Effects of plant sterol and stanol esters on oxyphytosterol concentrations and postprandial metabolism

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Effects of plant sterol and stanol esters
on oxyphytosterol concentrations
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The studies presented in this thesis were performed within NUTRIM School for Nutrition, Toxicology & Metabolism, which participates in the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences), accredited by the Royal Netherlands Academy of Arts and Sciences.

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CHAPTER 1

General Introduction

Introduction

Cardiovascular disease (CVD) is a collective term to describe disorders of the heart and blood vessels such as angina pectoris, myocardial infarction, stroke and transient ischemic attack. One of the underlying cause of CVD is atherosclerosis, which is a slow, chronic and continuing process that starts with the formation of fatty streaks at an early age and eventually results in plaque formation within the vessel walls [1]. Endothelial dysfunction and inflammatory responses play a central role in the development of atherosclerosis, resulting in an increased permeability of the endothelial layer. This allows atherogenic low-density lipoprotein (LDL) particles to migrate into the underlying intima layer, where they subsequently can be oxidized. Accumulated oxidized LDL particles in the endothelial wall will stimulate the expression of adhesion molecules, attracting monocytes that can migrate into the intima layer and can transformed into macrophages. Oxidized LDL particles are taken up by macrophages and will form lipid-loaded foam cells, creating a viscous cycle of inflammatory responses with secretion of cytokines and growth factors. Ultimately, an advanced complicated lesion is formed, covered by a fibrous cap consisting of fibrous tissue, smooth muscle cells, platelets and macrophages, which can obstruct arterial blood flow. When an atherosclerotic lesion ruptures, it can form a thrombus and cause a myocardial or cerebrovascular infarction [2, 3].

The risk to develop atherosclerotic lesions is influenced via many factors, such as cigarette smoking, high blood pressure, diabetes mellitus and obesity. Another important CVD risk factor is an unfavourable lipoprotein profile, which is characterized by increased LDL-cholesterol (LDL-C) concentrations, increased triacylglycerol (TAG) concentrations and/or decreased high-density lipoprotein cholesterol (HDL-C) concentrations. For this reason, reducing LDL-C concentrations is the major target in CVD prevention [4]. Elevated serum LDL-C concentrations can be lowered via lifestyle changes and if necessary via pharmacological treatment. The most widely prescribed drugs are statins, which are able to lower LDL-C concentrations up to 50%. More dramatic decreases in LDL-C concentrations can be achieved by targeting PCSK9, which is a protein secreted by hepatocytes and regulates LDL receptor breakdown. Antibodies to PCSK9 are in clinical trial development and LDL-C reductions of 60% were seen in hypercholesterolemic patients, while reductions up to 70% were achieved in statin-treated patients [5, 6].

Subjects suffering from mild hypercholesterolemia do not qualify for pharmacological treatment and are able to achieve more modest LDL-C reductions through dietary changes. In addition, subjects on statins not meeting their LDL-C goals or subjects suffering from statin intolerance might also benefit from dietary changes to lower LDL-C concentrations [7]. Changing the composition of the diet and adding specific foods or food components (so-called functional foods) to the daily diet improves an unfavourable lipoprotein profile. Nowadays, functional foods are widely incorporated with a variety of health-promoting activities.

Plant sterols and plant stanols as cholesterol-lowering functional foods

The enrichment of foods with plant sterol and plant stanol esters is one of the first examples of functional foods that demonstrated a clear health benefit, i.e. lowering serum total and LDL cholesterol concentrations. Plant sterols and plant stanols share structural similarities with cholesterol. They consist of a steroid nucleus with a double bond present at C5-C6 while only the side-chain configuration differs. Plant stanols are the saturated derivative of plant sterols (**Figure 1.1**). Plant sterols and plant stanols are naturally occurring constituents of plants and are present in our daily diet. They are found in vegetable oils, such as corn oil, soybean oil, and rapeseed oil and they are also present in cereals, nuts, fruit and vegetables. The average intake of plant sterols and plant stanols in Western countries is approximately 300 mg/day, but can be as high as 600 mg/day in vegetarians. The most abundant plant sterols in the human diet are β -sitosterol, campesterol and stigmasterol, while plant stanols are less abundant and consist mainly of sitostanol and campestanol [8]. Serum plant sterol and plant stanol concentrations are the result of intestinal absorption and biliary secretion, since humans cannot synthesize plant sterols and plant stanols. Their fractional absorption rate is very low (0.5-2.0% for plant sterols and 0.04-0.2% for plant stanols) and biliary secretion is high, which results in serum concentrations of plant sterols and plant stanols that are less than 1% of that of serum cholesterol. On average, plasma sitosterol concentrations range from 0.16 to 0.67 mg/dL and campesterol concentrations from 0.28 to 1.12 mg/dL [9], while plasma plant stanol concentrations are approximately 100 times lower and vary between 2 and 12 μ g/dL in the normal population [10].

Figure 1.1 Chemical structures of cholesterol, plant sterols and plant stanols.

The LDL-C lowering effect of plant sterols and plant stanols has been demonstrated in numerous studies and has resulted in the recommendations to consume plant sterol and plant stanol enriched products in current dietary guidelines [11]. Plant sterols or plant stanols are able to reduce serum LDL-C concentrations by decreasing intestinal cholesterol absorption, which results in an increased endogenous cholesterol synthesis rate and an up-regulation of LDL receptor expression. Large meta-analyses have shown that consuming approximately 2.5 g/day of plant sterols or plant stanols lowers serum LDL-C concentrations up to 10%. At doses up to 3.0 g/day, plant sterols and plant stanols have been shown to have a comparable LDL-C lowering efficacy, while there are discrepancies between the efficacy of plant sterols and plant stanols at higher intakes. However, studies specifically designed to answer this question are lacking and therefore conclusions regarding relative efficacy of plant sterols and plant stanols at higher intakes cannot be drawn. Plant sterol and plant stanol enriched products have been shown to lower LDL-C concentration in different populations and incorporated into different matrices as extensively reviewed [11, 12]. While reduced LDL-C concentrations after plant sterol and plant stanol consumption have been unequivocally proven, there is a lack of hard endpoint studies evaluating the clinical benefit from the consumption of plant sterol and plant stanol enriched products.

Potential atherogenicity of plant sterol and plant stanol consumption

Consuming plant sterol enriched products will increase serum plant sterol concentrations, while the consumption of plant stanols will decrease plant sterol concentrations. A recent meta-analysis reported average increases in sitosterol concentrations of 31% (CI: 26-37%) and increases in campesterol concentrations of 37% (CI: 29-45) after consumption of plant sterol enriched products [13]. Phytosterolemia is a rare genetic sterol storage disease that is characterized by dramatic increases in serum plant sterol concentrations, ranging from 10-65 mg/dL, while serum cholesterol concentrations are normal or only slightly increased. Patients suffering from this disease often develop tendon and tuberous xanthomas and some patients develop premature atherosclerosis, while other patients do not show any signs of CVD, complicating the association between increased plant sterol concentrations and CVD risk [14]. The disease is caused by a mutation in the ABCG5/8 half transporters, resulting in a decreased secretion of absorbed sterols from the enterocytes back into the lumen, as well as a reduction from the liver into bile. In healthy subjects, increases after plant sterol consumption remain within the normal range (<1.0 mg/dL) and are only marginal as compared to plant sterol concentrations observed in phytosterolemic subjects. However, the potential impact of these elevated circulating plant sterol concentrations on cardiovascular risk is currently hotly debated. Several prospective and cross-sectional epidemiological studies evaluated the potential relationship between circulating plant sterol concentrations and CVD risk with contradictory results. Genser et al. performed a systematic review based upon 17 epidemiological studies (n=11,182) and did not find an

association between serum concentrations of plant sterols and elevated risk of CVD [15]. Serum plant sterol concentrations are surrogate markers for intestinal absorption and might underlie the potential association between increased plant sterol concentrations and CVD risk as shown by Silbernagel et al. in a recent study [16]. They identified an atherogenic role for high intestinal cholesterol absorption rather than for increased circulating plant sterol concentrations, based on the cholestanol-to-cholesterol ratio. Cholestanol is a derivative of cholesterol and produced by the liver and often used as a surrogate marker for cholesterol absorption. However, the validity of this marker has recently been questioned since cholestanol is also present in diet (less than 2 mg/day) and it does not reflect cholesterol absorption under certain conditions such as impaired biliary secretion [17, 18]. Although cholestanol concentrations correlated with fraction cholesterol absorption in a recent overview of plant stanol studies, individual studies do not always show this correlation. Silbernagel et al. implied that increased plant sterol concentrations are merely a marker for high cholesterol absorption rates without being atherogenic themselves. However, since this assumption heavily relies on serum cholestanol concentrations as a surrogate marker for cholesterol absorption, verification in future research is desirable.

Oxidation of cholesterol, plant sterols and oxysterol concentrations

Besides plant sterols acting as a surrogate marker, another possibility to explain the potential association between plant sterol concentrations and CVD risk might be increases in plasma oxysterol concentrations. Plant sterols are – analogue to cholesterol – susceptible to oxidation and can result in the formation of oxysterols, while plant stanols do not have a double bond in their ring structure and can therefore not be oxidized.

Oxysterol and oxysterol concentrations can result from dietary absorption or from endogenous oxidation from non-oxidized sterols. Plant sterols most likely can only be oxidized at the 7-position of the sterol ring, while cholesterol can be oxidized both at the sterol ring and on their side-chain [19]. **Figure 1.2** shows the most important oxysterol and oxysterol structures present in most tissues. In general, ring-oxidation products are formed non-enzymatically, e.g. through reactive oxidative species (ROS)-mediated oxidation while side-chain oxidation products are formed enzymatically. Exceptions to this are 7 α -OH-sterols and 25-OH-cholesterol, which both can be formed enzymatically and non-enzymatically. ROS-mediated oxidation of a cholesterol or a plant sterol molecule starts with the abstraction of an allylic hydrogen atom at carbon-7 of the sterol ring. The generated radical can further react with oxygen to form a cholesterol or plant sterol peroxy radical and after further abstraction of hydrogen, the relatively stable 7 α - and/or 7 β -hydroperoxides are formed. These hydroperoxides products can be reduced to form 7 α - and or 7 β -OH products or be dehydrated to form 7-keto-products, which are the major non-enzymatic oxysterols and oxysterols present in the human body [20]. For cholesterol, 24-, 25- and 27-OH-cholesterols are generated by enzymatic oxidation of

the side-chain. 27-OH-cholesterol is produced by sterol 27-hydroxylase (CYP27A1), a P450 enzyme mostly expressed in the liver and macrophages that is involved in many processes, including the breakdown of cholesterol to bile acids. Cholesterol 24-hydroxylase (CYP46A1) is also a P450 enzyme, located in neural cells of the brain and retina and is able to produce 24-OH-cholesterol. 25-OH-cholesterol is a regulator of the sterol regulatory element binding protein (SREBP) pathway for cholesterol synthesis and is produced both non-enzymatically as well as enzymatically by cholesterol 25-hydroxylase [21]. Oxysterols formed through P450-oxidation are involved in functional processes in the body, such as sterol synthesis/breakdown and the metabolism of potential harmful compounds. On the other hand, ROS-mediated oxysterols mainly reflect oxidation status of the body and will often be accompanied by many other, potentially toxic, oxidized compounds [22]. Regarding plant sterols, oxidation only occurs at the 7-position of the ring structure, indicating that the majority of oxidized plant sterols will result from radical oxidation by ROS and not through enzymatic oxidation. However, information regarding oxysterol metabolism is very scarce and enzymes responsible for plant sterol oxidation might be identified in future research.

Figure 1.2. Plant sterol and cholesterol oxidation products. In general, cholesterol and plant sterol oxidation products oxygenated at the sterol ring are produced non-enzymatically, while cholesterol oxidation products oxygenated at the side chain require enzymes. 25- and 7α-OH-cholesterols can be produced enzymatically and non-enzymatically.

Besides in vivo formation, oxysterols are also found in food products, such as heated vegetable oils, infant milk formulas, parenteral nutrition, French fries, potato chips

and coffee beans. However, the main source of dietary oxyphytosterols are plant sterol enriched products, where one serving can provide up to 1.7 mg oxyphytosterols per day if a recommended daily intake of 20-25 gram of spread is consumed. This exceeds the intake of other oxyphytosterol-containing products, such as heated vegetable oils, where oxyphytosterol intake was estimated to be 0.7 mg per day or less [23-26]. Circulating oxyphytosterols have been identified in phytosterolemic subjects [27], in healthy subjects [28, 29] and in aortic tissue of CVD patients [30]. Concentrations in healthy subjects are very low, i.e. a range of 0.3-4.5 ng/mL, which is 10-100 times lower than plasma oxysterol concentrations. Whether circulating oxyphytosterols originate from dietary absorption, endogenous oxidation from non-oxidized plant sterols or a combination of both remains to be determined. Since there is hardly anything known regarding oxyphytosterols (i.e. absorption, endogenous oxidation of plant sterols and dietary influence), we carried out two intervention studies to increase our basic knowledge on oxyphytosterol metabolism. In a large randomised cross-over trial, we examined the effects of consuming plant sterol or stanol ester enriched margarines on fasting and postprandial plasma oxyphytosterol concentrations in healthy subjects (chapter 3 and 5). We also investigated the difference between subjects with low and high plasma oxyphytosterol concentrations to increase our understanding of potential determinants of circulating oxyphytosterol concentrations (chapter 4). In a subsequent study, we also evaluated whether plasma oxyphytosterol concentrations are influenced by antioxidant supplementation in a population characterized by increased oxidative stress (chapter 7).

