG. Acute regulation of VLDL assembly and secretion is controlled by insulin that suppresses VLDL in the postprandial state. In IR, this suppression is reduced, both postprandial and in the fasting state, resulting in hypertrygliceridaemia. Hepatic TG content positively correlates with hepatic VLDL secretion (Fabbrini *et al*. 2009). The determinants of hepatic TG content are the free FA flux from adipose tissue lipolysis, dietary TG in CMRs and DNL. In the metabolic syndrome patients the contribution of CMRs and DNL are larger than in healthy individuals (Donnelly *et al.* 2005, Timlin *et al.* 2005).

Here, a number of hypotheses are presented on the possible mechanisms behind the serum fasting TG lowering effect of PS. They relate to hepatic TG content, hepatic VLDL assembly and secretion, and low-grade hepatic inflammation related to IR. These hypotheses are summarized as: 1) PS reduce intestinal absorption of dietary FA, 2) PS reduce hepatic uptake of TG-rich CM remnants and/or DNL, 3) Reduced VLDL-TG secretion by PS is an indirect effect resulting from their LDL-cholesterol lowering, 4) PS reduce levels of proteins involved in hepatic assembly and secretion of VLDL and 5) PS have an anti-inflammatory effect on activated Kupffer cells, reducing the expression of TNFα and IL-6 and alleviating their induction of IR.

A recent and yet unpublished study by Plat *et al*. investigated the effect of PS on postprandial rather that fasting serum TG in healthy subjects (personal communication, Trautwein). There, PS given at 3 gram per day for 4 weeks and after a fat rich meal, did not lower postprandial serum TG. This could imply that PS did not interact with absorption of dietary TG, or with hepatic uptake of dietary TG-rich CMRs. In addition, if the fasting serum TG lowering effect of PS is really specific for VLDL-1 only, the change in total TG after a high fat meal is probably also difficult to detect, especially in healthy subjects where the effect is small. Since the data of this study are not yet available, it is currently difficult to draw specific conclusions on the difference between the PS effect on fasting versus postprandial serum TG.

The fact that metabolic syndrome patients can develop non-alcoholic fatty liver disease, suggests that increased VLDL secretion in these individuals is insufficient to compensate for hepatic TG accumulation (Choi *et al.* 2011). Studies have suggested that in IR, part of the lack of full compensation could result from ER-stress and the related uncoupled protein response (UPR), inhibiting apoB assembly and secretion (Ota *et al.* 2008, Rutkowski *et al.* 2008, Hotamisligil *et al.* 2010, Schroder *et al.* 2010). Therefore, the possibility exists that the observed PS-induced decrease in serum TG in individuals with high baseline TG levels, reflects a further impairment of VLDL secretion potential via PS-induced ER-stress. However, no studies pointing in that direction have been reported and further research is needed to address this issue.

8. References

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