Effects of plant sterol and plant stanol consumption on postprandial metabolism and sequential meal consumption

Atherosclerosis has been suggested to be a postprandial disease, where remnant lipoproteins play a large role and evidence is accumulating towards non-fasting TAG as another important CVD risk factor [31, 32]. Except for a couple of hours in the early morning, humans are in a non-fasting state for most part of the day. The (postprandial) transport of lipoproteins is shown in **Figure 1.3**. After consumption of a (fatty) meal, dietary triglycerides are hydrolysed via enzymatic lipases to free fatty acids and monoglycerides (MAGs) and, together with other lipophilic substances such as bile salts and cholesterol, are formed into mixed micelles. Fatty acids and MAGs are absorbed in the enterocytes and resynthesized to form TAGs in the endoplasmic reticulum. They are then packed with apolipoprotein (apo) B48 particles, A-1 and A-IV into chylomicrons, secreted into lymph and pass into the blood via the thoracic duct. In the circulation, chylomicrons acquire apoCII and apoE from HDL particles. Chylomicron-TAGs are hydrolysed by lipoprotein lipase (LPL) present on endothelial cells which uses apoCII as a co-factor, and the released FAs are absorbed by adipose or skeletal muscle and used for energy generation or storage as TAGs. Phospholipids, cholesterol and apolipoproteins are transferred to HDL particles and the remaining cholesterol-rich chylomicron is transferred into a remnant particle. Chylomicron remnants are taken up by

the liver via surface cell receptors (LDL-receptor via apoB and LDL-receptor related protein (LRP) via apoE), which completes the exogenous lipoprotein transport. On the other hand, the endogenous lipoprotein transport involves the production of TAG-rich VLDL particles by the liver. VLDLs contain predominantly TAGs, smaller amounts of cholesterol and apoB100, CII and E. Similar to chylomicrons, they undergo lipolysis via LPL, which results in the formation of intermediate-density lipoproteins (IDL) that can be taken up by the liver or be converted into LDL particles. VLDL and LDL particles are removed from the circulation via LDL-receptor and LRP-mediated clearance [33]. LDL-receptor mediated uptake is no longer possible when apoB100 is modified (for example through oxidative stress) and LDL is then taken up by scavenger receptors present on macrophages, contributing to the development of atherosclerosis [34]. HDL particles are responsible for reverse cholesterol transport (RCT). For this, nascent HDL particles acquire cholesterol from non-hepatic cells by interacting with ABCA1 and ABCG1, after which cholesterol is esterified by lecithin:cholesterol acyltransferase (LCAT) and mature HDL particles are formed. Cholesterol ester transfer protein (CETP) can transfer cholesterol esters from HDL to VLDL, LDL and chylomicron particles in exchange for

Figure 1.3. Schematic overview of the lipoprotein metabolism. LDLr: low-density lipoprotein receptor, LRP: lipoprotein receptor-related protein, SR-B1: scavenger receptor class B member 1, CM: chylomicron, VLDL: very low-density lipoprotein, IDL: intermediate low-density lipoprotein, LDL: low-density lipoprotein, HDL: high-density lipoprotein, LPL: lipoprotein lipase, CE: cholesterol ester, TAG: triglycerides, FFA: free fatty acids, CETP: cholesterol ester transfer protein, LCAT: lecithin-cholesterol acyltransferase.

TAG. The transfer of cholesterol from HDL-C to LDL-C predominates, with minor transfer to VLDLs and chylomicrons [35].

Postprandial lipemia is a normal response of the human body, which is determined by intestinal and hepatic TAG secretion (into chylomicron and VLDL particles), TAG clearance by specific lipases and remnant-receptor driven removal of particles from the circulation. This response is transitory, but can become pathological when the extent of the TAG response is increased after meal consumption, which will then result in an accumulation of TAG-rich lipoproteins (TRLs) and their remnants. An accumulation of TRLs in the circulation will result in smaller chylomicron and VLDL remnant particles with a higher density that are more atherogenic than their larger counterparts. Smaller high dense remnants can more easily infiltrate into the arterial wall, are more prone to oxidation and are cleared more slowly. In addition, postprandial accumulation of TAG-rich lipoproteins enhances the exchange of TAGs for cholesterol esters from LDL-C and HDL-C, leading to a TAG enrichment of both HDL-C and LDL-C, giving rise to the formation of smaller and denser HDL-C and LDL-C particles (see insert Figure 1.3) [36]. Recent guidelines state that increased non-fasting TAG concentrations are markers for CVD, but cut-off values are lacking. In addition, there is no consensus with respect to the optimal study design to assess postprandial lipemia. Dietary and lifestyle changes remain the first line of approach to reduce (non-fasting) TAG concentrations, but studies addressing postprandial lipid and lipoprotein responses after plant sterol and stanol ester intake are scarce. Since there is a need for more detailed knowledge regarding postprandial effects of plant sterol and stanol ester consumption on lipoprotein metabolism, we assessed the effects of plant sterol and stanol ester consumption on postprandial lipoprotein metabolism (chapter 6).

Outline thesis

This thesis describes the results of two human intervention studies, investigating the effects of plant sterol and plant stanol consumption in the fasting and postprandial state and evaluating the effect of antioxidant supplementation on plasma plant sterol oxidation products. In chapter 2, an overview of the available literature is provided on the use of plant sterols and plant stanols in the treatment of dyslipidaemia. Chapter 3 describes the effects of plant sterol and plant stanol consumption on fasting plasma oxyphytosterol concentrations in healthy human subjects. Potential factors that might explain inter-individual difference in plasma oxyphytosterol concentrations are investigated in chapter 4. In the postprandial study described in chapter 5, plasma oxyphytosterol concentrations are evaluated after consumption of a plant sterol or stanol enriched mixed meal in healthy subjects. In chapter 6, the effects of plant sterol or stanol consumption on postprandial metabolism are evaluated. In chapter 7, the results are described of a human intervention study, carried out in subjects with type 2 diabetes or impaired glucose tolerance, in which the effects of antioxidant supplementation on plasma oxyphytosterol concentrations were

studied. In chapter 8, the main results and conclusion are described and discussed and future directions within this field are addressed.

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Chapter 2

Plant sterols and stanols in the treatment of dyslipidaemia: new insights into targets and mechanisms related to cardiovascular risk

Sabine Baumgartner, Ronald P. Mensink, Jogchum Plat

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Abstract

Plant sterols and stanols are naturally occurring constituents of plants and as such normal components of our daily diet. The consumption of foods enriched in plant sterols and stanols may help to reduce low-density (LDL) cholesterol concentrations. Meta-analyses have shown that consuming approximately 2.5 gram plant sterols or stanols per day lowers serum LDL-cholesterol concentrations up to 10%, with little additional benefit achieved at higher intakes. However, recent studies evaluating plant stanol intakes up to 9 g/d have indicated that LDL-cholesterol concentrations can be reduced up to 17%, which suggests that more pronounced reductions can be achieved at higher intakes. Studies describing effects of high plant sterol intakes on serum LDL-cholesterol concentrations are not consistent. Besides the effects of higher than advocated intakes on serum LDL-cholesterol concentrations several topics will be discussed in this review. First, besides the well-characterized effect of plant sterols and stanols on serum LDL-cholesterol concentrations, evidence is now emerging of their effects on triacylglycerol metabolism, which makes them highly attractive for interventions in metabolic syndrome-like populations. Secondly, there is an ongoing debate whether increased plant sterol concentrations are associated with an increased cardiovascular disease risk or not. For this there are at least two possible explanations. First, the potential atherogenicity of increased plant sterol concentrations might be ascribed to the formation of plant sterol oxidation products (so-called oxyphytosterols) or secondly elevated serum plant sterol concentrations should only be seen as surrogate markers for characterizing subjects with a high intestinal cholesterol absorption. Finally, we will discuss recent studies, which suggest that plant sterols and stanols are able to improve endothelial dysfunction in subjects at risk, although evidence is limited and more research is needed.

Plant sterols and stanols

Dyslipidaemia is a major risk factor for developing cardiovascular disease (CVD), which includes hypercholesterolemia as well as hypertriglyceridemia. By lowering low-density lipoprotein (LDL) cholesterol concentrations, CVD risk is considerably reduced. It has been estimated that each percentage reduction in LDL-cholesterol can reduce CVD risk by 2% [1]. The impact of lowering circulating LDL-cholesterol concentrations on CVD risk can also be deduced from genetic studies. Carriers of the PCSK9 SNP (PCSK9^{142x} or PCSK9^{679x}), for example, have lifelong on average 28% lower serum LDL-cholesterol concentrations and an 88% lower CVD risk [2]. PCSK9 is involved in the degradation of the LDL receptor and the above-mentioned loss of function mutation consequently results in lower serum cholesterol concentrations [3]. The consumption of food products enriched with plant sterols and stanols may help to reduce increased LDL-cholesterol concentrations and as such the risk of CVD. Evidence for this LDL-cholesterol lowering effect has evolved so strongly over the past decade that plant sterols and stanols are now part of the NCEP guidelines, which encourages the daily incorporation of 2 g of plant sterols into a healthy diet low in saturated

fatty acids to reduce CVD risk for subjects with elevated LDL-cholesterol concentrations [4]. Plant sterols are naturally occurring constituents of plants and are common components of our daily diet. They are structurally related to cholesterol and the cellular functions of plant sterols in plants are analogous to those of cholesterol in animals. Both cholesterol and plant sterols consist of a steroid nucleus with a double bond present at C5-C6 and only the side-chain configuration differs. The average intake of plant sterols in Western countries is approximately 250 mg/day, mainly derived from vegetable oils, cereals, nuts, seeds, fruits and vegetables [5]. The most abundant plant sterols in the human diet are β -sitosterol, campesterol, and stigmasterol. Plant stanols are the saturated derivatives of plant sterols. The major plant stanols are sitostanol and campestanol, which are considerably less abundant in nature than plant sterols are [6]. Since humans are unable to synthesize plant sterols and stanols, their serum concentrations are the results of intestinal absorption and biliary secretion. In contrast to the absorption rate of cholesterol, which is around 50%, the absorption rate of plant sterols is much lower; i.e. about 10-15% for campesterol and campestanol, 4-7% for sitosterol and 1% for sitostanol [7]. Once present in the circulation, plant sterols and stanols are taken up by the liver and rapidly secreted into bile by hepatic ABCG5/G8 transporters. Although cholesterol is also secreted into bile, the rate of plant sterol and stanol secretion into bile is much faster [8]. Due to their low absorption and high biliary secretion serum concentrations of plant sterols and stanols are less than 1% of that of serum cholesterol [9]. On average, plasma sitosterol concentrations range from 0.12 to 0.62 mg/dL and campesterol concentrations from 0.27 to 1.08 mg/dL [10].

While the effect of plant sterols and stanols on serum LDL-cholesterol is well characterized and has been reviewed extensively [6, 7, 11, 12], evidence is emerging of effects beyond LDL-cholesterol lowering. Plant sterols have been studied in relation to immune modulation [13, 14], triacylglycerol metabolism [15] and cancer prevention [16]. In this review we will therefore only briefly discuss the effects of plant sterols and stanols on serum LDL-cholesterol concentrations and cholesterol metabolism. The focus will be on the effects of plant sterols and stanols on triacylglycerol metabolism. Furthermore, we will address recent studies suggesting that plant stanol esters have larger effects on serum LDL-cholesterol concentrations when provided in amounts higher than currently advocated. The issue of promoting higher daily plant sterol and / or stanol intakes is especially relevant regarding the ongoing debate whether elevated serum plant sterol concentrations are associated with cardiovascular risk or not. Two explanations for this possible relationship will be discussed. First, we will discuss the possible atherogenicity of plant sterols as a result from plant sterol oxidation, i.e. formation of the so-called oxysterols. Secondly, we will consider the possibility that serum plant sterols should only be considered as markers for cholesterol absorption, which means that subjects with elevated serum plant sterol concentrations absorb also more (dietary) cholesterol. Finally, we will briefly discuss the evidence whether plant sterol and stanol consumption influences endothelial function and characteristics of the microcirculation.

LDL-cholesterol lowering effect

Several meta-analyses have clearly indicated the LDL-cholesterol lowering effect of plant sterols and stanols. Here, we will provide a short overview of the main conclusions from these meta-analyses. The very first meta-analysis by Law [6], already published in 2000, summarized the results of 10 studies, in which plant sterols and stanols were mainly consumed when added to fat spreads. It was found that at doses of >2 g/d, the average reduction in LDL-cholesterol ranged from 0.43-0.54 mmol/L, or 9-14%. Three larger meta-analyses have been performed thereafter, which unanimously concluded that a non-linear dose-response relationship existed between the intake of functional foods enriched with plant sterols and stanols and decreases in serum LDL-cholesterol concentrations [7, 11, 12]. The meta-analysis by Katan et al. [7] identified 41 trials, with mainly fat-based foods such as spreads, mayonnaise and salad dressings. At an intake of 2 g/d, it was estimated that serum LDL-cholesterol was reduced by 10%, with little additional benefit at intakes higher than 2.5 g/d. An updated meta-analysis by Abumweis et al. [11] identified 59 studies that consisted of 95 relevant strata. Evidence was found for a dose-response effect with LDL-cholesterol reductions of respectively 0.29 mmol/L, 0.32 mmol/L and 0.42 mmol/L for plant sterol or stanol intakes of 1.5-2.0 g/d, 2.1-2.5 g/d and 2.5 g/d. This meta-analysis also highlighted the importance of the food matrix. Finally, Demonty et al. [12] aimed to quantify the dose-response relationship between LDL-cholesterol reductions and plant sterol or stanol intake, and the impact of different intervention characteristics such as plant sterol type (sterol vs. stanol) and food format. Eighty-four eligible trials with 141 strata were identified. The mean plant sterol / stanol intake was 2.15 g/d (range 0.45-9.00 g/d), which was consumed for 21 to 182 days. In 74 strata plant sterols were used while in 53 strata the experiments were conducted with plant stanols. The dose-response equation predicted a serum LDL-cholesterol lowering effect of 9% at the recommended daily intake of 2 g/d. In agreement with the estimation made by Katan et al. [7], the LDL-cholesterol lowering effect levelled off at an intake of 3 g/d and was 10.7%. Thus, also this meta-analysis indicated that intakes of plant sterols and stanols higher than 2.5 g/d offered little additional benefit. The results of these three large meta-analyses are in contrast with the outcomes of two recently published placebo-controlled intervention studies. Mensink et al. [17] showed a linear dose-response relationship between plant stanol intake and the reduction in LDL-cholesterol. Plant stanol intakes were 0, 3, 6 or 9 g/d and LDL-cholesterol decreased linearly up to 17.0% at the highest intake. This observation completely agreed with a study by Gylling et al., [18] who found a reduction of 17.4% after an intake of 8.8 g/d of plant stanols. Results from studies with high plant sterol intakes, are less consistent, Ayesh et al. [19] found an LDL-cholesterol reduction of 23% at an intake of 8.6 g/d of plant sterols. This effect may however been slightly overestimated, as the cholesterol content of the control and experiment margarines were not comparable. Clifton et al. [20] provided 6.6 g/d of plant sterols to 35 subjects and reported an LDL-cholesterol reduction of 12.4%. In contrast, Davidson et al. [21] found no additional effect on LDL-cholesterol when an intake of 9 g/d of plant sterols was compared

with that of 3 g/d. The characteristics of the described studies performed with a high dose plant sterol or stanol intake are given in **table 2.1**.

Table 2.1. Characteristics of intervention studies performed with high dose intake plant sterols or stanols

Design and treatment period	Population	Type of sterol	Food product	Daily intake (g)	Change in TCH (mmol/L, %)*	Change in LDL-C (mmol/L, %)*
Ayesh et al., 1999 [19]	Parallel, men 3 weeks, women 4 weeks	Plant sterol	Margarine	8.6	-0.76, 15.1	-0.58, 22.3
Clifton et al., 2004 [20]	Parallel, 6 weeks	Plant sterol	Bread, breakfast cereal and spread	6.6	-0.55, 8.4	-0.55, 12.4
Davidson et al., 2001 [21]	Parallel, 8 weeks	Plant sterol	Spread and salad dressing	3 (n=21) 6 (n=19) 9 (n=23)	-0.16, 3.0 -0.34, 6.3 -0.28, 5.3	-0.01, 0.3 -0.16, 4.8 -0.14, 4.2
Gylling et al., 2009 [18]	Parallel, 10 weeks	Plant stanol	Margarine and oat-based drink	8.8	-0.69, 12.0	-0.60, 17.4
Mensink et al., 2010 [17]	Parallel, 4 weeks	Plant stanol	Margarine and soy-based yoghurt	3 (n=24) 6 (n=22) 9 (n=25)	-0.35, 5.3 -0.48, 7.2 -0.79, 12.1	-0.32, 7.3 -0.51, 11.8 -0.72, 17.0

*Change from baseline when compared to control group
Abbreviations: TCH=total cholesterol concentrations, LDL-C=low-density cholesterol concentrations

Up till now, 5 studies have provided far more plant sterols or stanols than currently advocated and it is important to monitor the effects of these high intakes on fat-soluble antioxidant concentrations. Several studies have found reduced concentrations of especially the lipophilic hydrocarbon carotenoids, like β -carotene and lycopene after plant sterol and stanol intake [22]. However, most high-dose studies did not show larger reduction in antioxidant concentrations than found at recommended intakes. Moreover, several parameters reflecting liver and kidney function were unchanged during the intervention periods. More studies for a longer duration and in other population groups are however warranted before advocating higher intakes than currently recommended. The high intake studies carried out so far should be regarded as proof-of-principle experiments, in which it is shown that LDL-cholesterol lowering can be extended over the currently advocated intakes, although evidence regarding it is still contradictory.

Plant sterols and stanols are currently incorporated into a wide variety of food products. There is an ongoing discussion concerning the types of food that yields the most efficient LDL-cholesterol reduction. Abumweis et al. reported a larger reduction in LDL-cholesterol when plant sterols and stanols were incorporated into food products like fat spreads, mayonnaise and salad dressing or milk and yoghurt as compared with food products, such as cereal and chocolate bars, orange juice, non-fat beverages and bread [11]. On the other hand, the meta-analysis by Demonty et al. indicated that the food characteristics (fat-based vs. non fat-based formats, dairy vs. non-dairy formats, free plant sterol/stanols vs. esterified forms, and plant sterols vs. stanols) did not influence its LDL-cholesterol lowering efficacy [12]. The frequency of intake may also be important. Plat et al. compared the effects of once per day compared to a 3-times/d intake of plant stanols and reported no difference in LDL-cholesterol lowering between the treatments [23]. Demonty et al., however, concluded that most evidence pointed towards a larger efficacy of plants sterols and stanols when consumed at multiple time points [12]. In this respect, when consumed once a day, the time of consumption may be essential. Abumweis et al. showed that consumption of a single dose of plant sterols in the morning failed to lower LDL-cholesterol concentrations [24]. It seems that plant sterols or stanols should be consumed during or after a meal, preferably a larger meal like lunch or dinner [11].

Effects beyond LDL-cholesterol lowering

Besides the well-characterized LDL-cholesterol lowering effects, studies are emerging showing that plant sterols and stanols can also affect other lipid parameters. Until recently, almost all studies reported that plant sterol or stanol intake did not change serum high-density (HDL) cholesterol or triacylglycerol (TAG) concentrations. In some studies, slight changes were found, but these changes were most likely due to other dietary changes and not to plant sterol or stanol intake per se [25]. However in a recent meta-analysis, Naumann and co-workers analysed data from five well-controlled intervention studies with

plant stanols performed at their department and reported that changes in HDL cholesterol depended on baseline concentrations. In subjects with low concentrations at the start of the study, slight increases were observed, while decreases were seen in subjects with high baseline concentrations. Without this differentiation, the mean change in HDL cholesterol in the intervention groups was zero. Results of this meta-analysis also suggested that plant stanols lowered fasting TAG concentrations, especially in individuals with elevated baseline TAG concentrations [26]. A reason why this effect was not observed in earlier studies could have been due to a lack of statistical power, as most participants in the trials had normal to low baseline TAG concentrations. Therefore, Theuvsen et al. [15] designed a study to specifically evaluate the effect of plant stanol supplementation on serum TAG changes in a population with elevated fasting serum TAG concentrations. **Figure 2.1** indeed shows that serum TAG concentrations hardly changed in subjects with baseline TAG concentrations <2.3 mmol/L (change of 0.09 mmol/L compared to controls), while a significant TAG reduction was observed in subjects with higher baseline TAG concentrations (change of -0.53 mmol/L compared to controls).

Figure 2.1. Changes in TAG concentrations (mmol/L) for subjects with moderate (1.7-2.3 mmol/L) and high baseline (>2.3 mmol/L) concentrations.

Also in a study performed with metabolic syndrome patients, who are characterized by increased serum TAG concentrations, a significant reduction of 32% in fasting serum TAG concentrations was found after consumption for 9 weeks of 2 g/d of plant stanols provided via a yoghurt drink [27]. These findings show that functional foods enriched with plant stanols are not only useful to lower serum LDL-cholesterol concentrations, but may also be beneficial to lower serum TAG concentrations in specific population groups. These effects may not be exclusive for plant stanol esters. A daily intake of 1.6 g of plant sterols provided via a low-fat drinkable yoghurt reduced serum TAG concentrations with 12.9% in

hypercholesterolemic subjects with normal TAG concentrations [28]. In addition, Judd et al. showed that 3.6 g/d of plant sterols reduced not only serum LDL-cholesterol, but also serum TAG concentrations in healthy volunteers [29]. Thus, these two studies indicate that plant sterols may reduce serum TAG concentrations when provided in a low-fat format. More interestingly, these effects were found in populations without elevated baseline serum TAG concentrations, which contrasts the findings of Naumann et al., [26] suggesting that effects were hardly present in subjects with normal serum TAG concentrations. It should, however, be taken into account that at least 50 other studies with plant sterol esters in normotriglyceridemic subjects did not show these effects. **Table 2.2** shows an overview of the described studies that showed an effect of plant sterol or stanol ester consumption on serum TAG concentrations. A meta-analysis with all published studies that measured serum TAG concentrations after plant sterol or stanol consumption is lacking. It would be valuable to carry out such a meta-analysis according to the same principles as has been used to examine effects on LDL-cholesterol concentrations [11], also focusing on factors like the food matrix and intake frequency.

The most widely accepted mechanism via which plant sterols and stanols reduce LDL-cholesterol is through the displacement of cholesterol from mixed micelles, since plant sterols and stanols are more hydrophobic than cholesterol [30]. This replacement reduces micellar cholesterol concentrations and consequently cholesterol absorption. It has also been shown in Caco-2 cells that plant stanols were able to up-regulate the expression of ABC-transporters, which could lead to an increased excretion of cholesterol by the enterocyte back into the lumen [31]. Each of these two proposed mechanisms will result in a reduced intestinal cholesterol absorption and thus in a reduced cholesterol flux through the chylomicrons to the liver. A reduced flux to the liver will lead to a compensatory increase in hepatic endogenous cholesterol synthesis and an up-regulated hepatic LDL receptor expression, ultimately resulting in reduced serum LDL-cholesterol concentrations. A possible mechanism via which plant sterols and stanols might lower serum TAG concentrations is by reducing hepatic production of large TAG-rich VLDL-1 particles. This hypothesis was evaluated by Plat et al. [32], who showed in two independent studies that plant stanol esters significantly lowered serum VLDL-1 particle concentrations. One study was carried out in normocholesterolemic subjects, and the other study in subjects with the metabolic syndrome subjects. Obviously, effects were much more pronounced in the metabolic syndrome subjects, who had higher circulating VLDL-1 particle concentrations [32]. Although supportive, these data do not prove that the postulated hypothesis that the serum TAG-lowering effect of plant stanols is caused by a reduction in the hepatic production of large TAG-rich VLDL-particles. It is also possible that serum VLDL-1 particle concentrations are reduced via an increased clearance rate of VLDL-1. This, however, demands further study. The mechanism by which ezetimibe, like plant sterols and stanols a potent intestinal cholesterol absorption inhibitor, lowers serum TAG concentrations in humans involves a reduced production of VLDL particles, thereby lowering VLDL pool size [33, 34] with a concomitant reduction in hepatic lipid

Table 2.2. Characteristics of studies showing an effect of plant stanol or stanol consumption on TAG concentrations

Panel A. Meta-analysis by Naumann et al*													
Daily intake (g)		Predicted change in TCH				Predicted change in LDL-C				Predicted change in TAG			
		Baseline	Baseline	Baseline	Baseline	Baseline	Baseline	Baseline	Baseline	Baseline	Baseline	Baseline	Baseline
		4.0	6.0	8.0	mmol/L	mmol/L	mmol/L	mmol/L	mmol/L	mmol/L	mmol/L	mmol/L	mmol/L
2	mmol/L	-0.17	-0.27	4.4	4.6	6.9	6.8	6.8	1.0	3.8	4.7		
	%	4.2	4.4										
3	mmol/L	-0.25	-0.40	-0.55	-0.31	-0.41	-0.51	-0.21	-0.02	-0.40	-0.21		
	%	6.2	6.7	6.9	10.3	10.2	10.1	1.5	5.7	7.1			
4	mmol/L	-0.33	-0.54	-0.74	-0.41	-0.55	-0.68	-0.02	-0.15	-0.28			
	%	8.3	8.9	9.2	12.7	13.6	13.5	2.0	7.6	9.5			
*Meta-analysis (2008) was performed on 5 studies (n=301), which individually did not show an effect on TAG concentrations. Foods were provided via margarines and low-fat yoghurts and treatment periods ranged from 21-56 days													
Panel B. Intervention studies													
Reference	Design and treatment period	Population	Type of sterol	Food product	Daily intake (g)	Change in TCH (mmol/L, %)*	Change in LDL-C (mmol/L, %)	Change in TAG (mmol/L, %)					
Judd et al, 2002 [29]	Cross-over, 3 weeks	N=53	Plant sterol	Salad dressing	3.6	-0.36, 7.5	-0.32, 10.8	-0.22, 7.8					
Plana et al, 2008 [28]	Parallel, 6 weeks	N=83	Plant sterol	Yoghurt drink	1.6	-0.36, 6.1	-0.36, 9.5	-0.20, 12.9					
Plat et al, 2009 [27]	Parallel, 9 weeks	N=36	Plant stanol	Yoghurt drink	2	-0.81, 10.9	-0.98, 15.5	-0.46, 31.7					

*Change from baseline when compared to control group
Abbreviations: TCH=total cholesterol concentrations, LDL-C=low-density cholesterol concentrations, TAG=triacylglycerol concentrations

content [35]. Whether plant sterols and stanols also affect intra-hepatic fat accumulation warrants further research.

A reduction of 32% in serum TAG concentrations, as seen in the study by Plat et al., [27] is in the same magnitude as described for fish oils [36, 37]. Fish oils are nowadays one of the very few nutritional strategies to lower serum TAG concentrations. An interesting question is whether effects of plant sterols and stanols on serum TAG concentrations are additional to the effects of fish oils. Demonty et al. performed a study in which they compared a diet rich in plant sterols esterified with sunflower oil, a diet rich in plant sterols esterified with fish-oil and a diet rich in regular fish oil supplementation. No change in TAG concentration was seen after the diet enriched with plant sterols esterified with sunflower oil, while a marked decrease was seen after the fish-oil supplementation and an even more pronounced decrease was seen after the diet enriched with fish-oil esters of plant sterols. The authors attribute this stronger TAG decrease of the combination therapy to the plant sterol content of the intervention. However, it is difficult to make this conclusion, as the design of the study does not allow making these assumptions. The composition of the fish-oil supplementation was not the same for both interventions; the combination therapy contained, next to plant sterols, also a much higher content of fish oils. Therefore, it is not possible to determine whether the additional TAG reducing effect in the combination therapy stems from the additional plant sterol or higher fish oil content of the intervention. More studies are warranted to provide evidence that even larger effects in serum TAG concentrations can be achieved by combination strategies and to help find mechanistic explanations underlying the observed effects. Finally, Ford et al. [38] estimated that about one-third of the population is characterized by elevated fasting serum TAG concentrations. Moreover, Sarwar et al. [39] identified in 29 prospective studies that elevated fasting TAG concentrations are an independent risk factor for CVD. The high prevalence and increased risk of elevated TAG concentrations indicates the need and potential clinical benefit for TAG lowering interventions.

Potential atherogenicity

Among others, Naumann et al. have shown that the increase in serum plant sterol concentrations is positively related to plant sterol intake, while plant stanol ester consumption lowers serum plant sterol concentrations [40]. The implication of increased serum plant sterol concentrations is not clear, but speculations about a potential undesirable effect were first brought up after the identification of the disease phytosterolemia. Phytosterolemia is a rare autosomal-recessively inheritable sterol storage disease that has only been identified in ± 200 patients worldwide (personal communication D. Lütjohann). The disease is characterized by very high plant sterol concentrations ranging from 10-65 mg/dL while the reference range in non-affected subjects is below 1 mg/dL. Despite high serum plant sterol concentrations, serum cholesterol concentrations are normal or only slightly

increased. Patients with this disease exhibit tendon and tuberous xanthomas and have a strong predisposition to develop premature coronary atherosclerosis [41]. The cause of this disease is a mutation in the ABCG5/8 half transporters, which results in a decreased secretion of absorbed sterols from the enterocytes back into the lumen, as well as a reduced plant sterol excretion from the liver into bile [42]. The fact that these patients are confronted with severe vascular diseases at a relatively young age, despite normal cholesterol concentrations, raised the issue about the atherogenicity of increased plant sterol concentrations. Although many clinical trials have unequivocally shown that plant sterols and stanols lower serum LDL-cholesterol concentrations, effects on clinical endpoints related to CVD are not clear. Of course, the reduction in LDL-cholesterol is a strong indication that CVD risk will be lowered. However, the potential association between elevated plant sterol concentrations and CVD risk might influence the outcome.

The potential association between circulating plant sterol concentrations and CVD risk has been investigated in several prospective and cross-sectional epidemiological studies with contradictory results. Some large prospective cohort studies have shown that elevated concentrations of plant sterols are associated with an increased risk of CVD [43, 44]. Elevated plant sterol concentrations have also been associated with a family history of premature CVD in hypercholesterolemic subjects [45, 46]. More recent studies have also related increased serum plant sterol concentrations with an increased mortality risk. For example, Silbernagel et al. [47] have shown that serum campesterol concentrations were associated with increased all cause and cardiovascular mortality in a large cohort of subjects who were referred to the hospital for an angiogram. In addition, Teupser et al. [48] have observed that single nucleotide polymorphisms (SNPs) in the gene encoding for ABCG8 were not only associated with increased serum campesterol concentrations, but also with a risk for coronary artery disease. This indicates that genetic variants affecting plant sterol metabolism may also be related to the susceptibility to develop coronary artery disease. However, despite this list of studies suggesting possible atherogenic effects of plant sterols, a number of other prospective studies did not find an association between serum plant sterol concentrations and CVD risk [49, 50], while two studies even indicated that elevated plant sterol concentrations were associated with a decreased CVD risk [51, 52]. In **Table 2.3 and 2.4**, an overview is provided in which characteristics are given of the studies that have examined the potential association between increased plant sterol concentrations and CVD risk.

It has been postulated that the ongoing controversy about the potential atherogenicity of plant sterols may be due to the formation of oxyphytosterols, the oxidized forms of plant sterols. Oxyphytosterols can be formed during physical processes, such as heating and radiation, by non-enzymatic processes, such as reactions with reactive oxygen species (ROS) or enzymatically by specific cytochrome P450 mono-oxygenases. The side-chain of cholesterol can be oxidized enzymatically and the nucleus non-enzymatically, the latter probably being a radical driven process. For plant sterols, only the nucleus can be oxidized

Table 2.3. Characteristics of prospective cohort studies investigating the potential atherogenicity of increased plant sterol concentrations

Authors	Population (age, years)	Mean follow-up (years)	Endpoint	Concentration plant sterols (mg/ dL)	Outcome parameter	Main conclusion
Fassbender et al., 2008 [51]	1192 men and women 125 events (65-89)	10	Coronary heart disease (CHD)	<i>Cases</i> sitosterol: 0.30, campesterol: 0.32 <i>Controls</i> sitosterol: 0.34, campesterol: 0.36	Odds ratio (95% CI) sitosterol: 0.78 (0.62-0.98), $p<0.05$	Significantly lower serum plant sterol concentrations in patients with CHD, suggesting a neutral or beneficial effect
Silbernagel et al., 2010 [47]	887 men and women 94 events (63 ± 11)	7	Cardiovascular (CV) mortality	Not reported	Hazard ratio camp:chol ratio 1 st tertile: 1.0 2 nd tertile: 1.39, NS 3 rd tertile: 2.06, $p<0.01$	High cholesterol absorption might be predictive for CV mortality
Strandberg et al., 2010 [52]	232 men 101 events (57-64)	22	Total mortality	<i>Cases</i> sitosterol: 0.29 campesterol: 0.42 <i>Controls</i> sitosterol: 0.32 campesterol: 0.47	Hazard ratio (95% CI) highest vs lowest tertile sito:chol ratio: 0.51 (0.30-0.87), $p<0.05$	Higher sitosterol-to- cholesterol ratio was associated with a decreased mortality risk

NS=non significant

Table 2.4. Characteristics of case-control studies investigating the potential atherogenicity of increased plant sterol concentrations

Authors	Population (age, years)	Disease	Concentration plant sterols (mg/dL)	Outcome parameter	Main conclusion
Assmann et al., 2006 [43]	159 patients 318 controls (35-65)	Coronary event, defined as sudden cardiac death or a definite fatal or nonfatal myocardial infarction	<i>Cases</i> sitosterol: 0.20 campesterol: 0.41 <i>Controls</i> sitosterol: 0.18 campesterol 0.36	Hazard ratio sitosterol >0.22mg/dl: 1.81, $p=0.015$ sitosterol >0.22mg/dl + global risk >20%: 3.16, $p=0.032$	High serum sitosterol concentration associates with an increased occurrence of coronary events, especially in men at high global risk of coronary heart disease
Matthan et al., 2009 [44]	155 patients 414 controls (68, matched)	Cardiovascular disease (CVD)	<i>Cases</i> sitosterol: 0.23 campesterol: 0.32 <i>Controls</i> sitosterol: 0.21 campesterol: 0.27	Odds ratio sitosterol: 1.86, $p<0.05$ campesterol: 2.47, $p<0.05$	Disturbances in cholesterol homeostasis are associated with an increased CVD risk
Pinedo et al., 2006 [50]	758 men and women 373 events (65, matched)	Coronary artery disease (CAD)	<i>Cases</i> sitosterol: 0.21 campesterol: 0.31 <i>Controls</i> sitosterol: 0.21 campesterol: 0.32	Odds ratio (95% CI) In highest tertile sitosterol: 0.79 (0.56-1.13), NS campesterol: 0.97 (0.68-1.39), NS	Higher concentrations of sitosterol and campesterol are not adversely related to CAD

Table 2.4. Continued

Rajaratnam et al., 2000 [76]	48 patients 61 controls (50-55)	Coronary artery disease (CAD)	Cases sitosterol: 0.33 campesterol: 0.61 <i>Controls</i> sitosterol: 0.27 campesterol: 0.49	Odds ratio sito:chol ratio: 1.01, $p < 0.05$ camp:chol ratio: 1.01, $p < 0.05$	Increased sitosterol- and campesterol- to-cholesterol ratio's, indicating high cholesterol absorption, are associated with CAD
Silbernagel et al., 2009 [58]	Friesinger Score (FS) A (0-1): N= 502 (58 ± 12) B (2-4): N= 553 (64 ± 10) C (5-8): N= 793 (64 ± 10) D (9-15): N= 592 (65 ± 9)	Severity of coronary artery disease (CAD) as assessed by the FS	Not reported	Difference in camp:chol ratio (%) in FS-categories A - B 3.6, $p = 0.26$ C 5.4, $p = 0.09$ D 7.8, $p = 0.03$	High cholesterol absorption, indicated by increased campesterol-to- cholesterol ratio is associated with more severe CAD
Sudhop et al., 2002 [45]	26 patients 27 controls (46-77)	Having a positive family history of coronary heart disease (CHD)	<i>FH+</i> sitosterol: 0.40 campesterol: 0.50 <i>FH-</i> sitosterol: 0.31 campesterol: 0.38	Mean difference sitosterol: 0.09, $p < 0.01$ campesterol: 0.12, $p < 0.05$	High campesterol and sitosterol concentrations are an additional risk factor for CHD

Table 2.4. Continued

Sutherland et al., 1998 [77]	44 patients (56 ± 7)	Extent and severity of coronary artery disease (CAD)	Cases sitosterol: 0.22 campesterol: 0.46	Correlation (r) log sitosterol&no.lesions >50% stenosis: $r=0.312$, $p<0.05$ log sito:chol&no.lesions >50% stenosis: $r=0.319$, $p<0.05$	High sitosterol concentration, as well as an increased sitosterol-to- cholesterol ratio is associated with an increased extent and severity of CAD
Wilund et al., 2004 [49]	532 patients 2010 controls (30-67)	Coronary atherosclerosis as assessed by coronary artery calcium (CAC)	Cases sitosterol: 0.13* campesterol: 0.22* Controls sitosterol: 0.13* campesterol: 0.23*	Correlation (r) sitosterol&CAC: $r=-0.025$, $p=0.27$ campesterol&CAC: $r=-0.010$, $p=0.63$	No association between concentrations of sitosterol or campesterol and CAC
	1032 patients 1792 controls (30-65)	Having a positive family history (FH) of coronary heart disease (CHD)	FH+ sitosterol: 0.13* campesterol: 0.23* FH- sitosterol: 0.13* campesterol: 0.23*	Mean difference no difference in mean sitosterol and campesterol concentrations	No association between concentrations of sitosterol or campesterol and CHD family history
Windler et al., 2009 [78]	186 patients 231 controls (64, matched)	Coronary heart disease (CHD)	Cases sitosterol: 0.15 campesterol: 0.24 Controls sitosterol: 0.17 campesterol: 0.27	Odds ratio (95% CI) sitosterol: 1.02 (0.77- 1.43) campesterol: 1.03 (0.87- 1.26)	Sitosterol and campesterol concentration do not seem to have an impact on CHD

*Concentration expressed as median values - NS=non significant

at the double bond at the C5-C6 position [53]. Due to sterical hindrance because of different structures, the side-chains of plant sterols is - unlike the side chain of cholesterol - not accessible for oxidizing enzymes and can, as far as known yet, not be oxidized [54]. The ring-oxidation products of cholesterol are potentially atherogenic, as indicated by animal studies [55]. Plant stanols do not have a double bond in the steroid nucleus and can therefore not be oxidized [53]. Interest in oxyphytosterol concentrations as mediators of CVD risk originates from the presence of elevated oxyphytosterol concentrations in serum of phytosterolemic patients [56]. In this study, it was shown that about 1.4% of sitosterol was present in its oxidized form. No oxyphytosterols could be identified in serum of healthy volunteers, which was probably due to a lack of sensitivity of the method. In a later study, Grandgirard et al. used a quantification method with a lower detection limit and was able to identify oxyphytosterols in plasma from healthy volunteers as well [57]. The question now is whether oxyphytosterols are atherogenic as has been suggested for oxysterols, or not. In vitro experiments from our group using human coronary arterial endothelial cells (HCAECs) have indicated that especially oxyphytosterols increased MCP-1 expression (unpublished results). Therefore the question whether the existing discrepancy in associations between elevated plant sterol concentrations and CVD risk is related to differences in oxyphytosterol concentrations, certainly deserves attention.

A second possible explanation for the association between elevated serum plant sterol concentrations and CVD risk relates to the finding that increased serum plant sterol concentrations are a marker for increased intestinal cholesterol absorption. Recently, Silbernagel et al. have determined concentrations of cholesterol absorption and synthesis markers in a large cohort of subjects undergoing angiography [58]. In this cohort, increased concentrations of both campesterol and cholestanol (markers of fractional cholesterol absorption) were associated with an increased severity of coronary artery disease (CAD), while a negative association was found for the cholesterol synthesis marker lathosterol. As this association was also evident for cholestanol, also a marker for fractional cholesterol absorption, a specific atherogenic role of (oxidized) plant sterols may be not likely, whereas it is more likely that the higher fractional intestinal cholesterol absorption may be the underlying reason for this association. This study also identified a positive association between an increased campesterol-to-lathosterol ratio and the severity of CAD. Since the campesterol-lathosterol ratio combines cholesterol absorption and synthesis, the question remains what the merit is of this ratio? Weingärtner et al. [59] found that the ratio campesterol-to-lathosterol was associated with CAD severity in patients with severe aortic valve stenosis. However, in this particular study the increase in the ratio was driven by a continuous lower lathosterol concentration, instead of higher cholesterol absorption markers, for which no differences were found. This means that a one-directional change of this ratio can thus result from two different processes. Since these two studies indicated a positive association between this ratio and CAD severity, by means of two different underlying and causative metabolic changes, no real value can be ascribed to this ratio. To our opinion, changes in

the absolute markers are favourable over using the described ratio. This implies that the finding by Silbernagel et al., [58] in which the so-called ‘absorbers’ display an increased severity of CAD, still holds true. Weingärtner and co-workers also isolated aortic valve cusps via elective aortic valve replacement and found a positive correlation between plant sterol concentrations in aortic valve cusps and the degree of aortic valve stenosis [59]. In an earlier study, this group also observed a positive association between the intake of plant sterols with sitosterol and campesterol concentrations in both serum and in aortic valve cusps [60]. Although these associations do not prove causality, they may suggest some role or another for plant sterols in the pathogenesis of aortic valve disease. However, in another study Weingärtner et al. [61] found no correlation between cholesterol absorption markers and carotid intima-media thickness (cIMT), while they did find a significant correlation between cholesterol synthesis markers and cIMT. This contrasts the suggestion that (sterol) absorbers as identified by elevated plant sterol concentrations are at risk. Thus, the question remains what causes the apparent inconsistency between studies examining the association between serum plant sterols and CVD risk. It has been suggested that age could play a role. Strandberg et al. have suggested that higher plant sterol concentrations may increase CVD risk at a younger age, but could be protective in older subjects [52]. In that prospective study in middle-aged men with an average of 61 years, higher plant sterol concentrations at baseline were associated with a decreased CVD risk 22 years later. Also the LASA study showed a reduced CVD risk associated with increased plant sterol concentrations at an older age [51]. In LASA, the average age of the subjects was 75 years, which is higher than in most other studies. The suggestion that increased plant sterol concentrations may protect at an older age should however be considered carefully, since it can also be argued that subjects with a low cholesterol absorption (low plant sterol concentrations) and a high cholesterol synthesis may have died before inclusion in these studies. Therefore, Strandberg et al. [52] concluded that the relationship between non-cholesterol sterols and CVD risk might be an example of ‘reverse epidemiology’. This paradox states that factors which are harmful at younger age, such as overweight and high LDL-cholesterol concentrations, might look beneficial at older age [62]. Although other studies in which a negative association between serum plant sterols with CVD risk was observed were performed in younger populations [61], this concept is still very interesting to explore into more detail.

Endothelial function

Recent evidence suggests that consumption of plant sterols or stanols might affect endothelial function. This effects might either be caused by the plant sterols directly or indirectly as a consequence of the reduced serum LDL-cholesterol concentrations. Unfortunately, the number of studies that have investigated these effects is limited. Endothelial dysfunction is a reflection of an early, but still reversible phase in the development of atherosclerosis and studies have indicated that the presence of endothelial dysfunction is predictive for future

cardiovascular events. In other words, preservation or improvement of endothelial function in response to treatment may decrease the risk for future cardiovascular events. Endothelial function can be determined via a wide variety of available techniques and flow-mediated dilatation (FMD) is probably the most commonly used and validated technique to measure endothelial function [63]. It is well known that statin therapy reduces LDL-cholesterol, which is associated with normalization of endothelial function [64]. It is however also possible, that effects are due to the pleiotropic effects of statins [65]. Effects of plant sterols and stanols on endothelial function have been examined in several studies. Results, however, are not conclusive. De Jongh et al. [66] performed a study in 41 children (5-12 years old), which were diagnosed with heterozygous familial hypercholesterolemia and had already impaired endothelial function. Although the consumption of 2.3 g/d of plant sterols for 4 weeks decreased serum LDL-cholesterol concentrations by 14%, this decrease was not associated with an improvement in endothelial function. Jakulj et al. [67] evaluated the effect of plant stanol supplementation on endothelial function in 42 children (7-12 years old) with heterozygous familial hypercholesterolemia. Serum LDL-cholesterol concentrations decreased with 9.2% after a 4 week intervention in which the children consumed 2.0 g/d of plant stanols. Again, despite a significant reduction in serum LDL-cholesterol, endothelial function did not improve. Although these two studies do suggest that plant sterol and stanol consumption is an effective strategy to lower serum LDL-cholesterol in children with familial hypercholesterolemia, both studies did not provide any evidence for an effect on endothelial function. In line with these two studies in children, Hallikainen et al. [68] investigated the effects of consuming 2.0 g/d of plant stanol and sterol esters for 10 weeks on endothelial function in hypercholesterolemic adult subjects. The intervention reduced LDL-cholesterol by 9-12% as compared to controls, but there was no change in endothelial function as measured by FMD. Brachial artery diameter was also determined, which did not change after the control treatment or the plant stanol treatment. The brachial artery diameter was however diminished after the plant sterol treatment, but the clinical relevance of such a reduction remains unclear. Also Raitakari et al. [69] assessed in hypercholesterolemic subjects FMD and carotid arterial compliance to determine the effect of consuming 2 g/d of plant stanols for a period of 12 weeks on endothelial function and arterial elasticity. The intervention reduced the serum LDL-cholesterol by 9%, but FMD and arterial elasticity did not change. However, subgroup analyses showed that endothelial function and arterial elasticity were improved in subjects who were characterized with below average values for these parameters at the start of the study. Similar results were found by De Jong et al., [70] who investigated long-term (85 weeks) plant sterol or stanol ester consumption on endothelial function and arterial stiffness in patients on statin treatment. They did not find an effect on vascular function in the whole study population, but in a subgroup of patients with more pronounced CVD, as indicated by high baseline levels of matrix metalloproteinase-9, an improvement in endothelial function and arterial stiffness was observed. These results seem promising for a possible role of plant sterols and stanols in improving vascular

function for subjects with suboptimal vascular function. The possible effects of plant sterols and stanols on endothelial function are only minor in comparison to the effects of statins, which have a strong ability of improving endothelial function. Plausible explanations for this discrepancy in improving endothelial function between statins and plant sterols and stanols could be ascribed to the more potent LDL-cholesterol lowering effects of statins as compared to those of plant sterols and stanols [66]. Another possibility is that statins exert pleiotropic effects on the vasculature [71]. Since these pleiotropic effects have not been found for plant sterols and stanols, this latter option could be a likely explanation for the difference in potency between statins and plant sterols and stanols regarding their ability to improve endothelial function.

Besides effects on the larger vessels as measured via techniques like FMD, plant sterol and stanol consumption might change the microcirculation. Cardiovascular disease is known to be a disease of the large vessels, but evidence is accumulating that disturbances in the microcirculation also predict future CVD risk [72]. Studies have shown that a larger retinal venular diameter is associated with several established atherosclerotic risk factors [73]. Until now, only one study has investigated the long-term effects of plant sterol and stanol consumption on the retinal vasculature. Kelly et al. [74] performed a study in which subjects were asked to consume 2.5 g/d of plant sterols or plant stanols for a period of 85 weeks. They identified a non-significant increase in venular diameter after consumption of plant sterols, when compared to the changes in the plant stanol and control group. Linear regression analysis however showed a significant positive association between increases in cholesterol-standardized serum campesterol concentrations and increases in venular diameter. The functional consequences of the findings in terms of CVD risk are however unknown. Kelly et al. observed an increase in venular diameter of 2.3 μm in the sterol group when compared to the change in both the stanol and control group. Kawasaki et al. [75] showed that subjects suffering from the metabolic syndrome had on average an increase of 4.69 μm in their venular diameter, when compared to healthy subjects. Ikram et al. estimated that an increase in venular diameter of 0.4 μm was associated with a one point increase in carotid plaque-score and that current smokers had on average a 10 μm larger venular diameter than non-smokers [73]. Therefore, these findings warrant further investigation in terms of the functional consequences of diet-induced changes in venular diameter.

To summarize, the role of plant sterols and stanols as cholesterol lowering agents is well characterized. Several meta-analyses have shown that consumption of 2.5 g/d of plant sterols and stanols can lower serum LDL-cholesterol concentrations up to ~10%, while recent studies have shown that additional benefit can be achieved at higher intakes. Besides their cholesterol lowering effect, evidence is now emerging that plant sterols and stanols are also able to exert beneficial effects on triacylglycerol metabolism, which makes them more attractive for metabolic syndrome-like populations. On the other hand, there is an

ongoing debate whether increased serum plant sterol concentrations are associated with an increased CVD risk. Finally. Some studies suggested that plant sterols and stanols might improve endothelial dysfunction in those at risk, although evidence is still limited and more research is needed.

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Chapter 3

Effects of plant sterol or stanol enriched margarine on fasting plasma oxyphytosterol concentrations in healthy subjects

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Abstract

Consumption of plant sterols and plant stanols reduces low-density lipoprotein cholesterol (LDL-C) concentrations. At the same time, plasma plant sterol concentrations will increase after plant sterol consumption, but decrease after plant stanol consumption. In contrast to plant stanols, plant sterols can undergo oxidation and form oxyphytosterols. Findings from *in vitro* and animal studies suggest that oxyphytosterols might be atherogenic. The objective was to examine whether plant sterol and stanol consumption changes fasting plasma oxyphytosterol concentrations. A randomised, double blind, cross-over study was performed in which 43 healthy subjects (18-70 years) consumed for 4 weeks a plant sterol enriched (3.0 g/d of plant sterols), a plant stanol enriched (3.0 g/d of plant stanols), and a control margarine separated by wash-out periods of 4 weeks. Oxyphytosterol concentrations were determined in BHT-enriched plasma via GC-MS. Compared to control, serum LDL-C concentrations were reduced after plant sterol (-8.1%; $p < 0.001$) and plant stanol consumption (-7.8%; $p < 0.001$). Plant sterol consumption did not change plasma oxyphytosterol concentrations. On the other hand, intake of the plant stanol margarine reduced 7 β -OH-campesterol by 0.07 ng/mL (~14%; $p < 0.01$) and by 0.07 ng/mL (~15%; $p < 0.01$) compared with the control and sterol margarines, respectively. When standardized for serum cholesterol, effects on these oxyphytosterols were comparable. In addition, plant stanol intake reduced cholesterol-standardized 7-keto-campesterol levels compared with plant sterol intake ($p < 0.05$). Daily consumption of a plant sterol enriched margarine does not increase oxyphytosterol concentrations, while plant stanol consumption may reduce the concentrations of the oxidative plant sterol metabolites 7 β -OH-campesterol and 7-keto-campesterol. This trial is registered at clinicaltrials.gov as NCT01559428.

Introduction

Plant sterols are structurally related to cholesterol and only the side-chain configuration at the C24 position differs. Thus, both components consist of a steroid nucleus with a double bond present at C5-C6. This double bond is missing in plant stanols, the hydrogenated form of plant sterols. Humans are unable to synthesize plant sterols and stanols, which mean that serum plant sterols are by definition diet-derived. On average, serum sitosterol concentrations range from 0.12 to 0.62 mg/dL and campesterol concentrations from 0.27 to 1.08 mg/dL [1]. Serum plant stanol concentrations are approximately 100 times lower and vary between 2 and 10 µg/dL in the normal population [2].

Although plant sterol and plant stanol ester enriched products lower serum (low-density lipoprotein cholesterol (LDL-C) concentrations to the same extent at the recommended intake of approximately 2.5 g/day, they have a different effect on serum plant sterol concentrations; i.e. increased plant sterol consumption will elevate serum plant sterol concentrations, while an increased plant stanol consumption will lower serum plant sterol concentrations [3]. The relevance of increased serum plant sterol concentrations relates in great detail to the ongoing debate whether plant sterol concentrations are positively associated with cardiovascular disease (CVD) risk or not. The atherogenicity of increased plant sterol concentrations was first suggested after the identification of the rare autosomal-recessively inheritable sterol storage disease phytosterolemia. These patients are characterized by extremely high plant sterol concentrations ranging from 10-65 mg/dL and a strong predisposition for premature coronary atherosclerosis [4, 5]. Moreover, some epidemiological studies have suggested that also in the general population plasma plant sterol concentrations are positively associated with an increased risk to develop CVD [6-8]. Results are however far from conclusive, since there are also numerous studies that did not show this association [9]. How these inconsistencies between study outcomes can be explained is not known. One possibility is that the potential association between circulating plant sterols and CVD risk may not relate to plant sterols per se, but to the fact that serum plant sterols are a marker for intestinal cholesterol absorption [10]. In other words, it is possible that an increased absorption of cholesterol associates with CVD risk [11]. Alternatively, it is possible that oxidation of plant sterols - i.e. the formation of oxyphytosterols - is important to explain the potential association between plant sterol concentrations and CVD risk. Like cholesterol, plant sterols can undergo oxidation, as they possess a double bond between C5-C6 in the steroid nucleus (**Figure 3.1**).

Figure 3.1. Non-enzymatically formed oxysitosterol structures. Similar structures are also formed for campesterol and other plant sterols.

Ring-oxidation products of cholesterol may be atherogenic [12-14], while not much is known about the biological effects of ring-oxysterols. Oxysterols first received attention as potential mediators of CVD risk after it was shown that about 1.4% of circulating sitosterol in serum of phytosterolemic patients was present in its oxidized form [15]. Plant stanols do not have a double bond in the steroid nucleus and can therefore not oxidize [16]. In light of the widespread use of plant sterol enriched products, it is important to identify whether an increased intake of plant sterols will result in increased plasma oxysterol concentrations. Another interesting question is whether an increased plant stanol intake will not only decrease serum plant sterol concentrations, but will also change those of the oxysterols. Therefore, the objective of this study was to examine the effects of consuming plant sterol or stanol ester enriched margarines on fasting serum oxysterol concentrations in healthy volunteers.

Subjects and Methods

Subjects

Subjects were recruited through announcements in local newspapers and university buildings in Maastricht, the Netherlands, between February and April 2010. They were invited for two screening visits if they met the following criteria: aged between 18 and 70 years, BMI between 20 and 30 kg/m², no active cardiovascular disease or severe medical condition that might interfere with the study, no use of lipid-lowering medication or a medically prescribed

diet, stable body weight during the last three months, and no consumption of plant sterol- or plant stanol enriched products in the previous month. During the screening visits, weight and height were measured and two fasting blood samples were drawn to determine plasma glucose and serum lipid and lipoprotein concentrations. Forty-seven subjects (18 male and 29 female) were enrolled in the study. Two subjects did not complete the study due to time constraints and difficulties performing the venipuncture. In addition, two subjects were excluded before the analyses of the results: one male subject was not fasted during one blood sampling day and one female subject did not comply with margarine consumption. Baseline characteristics are shown in **Table 3.1**. All participants gave written informed consent before entering the study. The protocol was approved by the medical ethical committee of the Maastricht University Medical Centre+ (MUMC+). This trial is registered at clinicaltrials.gov as NCT01559428.

Table 3.1. Baseline characteristics of the subjects (n=43)

Age (years)	41 ± 18
Male / Female (n)	17 / 26
BMI (kg/m ²)	24.8 ± 2.8
Weight (kg)	73.2 ± 11.0
Height (m)	1.72 ± 0.1
Glucose (mmol/L)	5.25 ± 0.54
Total cholesterol (mmol/L)	5.72 ± 1.12
LDL cholesterol (mmol/L)	3.53 ± 1.06
HDL cholesterol (mmol/L)	1.69 ± 0.37
Triacylglycerol (mmol/L)	1.14 ± 0.46

Values are means ± SD

Diet and design

Subjects participated in a randomised placebo-controlled crossover trial, which consisted of three intervention periods of 4 weeks, separated by washout periods of 4 weeks. They were allocated to the intervention periods in a randomised order, based upon a computer-generated table with random numbers. During each period, subjects were asked to replace their own spread with the test margarine (70% fat) of which 20 gram had to be consumed on a daily basis. The daily intake of 20 gram of margarine provided no, or 3.0 g of plant

sterols or stanols per day, referred to as the control, sterol or stanol period. The margarines were packed in tubs of 140 grams each, providing margarine for 7 days. The margarines were similar in colour and taste and the tubs were colour coded to blind both subjects and investigators. All volunteers received instructions to consume the margarines divided over two meals, i.e. at lunch and diner. The margarines were all rapeseed oil based with (saturated) soy sterols as source of the plant sterols / stanols. Plant sterols and stanols were provided as fatty acid esters by transesterification of free plant sterols and stanols with rapeseed oil fatty acids. The plant sterol ester mixtures contained mainly sitosterol ester (43%), campesterol ester (25%) and stigmasterol ester (20%). Plant stanol mixtures were obtained by saturation of these sterols, resulting in sitostanol ester (76%) and campestanol ester (22%) (Raisio group, Raisio, Finland). Plant sterol, plant stanol and oxyphytosterol content of the margarines are shown in **Table 3.2**.

Table 3.2. Plant sterol, plant stanol and oxyphytosterol content of the margarines¹

	Control margarine	Plant sterol margarine	Plant stanol margarine
Sitosterol	0	64.8	1.4
Campesterol	0	38.1	2.4
Stigmasterol	0	29.6	0
Sitostanol	0	3.8	114.3
Campestanol	0	1.7	33.0
7 α -OH-sitosterol	0.35	6.15	0.19
7 α -OH-campesterol	0.09	2.48	0.08
7 β -OH-sitosterol	0.80	11.39	0.51
7 β -OH-campesterol	0.30	2.77	0.09
7-keto-sitosterol	1.67	5.41	2.31
7-keto-campesterol	1.67	5.54	2.23

¹Plant sterol and stanol content in $\mu\text{g}/\text{mg}$ and oxyphytosterol content in ng/mg

Subjects visited the university at the start of each period, and after 3 and 4 weeks of intervention. They received 4 tubs of margarine at the start of each period, the amount needed for 4 weeks. At the end of each week, the used margarine tubs had to be set aside and returned to the department to be weighed to determine compliance. Subjects completed a validated food frequency questionnaire (FFQ) at the end of all three intervention periods [17], and a dietician checked these questionnaires to calculate energy and nutrient intakes using the Dutch food composition table. Subjects were asked not to change their habitual

diet, level of physical exercise or use of alcohol throughout the three intervention periods in the study.

Blood sampling

Blood was sampled after an overnight fast. The same person performed all venipuncture at approximately the same time of the day. Blood was sampled at the start of each period and after 3 and 4 weeks of intervention. A clotting tube (Becton, Dickinson and Company, Franklin Lakes, NY, USA) was sampled at each occasion and serum was obtained by low-speed centrifugation at 1300x g for 15 min at room temperature, at least half an hour after venipuncture. Serum was stored at -80 °C and used for analysis of lipid and (apo)lipoprotein concentrations, and for plant sterol and plant stanol concentrations. An EDTA tube (Becton, Dickinson and Company, Franklin Lakes, NY, USA) was sampled at the start of each period and after 4 weeks and plasma was obtained by low-speed centrifugation at 1300x g for 15 min at 4 °C, and then stored at -80 °C. To avoid auto-oxidation, oxyphytosterol concentrations were determined in butylated hydroxytoluene (BHT)-enriched plasma; for this 10 µl BHT (25 mg/mL ethanol) was added per 1 mL of EDTA plasma, immediately after centrifugation.

Analyses

Total cholesterol (CHOD-PAP method; Roche Diagnostics, Mannheim, Germany), high-density lipoprotein cholesterol (HDL-C) (CHOD-PAP method; Roche Diagnostics, Mannheim, Germany) after precipitation of apoB-containing lipoproteins with phosphotungstic acid and magnesium ions, and triacylglycerol (TAG) concentrations, with correction for free glycerol, were analysed in serum enzymatically (GPO-Trinder; Sigma-Aldrich Corp., St. Louis, MO, USA). All samples from one subject were analysed within one run at the end of the study. LDL-C concentrations were calculated according to the Friedewald equation [18]. Apolipoprotein concentrations (apoA-1 and apoB100) were analysed using highly sensitive immunoturbidimetric assays (Horiba ABX, Montpellier, France). Lipid and (apo) lipoprotein concentrations from weeks 3 and 4 were averaged for data analysis. Plant sterol (sitosterol, campesterol), plant stanol (sitostanol, campestanol), cholesterol precursor (lathosterol), cholestanol and oxyphytosterol concentrations from week 4 were analysed by gas-liquid chromatography-mass spectroscopy (GC-MS) as described previously [19, 20]. Plant sterol, plant stanol and lathosterol concentrations were expressed as $10^2 \times \mu\text{mol}/\text{mmol}$ total cholesterol. The measured oxyphytosterols were 7α -hydroxy(OH)-sitosterol, 7α -OH-campesterol, 7β -OH-sitosterol, 7β -OH-campesterol, 7-keto-sitosterol, and 7-keto-campesterol according to the procedure as described by Husche et al. (20) and expressed as ng/mL. Oxyphytosterol concentrations were standardized for cholesterol concentrations and expressed as nmol/mmol cholesterol.

Statistics

To detect a change of 17 ng/mL in plasma oxyphytosterol concentrations with a power of 80% and a known within-subject variation on the response of 34 ng/mL (20), a sample size of 47 subjects was necessary, given an anticipated dropout rate of 10%. All data are presented as means $\pm$ standard deviations (SD). Effects of the experimental products were evaluated by a univariate analysis of variance (ANOVA) followed by Bonferroni's correction for multiple comparisons. Results were considered to be statistically significant if $p < 0.05$. Results were analysed to assess whether gender effects or carryover effects existed, and both effects were not present in the data. All statistical analyses were performed using SPSS 18.0 for Mac Os X (SPSS Inc., Chicago, IL, USA).

Results

Dietary intakes

As shown in **Table 3.3**, the average daily intakes of total energy (MJ/day), protein (energy%), carbohydrates (CHO, energy%), total fat (energy%), fatty acids (saturated fatty acids (SAFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA), energy%), fibre (g/day), cholesterol (mg/day) and vitamin E (mg/day) did not differ between the three intervention periods.

Serum lipids and lipoproteins

Serum lipid and lipoprotein concentrations are shown in **Table 3.4**. Compared with the control period, serum total cholesterol concentrations decreased by 0.30 ± 0.51 mmol/L (-5.3%; $p < 0.001$) in the sterol period and by 0.29 ± 0.42 mmol/L (-5.3%; $p < 0.001$) in the stanol period. These reductions could almost completely be ascribed to decreases in serum LDL-C concentrations. In the sterol period, serum LDL-C concentrations decreased by 0.29 ± 0.43 mmol/L (-8.1%) and in the stanol period by 0.26 ± 0.41 mmol/L (-7.8%; $p < 0.001$ for both periods). Serum HDL-C concentrations were not different between the three periods. This shift towards a more beneficial partitioning of cholesterol was also reflected in the total cholesterol/HDL cholesterol ratio, which decreased in the sterol period by 0.14 ± 0.36 (-3.1%; $p < 0.05$) and in the stanol period by 0.18 ± 0.35 (-4.9%; $p < 0.01$). Serum apolipoprotein B100 concentrations were reduced in the sterol period by 0.05 ± 0.09 g/L (-4.4%) and in the stanol period by 0.05 ± 0.08 g/L (-5.1%; $p < 0.01$ for both periods). No differences were found in serum TAG and apolipoprotein A1 concentrations between the three periods.

Table 3.3. Daily dietary intake during the study

	Control period	Plant sterol period	Plant stanol period
Energy (MJ)	10.6 ± 3.4	10.6 ± 3.2	10.5 ± 3.2
Fat (energy%)	37.5 ± 7.2	37.4 ± 6.5	37.4 ± 6.6
SAFA ¹	12.1 ± 3.1	12.2 ± 3.0	11.9 ± 3.0
MUFA	14.4 ± 3.2	14.6 ± 2.7	14.6 ± 3.0
PUFA	7.9 ± 2.4	8.2 ± 2.0	8.0 ± 1.8
Protein (energy%)	15.5 ± 3.6	15.4 ± 3.4	15.4 ± 3.2
CHO (energy%)	45.3 ± 9.5	44.4 ± 7.5	44.6 ± 8.1
Alcohol (energy%)	2.9 ± 2.9	2.6 ± 2.6	2.8 ± 3.2
Fibre (g/day)	23.5 ± 7.8	24.0 ± 7.5	23.3 ± 7.6
Cholesterol (mg/day)	221 ± 111	209 ± 92	205 ± 77
Vitamin E (mg/day)	13.1 ± 5.3	13.6 ± 5.8	13.3 ± 5.7

Values are means ± SD. All subjects (n=43) received the three dietary periods in random order.

¹SAFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, CHO: carbohydrates

Serum plant sterols, plant stanols, lathosterol and cholestanol

Cholesterol-standardized serum plant sterol, plant stanol, lathosterol and cholestanol levels are shown in Table 3.4. Sitosterol levels increased in the sterol period by $86.6 \pm 199 \text{ } 10^2 \times \mu\text{mol}/\text{mmol}$ cholesterol (49%; $p < 0.01$) compared with the control period and by $138 \pm 248 \text{ } 10^2 \times \mu\text{mol}/\text{mmol}$ cholesterol (167%; $p < 0.001$) compared with the stanol period. For campesterol, these values were $132 \pm 137 \text{ } 10^2 \times \mu\text{mol}/\text{mmol}$ cholesterol (66%; $p < 0.001$) and $214 \pm 138 \text{ } 10^2 \times \mu\text{mol}/\text{mmol}$ cholesterol (179%; $p < 0.001$). In addition, campesterol levels decreased in the stanol period by $82.3 \pm 53.3 \text{ } 10^2 \times \mu\text{mol}/\text{mmol}$ cholesterol (37.3%) compared with the control period ($p < 0.001$).

Sitostanol levels increased in the stanol period by $182 \pm 114 \text{ } 10^2 \times \mu\text{mol}/\text{mmol}$ cholesterol (511%; $p < 0.001$) compared with the control period and by $170 \pm 117 \text{ } 10^2 \times \mu\text{mol}/\text{mmol}$ cholesterol (404%; $p < 0.001$) compared with the sterol period. For campestanol, these values were $103 \pm 70 \text{ } 10^2 \times \mu\text{mol}/\text{mmol}$ cholesterol (398%; $p < 0.001$) and $96 \pm 66 \text{ } 10^2 \times \mu\text{mol}/\text{mmol}$ cholesterol (295%; $p < 0.001$). Lathosterol levels, a marker for cholesterol synthesis, increased in the sterol period by $189 \pm 454 \text{ } 10^2 \times \mu\text{mol}/\text{mmol}$ cholesterol (24.1%; $p < 0.01$) and in the stanol period by $147 \pm 322 \text{ } 10^2 \times \mu\text{mol}/\text{mmol}$ cholesterol (17.5%; $p < 0.05$) compared with the control period. Cholestanol levels, a marker for cholesterol absorption, decreased

Table 3.4. Effect of consumption of plant sterol and plant stanol enriched margarines on serum lipid and lipoprotein concentrations, and on serum plant sterol, plant stanol and lathosterol levels

	Control period	Plant sterol period	Plant stanol period
Total cholesterol (mmol/L)	5.56 ± 1.07	5.26 ± 1.08 ¹	5.27 ± 1.11 ¹
LDL cholesterol (mmol/L)	3.36 ± 1.06	3.08 ± 1.00 ¹	3.10 ± 1.05 ¹
HDL cholesterol (mmol/L)	1.69 ± 0.39	1.65 ± 0.39	1.68 ± 0.39
Total cholesterol/HDL	3.46 ± 1.00	3.32 ± 0.93 ²	3.28 ± 0.96 ³
Triacylglycerol (mmol/L)	1.14 ± 0.40	1.18 ± 0.44	1.08 ± 0.41
ApoB100 (g/L)	0.97 ± 0.25	0.93 ± 0.24 ³	0.92 ± 0.26 ³
ApoA1 (g/L)	1.57 ± 0.31	1.55 ± 0.31	1.55 ± 0.27
Sitosterol*	140 ± 69	226 ± 255 ³	88 ± 35 ⁴
Campesterol	214 ± 83	346 ± 172 ¹	131 ± 59 ^{1,4}
Sitostanol	4.3 ± 3.3	5.5 ± 5.7	22.4 ± 11.7 ^{1,4}
Campestanol	2.8 ± 1.6	3.5 ± 2.0	13.1 ± 7.7 ^{1,4}
Lathosterol	115 ± 57	134 ± 56 ³	130 ± 55 ²
Cholestanol	165 ± 0.33	153 ± 32 ²	155 ± 32 ²

Values are means ± SD and *sterols are expressed as 10³ × μmol/mmol cholesterol. All subjects (n=43) received the three dietary periods in random order.

Significantly different compared with control period: ¹(*p*<0.001), ²(*p*<0.05), ³(*p*<0.01)

Significantly different compared with sterol period ⁴(*p*<0.001)

in the sterol period by 112 ± 292 10³ × μmol/mmol cholesterol (5.2%; *p*<0.05) and in the stanol period by 96 ± 205 10³ × μmol/mmol cholesterol (4.9%; *p*<0.05) compared with the control period.

Plasma oxyphytosterols

Absolute oxyphytosterol concentrations (ng/mL) in BHT-enriched EDTA plasma are shown in **Table 3.5**. 7β-OH-campesterol concentrations were decreased in the stanol period by 0.07 ± 0.17 ng/mL (13.6%) compared with the control period (*p*<0.01) and by 0.07 ± 0.14

ng/mL (15.0%) compared with the sterol period ($p<0.01$). Concentrations of the other oxyphytosterol were comparable between the three periods.

Cholesterol-standardized oxyphytosterol levels (nmol/mmol cholesterol) showed comparable results, i.e. a decreased 7 β -OH-campesterol level in the stanol period compared with the control and sterol period ($p<0.01$ and $p<0.05$, respectively). In addition, 7-keto-campesterol levels were reduced in the stanol period compared with the sterol period ($p<0.05$).

Table 3.5. Effect of consumption of plant sterol and plant stanol enriched margarines on plasma oxyphytosterol concentrations

A	Control period	Plant sterol period	Plant stanol period
7 α -OH-sitosterol	0.21 $\pm$ 0.09	0.22 $\pm$ 0.11	0.26 $\pm$ 0.35
7 α -OH-campesterol	0.09 $\pm$ 0.05	0.09 $\pm$ 0.06	0.08 $\pm$ 0.05
7 β -OH-sitosterol	1.17 $\pm$ 0.35	1.09 $\pm$ 0.52	1.09 $\pm$ 0.58
7 β -OH-campesterol	0.32 $\pm$ 0.16	0.32 $\pm$ 0.15	0.25 $\pm$ 0.11 ^{1,2}
7-keto-sitosterol	2.49 $\pm$ 0.58	2.35 $\pm$ 0.78	2.35 $\pm$ 0.60
7-keto-campesterol	0.48 $\pm$ 0.19	0.49 $\pm$ 0.28	0.44 $\pm$ 0.22
B			
7 α -OH-sitosterol	0.10 $\pm$ 0.05	0.11 $\pm$ 0.06	0.12 $\pm$ 0.16
7 α -OH-campesterol	0.04 $\pm$ 0.03	0.05 $\pm$ 0.03	0.04 $\pm$ 0.02
7 β -OH-sitosterol	0.54 $\pm$ 0.17	0.56 $\pm$ 0.26	0.50 $\pm$ 0.26
7 β -OH-campesterol	0.15 $\pm$ 0.08	0.17 $\pm$ 0.08	0.12 $\pm$ 0.05 ^{1,3}
7-keto-sitosterol	1.18 $\pm$ 0.34	1.22 $\pm$ 0.45	1.10 $\pm$ 0.30
7-keto-campesterol	0.23 $\pm$ 0.10	0.26 $\pm$ 0.15	0.21 $\pm$ 0.10 ⁴

Panel A: Values are means $\pm$ SD and are expressed as ng/mL. Panel B: Values are means $\pm$ SD and are expressed as nmol/mmol cholesterol. All subjects (n=43) received the three dietary periods in random order.

Significantly different compared with sterol period ¹($p<0.01$), ⁴($p<0.05$)

Significantly different compared with control period ²($p<0.01$), ³($p<0.05$)

Discussion

There is an ongoing debate whether there is a relation between circulating serum plant sterol concentrations and cardiovascular risk. Based on findings from animal studies [21-23], we hypothesized that plant sterols themselves are not, but become atherogenic, when oxidized. We here show that in healthy volunteers, despite the increase in non-oxidized plant sterols concentrations, absolute concentrations of oxyphytosterols do not increase after consumption of plant sterol enriched margarines. However, plant stanol consumption reduced 7 β -OH-campesterol concentrations and – when standardized for cholesterol also 7-keto-campesterol levels – compared with the control as well as with the plant sterol enriched margarine.

Oxyphytosterol concentrations in humans have only been determined in a few - merely cross-sectional - studies. In 2001, Plat et al. identified oxyphytosterols in serum of phytosterolemic patients, but not in serum of healthy subjects [15]. A few years later, Grandgirard et al. used a method with a lower detection limit and detected α - and β -epoxysitosterol, 7-keto-sitosterol and sitostanetriol in plasma of healthy subjects in a range of 5-57 ng/mL [24]. We found oxyphytosterol concentrations in a range of 0.08-2.49 ng/mL, but measured different oxyphytosterols. 7-keto-sitosterol, however, was measured in both studies and while we measured a mean concentration of 2.5 ng/mL, Grandgirard et al. found a mean concentration of 6.1 ng/mL. The amount BHT added to the samples differed between our and Grandgirard's study (1% vs. 0.05%), which might explain the discrepancies in oxyphytosterol concentrations. Menéndez-Carreño measured the same oxyphytosterols as we did, but found somewhat higher oxyphytosterol concentrations in serum of healthy volunteers (0.25 – 4.48 ng/mL) [25]. This difference between our results with Menéndez-Carreño's data is probably due to the use of different methods, emphasizing the need for standardization of oxyphytosterol analyses. Recently, Husche et al. determined oxyphytosterol concentrations in plasma from 16 volunteers, who were given plant sterol enriched margarine for 4 weeks, providing 3.0 gram of plant sterols per day. Oxyphytosterol concentrations did not change after plant sterol consumption, except for an 85% increase in 7 β -OH-sitosterol concentrations [20]. In our study 7 β -OH-sitosterol concentrations did not change after consumption of a plant sterol enriched margarine, despite the fact that baseline 7 β -OH-sitosterol concentrations were comparable between the two studies (1.20 ± 0.54 ng/mL vs. 1.17 ± 0.35 ng/mL). The study of Husche et al., however, was not placebo-controlled, which might have influenced the results. The range of the different oxyphytosterol concentrations in Husche's study and our study was comparable (0.07-3.01 ng/mL vs. 0.08-2.49 ng/mL) and this consistency in baseline oxyphytosterol concentrations may help to define normal serum values.

So far oxyphytosterol concentrations have been reported as absolute values in literature. The question arises whether concentrations, as for non-oxidized plant sterols, should be standardized for cholesterol concentrations or not. Plant sterols concentrations are standardized for cholesterol, as plant sterols are transported by lipoproteins. This may be

true for oxyphytosterols as well. When oxyphytosterols are standardized for cholesterol concentrations, conclusions were very comparable to those of the absolute changes, i.e. there was a significant decrease in 7 β -OH-campesterol concentrations in the stanol period compared to the sterol and control period. In addition, 7-keto-campesterol was significantly decreased in the stanol compared with the sterol period. Alternatively, oxyphytosterols levels can also be expressed as the proportion of circulating plant sterol concentrations, which more or less indicates the percentage of substrate that is oxidized. The percentage oxidized plant sterols of total plant sterols is the highest in the stanol period and the lowest in the sterol period, but this is mostly due to a change in plant sterol concentrations and not in oxyphytosterol concentrations. For this reason, it was chosen to only express oxyphytosterols in absolute and cholesterol-standardized levels (Table 5).

Theoretically, oxyphytosterols can be derived from absorption of oxidized plant sterols present in food, as well as from endogenous synthesis (either from enzymatic or non-enzymatic ring-oxidation processes). Since this study cannot distinguish between endogenously formed and diet-derived oxyphytosterols, we can only speculate about the origin of the measured circulating oxyphytosterols. Although phytosterolemia patients follow a strict plant sterol-poor (and consequently also oxyphytosterol poor) diet, they still have elevated plasma (oxy) phytosterol concentrations [4]. This could indicate that plasma oxyphytosterols originate from *in vivo* oxidation of the high circulating non-oxidized serum plant sterol concentrations. A second potential indication for the *in vivo* formation of oxyphytosterols might be found in the oxyphytosterol content of the margarines. Oxyphytosterols in the diet are mainly derived from plant sterol enriched products [16]. Our plant sterol enriched margarine contained ~34 ng oxyphytosterols per milligram margarine, while the oxyphytosterol content of the other two margarines was ~5 ng/mg margarine (Table 2). However, this higher oxyphytosterol content did not translate into increased serum concentrations, which might suggest that serum concentrations are not related to absorption of oxyphytosterols from the diet. On the other hand, while non-oxidized plant sterol concentrations increased, oxyphytosterol concentrations did not increase, which could imply that oxyphytosterols are not formed *in vivo* but origin from intestinal absorption. In this respect, the relative absorption of different oxyphytosterols might give more insight into the origin of circulating oxyphytosterols. The sterol enriched margarines contained twice as much sitosterol oxidation products as campesterol oxidation products, while concentrations of sitosterol oxidation products in plasma were four to five times as high as campesterol concentration products. This may suggest that sitosterol oxidation products are preferentially absorbed or formed *in vivo*. However, in humans, non-oxidized campesterol is better absorbed than non-oxidized sitosterol and serum concentrations are also higher [26]. Human data is lacking, but animal studies have consistently shown that – analogue to the non-oxidized sterols – campesterol oxidation products are better absorbed than sitosterol oxidation products [27, 28]. This may suggest that *in vivo* formation of plant sterol oxidation products is more likely than intestinal absorption, or perhaps that excretion of sitosterol oxidation products is low. To definitively

answer the question whether plasma oxyphytosterols result from absorption or from *in vivo* formation, future studies should be performed in which for example labeled non-oxidized plant sterols are consumed and subsequent enrichment in labeled plasma oxyphytosterols is measured. In addition, little is known about tissue distribution of oxyphytosterols and whether serum oxyphytosterol concentrations relate to tissue concentrations. These questions should be addressed in future studies to elucidate the physiological role of circulating oxyphytosterols in humans.

We observed a decrease in absolute 7 β -OH-campesterol concentrations or cholesterol standardized 7 β -OH-campesterol and 7-keto-campesterol after plant stanol enriched margarine consumption. Again, it is not known whether this is due to effects on *in vivo* formation or on absorption. Also, it may be a chance finding. However, if true, it is interesting that 7 β -OH-cholesterol (cholesterol oxidation product) is regarded as the most atherogenic oxysterol and has been identified as the strongest predictor of a rapid progression of carotid atherosclerosis in humans [29]. Whether 7 β -OH-campesterol might also be regarded as an atherogenic compound remains to be determined.

Potential differences in dietary oxyphytosterol intake between treatments cannot be eliminated as the study is performed in a free-living setting, but due to the crossover design and low oxyphytosterol content in daily diet this is not very likely.

In conclusion, we have shown that a daily consumption of a plant sterol enriched margarine for a period of 4 weeks does not increase plasma oxyphytosterol levels. We further found that plant

stanol consumption reduced the absolute and cholesterol-standardized 7 β -OH-campesterol levels and the cholesterol-standardized 7-keto-campesterol level. The reproducibility and possible clinical implication of these reductions remain to be determined and deserve further investigation.

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Chapter 4

Oxyphytosterol formation in humans: identification of high vs. low oxidizers

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Abstract

Animal studies suggest that oxyphytosterols are atherogenic. However, we have previously shown that plasma oxyphytosterol concentrations did not increase after consuming a diet enriched in plant sterol esters (3 g/day), while minor reductions were seen after consuming a plant stanol ester-enriched diet. Large variations in oxyphytosterol concentrations between individuals however existed. The aim of this study was to identify factors that may explain inter-individual differences in plasma oxyphytosterol concentrations. For this, 43 subjects consumed for 4 weeks in random order a plant sterol, stanol and control margarine. Plasma oxyphytosterol concentrations were determined in butylated hydroxytoluene (BHT)-enriched EDTA plasma via GC-MS and serum oxidized low-density lipoprotein (oxLDL) concentrations were analysed via ELISA. Trolox equivalent antioxidant capacity (TEAC) values, α -tocopherol concentrations and iron/copper status were determined to assess plasma oxidative and anti-oxidative capacity. Serum (non-oxidized) sitosterol and campesterol concentrations did not correlate with plasma oxysitosterol and oxycampesterol concentrations during any of the three dietary interventions. Moreover, plasma oxyphytosterol concentrations remained relatively stable over time. Six subjects could be arbitrarily classified as having consistent low or high plasma oxyphytosterol concentrations, which was also reflected in oxLDL concentrations. However, oxidative and anti-oxidative capacity markers, such as iron/copper status, α -tocopherol concentrations and TEAC values, could not explain these differences. In conclusion, subjects seem to have consistent plasma oxyphytosterol concentrations, which resulted in the identification of 'low and high oxidizers'. Differences, however, could not be attributed to the oxidative and anti-oxidative capacity markers analysed.

Introduction

Cholesterol oxidation products (oxysterols) can be derived from dietary intake and from *in vivo* oxidation by specific enzymes or by reactive oxygen species (ROS) [1]. Increased oxysterol concentrations are found in plasma of patients suffering from cardiovascular disease (CVD) [2] and in atherosclerotic plaques [3]. Further, a positive correlation has been observed between ROS-induced oxysterol concentrations in serum and established CVD risk factors, such as obesity and increased triglyceride concentrations [4]. In addition, recent studies have also suggested that oxysterols contribute to the development of degenerative diseases such as age-related macular degeneration [5]. Although these findings are suggestive, causal relations between elevated plasma oxysterol concentrations and pathological conditions have not been established so far.

Plant sterols are structurally related to cholesterol and can be oxidized to oxysterols [6]. While the biological role of oxysterols has been studied into large detail [1, 7], studies evaluating the formation, kinetics and (patho)physiological effects of oxysterols are scarce. Oxysterols and oxysterols exert cytotoxic and apoptotic effects *in vitro*, as indicated by an inhibition of cell growth and a reduction of cell viability [8]. Oxysterols may be more cytotoxic than oxysterols [9], while pathways underlying the cytotoxicity of oxysterols and oxysterols seem to be different, i.e. antioxidants protected against the damaging effects of oxysterols, but not against the toxicity of oxysterols [10]. Studies in rodents have demonstrated that oxysterols and oxysterols accumulate in tissues, but discrepancies exist regarding their potential atherogenicity [11-14].

What is clearly lacking is a comprehensive understanding of the aetiology behind the formation of oxysterols and their effects in humans. Circulating oxysterol concentrations have only been measured occasionally in human studies [15-18] and large variations in serum oxysterol concentrations are observed when comparing the results of these studies. In a previous study, we have shown that plasma oxysterol concentrations in healthy volunteers did not increase after consuming a diet enriched in plant sterol esters (3 g/day) for 4 weeks, while minor reductions were seen after consuming a plant stanol ester enriched [19]. More importantly, we identified large variations in oxysterol concentrations between healthy human subjects, which remained stable over time. The aim of this study was now to identify factors that relate to these inter-individual differences in plasma oxysterol concentrations.

Subjects and Methods

Subjects, diets and design

The protocol of this placebo-controlled double-blind dietary intervention study has been reported into detail [19]. Briefly, subjects were recruited in Maastricht and surroundings. Subjects met the following criteria: 18-70 years of age; body mass index (BMI) between 20-30 kg/m²; no active cardiovascular disease or severe medical condition during the past 5

years that might interfere with the study; no use of lipid-lowering medication or a medically prescribed diet; stable body weight during the last three months; and no consumption of plant sterol or plant stanol enriched products in the previous month. In addition, serum total cholesterol concentrations were <7.8 mmol/L; serum triglyceride concentrations <3.0 mmol/L and plasma glucose concentrations <6.1 mmol/L, as determined during two screening visits. Baseline characteristics are shown in **Table 4.1**. All participants gave written informed consent before entering the study. The protocol was approved by the medical ethical committee of the Maastricht University Medical Centre+ (MUMC+), and the study was registered at clinicaltrials.gov as NCT01559428.

Table 4.1. Baseline characteristics

Age (y)	41 ± 18
Male / female (n)	17 / 26
BMI (kg/m ²)	24.8 ± 2.8
Total cholesterol (mmol/L)	5.72 ± 1.12
LDL cholesterol (mmol/L)	3.53 ± 1.06
HDL cholesterol (mmol/L)	1.69 ± 0.37
Triacylglycerol (mmol/L)	1.14 ± 0.46

Values are means ± SD

The study had a crossover design and consisted of three intervention periods of 4 weeks, separated by washout periods of 4 weeks. Subjects were stratified for gender and allocated to the intervention periods in a randomised order, based upon a computer-generated table with random numbers. During each intervention period, subjects were asked to replace their own spread with the test margarine (70% fat) of which 20 gram had to be consumed on a daily basis. The daily consumption of 20 gram of margarine provided no, or 3.0 gram of plant sterols or stanols (provided as fatty-acid esters), referred to as control, sterol or stanol period. Margarine composition has previously been described into detail [19].

Blood sampling

Blood was sampled after an overnight fast after 3 and 4 weeks of intervention. A clotting tube (Becton, Dickinson and Company, Franklin Lakes, NY, USA) was sampled and serum was obtained by low-speed centrifugation at 1300x g for 15 min at room temperature, at least half an hour after venipuncture. Serum was stored at -80 °C and used for analysis of lipid and lipoprotein concentrations, of plant sterol and plant stanol concentrations, of oxidized low-density lipoprotein (oxLDL) concentrations, of α -tocopherol (vitamin E)

concentrations, of iron status parameters, i.e. total iron, ferritin and transferrin and of copper status parameters, i.e. copper and ceruloplasmin. An EDTA and NaF tube (Becton, Dickinson and Company, Franklin Lakes, NY, USA) were sampled. EDTA and NaF plasma was obtained by low-speed centrifugation at 1300x g for 15 min at 4 °C, and then stored at -80 °C. To avoid auto-oxidation, oxyphytosterol concentrations were determined in butylated hydroxytoluene (BHT)-enriched EDTA plasma; for this 10 µl BHT (25 mg/mL ethanol) was added per 1 mL of EDTA plasma, immediately after centrifugation. The trolox equivalent antioxidant capacity (TEAC) assay was performed in de-proteinated NaF plasma; for this 10% TCA was added to the plasma (1:1) followed by centrifugation (13.000 rpm, 5 minutes at 4 °C).

Analyses

All analyses were performed in samples from week 4 of each intervention period, and samples from one subject were always analysed within one run to avoid bias caused by between run variations. Serum total cholesterol, plant sterol (sitosterol, campesterol) and plant stanol (sitostanol, campestanol) concentrations were analysed by gas-liquid chromatography-mass spectroscopy (GC-MS) as described previously [17, 20]. Plant sterol and plant stanol concentrations were expressed as $10^2 \times \mu\text{mol}/\text{mmol}$ total cholesterol. The measured oxyphytosterols were 7 α -hydroxy(OH)-sitosterol, 7 α -OH-campesterol, 7 β -OH-sitosterol, 7 β -OH-campesterol, 7-keto-sitosterol, and 7-keto-campesterol by GC-MS according the procedure as described by Husche et al. [17] and expressed as ng/mL. Oxyphytosterol concentrations were also standardized for cholesterol concentrations and expressed as nmol/mmol cholesterol. OxLDL concentrations were analysed by a commercially available sandwich ELISA (Mercodia, Uppsala, Sweden) and α -tocopherol concentrations by reversed-phase HPLC as described before [21]. The TEAC value is a measurement for the total antioxidant status, relating the free radical scavenging properties of a solution or a compound to that of the synthetic antioxidant trolox. The assay was performed as previously described [22]. Total iron concentrations were determined with a fixed-timed endpoint assay on a Beckman Coulter LX20 PRO Clinical Chemistry analyser (Beckman Coulter, Fullerton, USA). Transferrin and ceruloplasmin concentrations were analysed by particle-enhanced immunonephelometry on the BN Prospect from Siemens Healthcare Diagnostics (Siemens, Munich, Germany). The transferrin saturation (%) was calculated as $\text{Iron } (\mu\text{mol/l}) / (\text{transferrin } (\text{g/L}) * 25) * 100\%$. Ferritin concentrations were determined using an immunoturbidimetric assay on a Cobas 6000 system (Roche Diagnostics, Basel, Switzerland) and copper concentrations using the Thermo Scientific XSERIES 2 ICP-MS (Inductive Coupled Plasma-Mass Spectrometry) (Thermo Fischer Scientific, Florida, US).

Statistics

Effects of the experimental products were evaluated by a univariate analysis of variance (ANOVA) followed by Bonferroni's correction for multiple comparisons with subject number as a random factor. Pearson correlation coefficients were used to examine linear relationships between parameters. Results were considered statistically significant if $p < 0.05$. All statistical analyses were performed using SPSS 18.0 for Mac Os X (SPSS Inc., Chicago, IL, USA).

Results

Serum plant sterols and plasma oxyphytosterols

As reported previously [19], cholesterol-standardized serum sitosterol and campesterol levels increased in the sterol period compared with the control period (49%; $p < 0.01$ and 66%; $p < 0.001$, respectively) and the stanol period (167%; $p < 0.001$ and 179%; $p < 0.001$, respectively). In addition, cholesterol-standardized serum campesterol levels decreased in the stanol period compared with the control period (37%; $p < 0.001$). Plasma oxyphytosterol levels were not changed after consumption of the plant sterol enriched margarine. On the other hand, plant stanol consumption reduced cholesterol-standardized 7 β -OH-campesterol levels by 12% ($p < 0.05$) and 21% ($p < 0.01$) compared with the control and sterol period respectively, and 7-keto-campesterol levels by 12% ($p < 0.05$) compared with the sterol period.

Serum (non-oxidized) sitosterol concentrations did not correlate with plasma oxysitosterol (oxidized sitosterol) concentrations ($r = 0.08$; $p = 0.45$) during the control period. This was also true for serum campesterol and plasma oxycampesterol concentrations ($r = 0.12$; $p = 0.59$). Differences in serum plant sterol concentrations between the control period and sterol or stanol period did not correlate with those in plasma oxyphytosterol concentrations ($r = 0.23$; $p = 0.13$ and $r = 0.01$; $p = 0.96$ respectively). **Figure 4.1** (Panel A) shows individual differences in plasma oxyphytosterols (sum of oxysitosterol and oxycampesterol) and serum plant sterols (sum of sitosterol and campesterol) concentrations between the sterol and control periods, while panel B shows these differences between the stanol and control periods.

The individual changes in oxyphytosterol concentrations were ranked from the largest decrease to the highest increase (upper panels) and the accompanying changes in plant sterol concentrations in the same individual are plotted in the lower panels. As expected, the patterns of changing plasma oxyphytosterol concentrations and serum plant sterols concentrations did not correspond, i.e. an increase in serum plant sterol concentrations is not by definition accompanied by an increase in plasma oxyphytosterol concentrations (Figure 4.1, panel A). Similarly, a decrease in serum plant sterol concentrations was not accompanied by a corresponding decrease in plasma oxyphytosterol concentrations in the same individual (Figure 4.1, panel B).

Figure 4.1. Individual differences between sum oxyphytosterol concentrations and sum plant sterol concentrations after plant sterol (panel A) and plant stanol consumption (panel B) when compared to control period (n=43).

Plasma oxysitosterol concentrations at the end of the control and sterol period were significantly correlated (**Figure 4.2**; panel A, $r=0.51$; $p<0.001$), and this was also true for serum oxycampesterol concentrations (panel B, $r=0.45$, $p<0.01$). Significant correlations between dietary periods were also seen for the individual oxidation products (7 α -OH, 7 β -OH and 7-keto) of sitosterol and campesterol (data not shown). This suggests that differences in plasma oxyphytosterol concentrations between subjects were consistent during the different dietary interventions. By comparing the various oxyphytosterol correlation graphs (of all six individual oxyphytosterols, during all three dietary interventions), three subjects could arbitrarily be classified as having consistent low plasma oxyphytosterol concentrations and three subjects as having consistent high plasma oxyphytosterol concentrations.

To further explore this difference in oxyphytosterol concentrations between these so-called low and high oxidizers, serum oxLDL concentrations were determined. **Figure 4.3** shows oxyphytosterol and oxLDL concentrations in the entire group and in the low and high oxidizers in the control period. In line with the findings for plasma oxyphytosterol concentrations, similar differences between low and high oxidizers in oxLDL concentrations were shown. Figure 3 illustrates that differences in oxyphytosterol concentrations are also reflected in oxLDL concentrations, although the groups are somewhat overlapping. The correlation between sum oxyphytosterol and oxLDL concentrations in the control period did not reach significance ($r=0.13$, $p=NS$). On the other hand, there was a significant correlation between

sum oxyphytosterol and oxLDL concentrations in the sterol period ($r=0.36$; $p<0.05$) and stanol period ($r=0.32$; $p<0.05$). More specifically, these significant correlations were only present for 7β -OH-sitosterol, 7β -OH-campesterol, 7-keto-sitosterol and 7-keto-campesterol (ROS-induced oxyphytosterols) and not for 7α -OH-sitosterol and 7α -OH-campesterol, which are in contrast to the other oxyphytosterols not only formed via ROS but also enzymatically produced (data not shown).

Figure 4.2. Correlations between sum oxycampesterol concentrations (panel A, $r=0.51$; $p<0.001$) and between sum oxytosterol concentrations (panel B, $r=0.45$; $p<0.01$) in control and sterol period ($n=43$).

Anti-oxidative and pro-oxidative markers

Plasma α -tocopherol concentrations, TEAC values and iron and copper status markers were determined to identify factors that could relate to circulating plasma oxyphytosterol concentrations. As shown in **Table 4.2**, plasma α -tocopherol concentrations decreased in the stanol period by $3.73 \pm 10.6 \mu\text{mol/L}$ compared with the control period (7.9%; $p<0.05$), while TEAC values and iron and copper status did not differ between the three dietary periods. This implies that a possible correlation between any of these parameters and plasma oxyphytosterol concentrations cannot be attributed to diet-induced changes in these anti-oxidative and pro-oxidative markers. No correlations between plasma oxyphytosterol

concentrations with α -tocopherol concentrations or TEAC values, both accepted markers of anti-oxidative capacity, were observed during any of the three dietary periods.

Figure 4.3. Oxidative stress markers in low and high oxidizers vs. group average.

¹Oxyphytosterol is the sum of 7α -sitosterol, 7β -OH-sitosterol, 7-keto-sitosterol, 7α -campesterol, 7β -OH-campesterol and 7-keto-campesterol concentrations. All analyses have been performed in each individual in the control period.

Next, iron status, a contributor to metal-induced oxidation *in vivo*, was determined by measuring total iron, ferritin and transferrin saturation. Correlations between all three iron status markers and oxLDL, and between iron status markers and oxyphytosterol concentrations during the control period are shown in **Figure 4.4**. In line with expectations, there were positive correlations between all three iron status markers and plasma oxLDL concentrations (total iron $r=0.37$; $p<0.05$, ferritin $r=0.37$; $p<0.05$, transferrin saturation $r=0.35$; $p<0.05$). These correlations were comparable during all three dietary periods, except for oxLDL and ferritin concentration during the sterol period, which showed no statistically significant correlation ($r=0.15$; $p=0.34$). In contrast, no correlations were observed between any of the three iron status markers and oxyphytosterol concentrations during each of the three dietary periods. Finally, copper and ceruloplasmin (copper-binding protein)

concentrations did not correlate with oxLDL concentrations or with plasma oxyphytosterol concentrations during any of the three dietary periods.

Table 4.2. Effect of consumption of plant sterol and plant stanol enriched margarines on plasma α -tocopherol concentrations, TEAC values and on iron and copper status markers

	Control period	Plant sterol period	Plant stanol period
α -tocopherol ($\mu\text{mol/L}$)	44.5 $\pm$ 12.5	41.9 $\pm$ 13.3	40.8 $\pm$ 15.3 ¹
TEAC values	658.5 $\pm$ 101.9	676.0 $\pm$ 118.9	675.7 $\pm$ 102.1
Total iron ($\mu\text{mol/L}$)	17.9 $\pm$ 7.7	17.1 $\pm$ 7.5	16.7 $\pm$ 7.4
Ferritin ($\mu\text{g/L}$)	62.3 $\pm$ 51.8	65.8 $\pm$ 51.7	63.2 $\pm$ 54.1
Transferrin saturation (%)	30.7 $\pm$ 16.7	28.7 $\pm$ 14.2	28.3 $\pm$ 15.1
Copper ($\mu\text{mol/L}$)	18.4 $\pm$ 8.0	19.0 $\pm$ 8.0	18.5 $\pm$ 7.6
Ceruloplasmin (g/L)	0.30 $\pm$ 0.11	0.31 $\pm$ 0.11	0.31 $\pm$ 0.10

Values are means $\pm$ SD. All subjects (n=43) received the three dietary periods in random order.

¹Significantly different compared with control period ($p < 0.05$)

Figure 4.4. Correlations between iron status markers and oxLDL concentrations (panel A) and between iron status markers and sum oxyphytosterol concentrations (panel B) in control period ($n=43$).

Discussion

This study demonstrates that in healthy volunteers plasma oxyphytosterol concentrations do not correlate with serum non-oxidized plant sterol concentrations. This lack of correlation was expected, as we have earlier found in the same study that an increased consumption of plant sterols does not increase plasma oxyphytosterol concentrations [19]. However, what we also showed is that plasma oxyphytosterol concentrations remained relatively stable during the 20 week study period. This consistency in low or high plasma oxyphytosterol concentrations was also reflected in serum oxLDL concentrations, a frequently used oxidative stress marker, which suggests a relationship between plasma oxyphytosterol concentrations and oxidative stress. Although the evidence is scarce, it is possible that oxyphytosterols – just like oxysterols – are atherogenic components. In animal studies, oxysterols and oxyphytosterols have been shown to accumulate in serum and liver, but the implications of this are still under debate [11-14]. What is lacking is a clear insight in the factors that influence formation of oxyphytosterols in humans, as well as the exact locations of oxidation within the human body. To identify factors that relate to these inter-

individual differences in plasma oxyphytosterol concentrations, we searched for similarities and differences between oxLDL and oxyphytosterol concentrations.

In vivo oxidation is often catalysed by iron ions. Ferrous ions (Fe^{2+}) generate highly reactive hydroxyl radicals from hydrogen peroxide. These can oxidize lipids, nucleic acids, and proteins [23]. Increased iron stores are associated with oxidative stress, but associations between iron stores with oxidative stress-related diseases, such as type II diabetes and cardiovascular diseases remain contradictory [24, 25]. We here show a positive correlation between iron stores and serum oxLDL concentrations. However, plasma oxyphytosterol concentrations were not correlated with iron stores. This suggests that oxidation of plant sterols and oxidation of LDL particles are not – at least not entirely – regulated by the same mechanisms. This might in part be explained by the fact that serum oxLDL concentrations represent essentially a fatty acid oxidation process and the antibody used binds to malondialdehyde (MDA)-modified apolipoprotein B particles. In contrast, oxidation of plant sterols represents sterol oxidation. Sterols are less susceptible to oxidative attack than polyunsaturated fatty acyl moieties, and will therefore be accompanied by other oxidized compounds [1]. Nevertheless, plasma oxycholesterol concentrations were positively associated with serum ferritin concentrations in a large cohort of Finnish men [26]. This is the first study that relates iron stores to plasma oxyphytosterols concentrations and our findings suggest that oxidation of plant sterols – in contrast to oxycholesterol and oxLDL formation – might not be iron-mediated. In contrast to the positive correlations between iron markers and oxLDL concentrations, we did not find this correlation for markers of copper status. As for iron, there was also no correlation between copper markers and oxyphytosterol concentrations. Epidemiological data regarding the role of copper in oxidative processes and CVD risk are not consistent and are still a matter of debate [27]. Copper is part of superoxide dismutase (SOD); an enzymatic antioxidant and copper deficiency (e.g. decreased SOD status) is associated with specific pathologies [28]. On the other hand, not only deficiencies but also copper overload is associated with inflammation and markers of oxidative stress in the body [29]. Ceruloplasmin is the major copper-containing plasma protein and depending on the circumstances can act as an antioxidant or pro-oxidant [27]. The fact that in this study serum copper and ceruloplasmin concentrations did not correlate with serum oxLDL or plasma oxyphytosterol concentrations seems in contrast with *in vitro* studies in which oxidation tendency of LDL particles is studied by means of copper-ions [30]. However, there is strong evidence that different oxidative products are generated during *in vitro* copper-induced oxidation of LDL compared with *in vivo* oxidation of these particles [1]. As described before, the role of copper in *in vivo* oxidative processes is contradictory and our results suggest that in this population of healthy volunteers, copper does not play a role (anti-oxidative or pro-oxidative) in the oxidation of LDL or plant sterols.

Both TEAC values and α -tocopherol concentrations, markers for anti-oxidative capacity, were not associated with plasma oxyphytosterol concentrations, indicating that a relationship between oxyphytosterols and oxidative stress is not straightforward. On the other hand,

serum oxLDL concentrations, a validated marker for oxidative stress, also did not correlate with TEAC values. Although these correlations imply that there is no association between anti-oxidative capacity and oxyphytosterol concentrations, it must be kept in mind that these are cross-sectional correlations and it would be interesting to assess oxyphytosterol concentrations after antioxidant supplementation.

Altogether the obtained results imply that for now, we cannot answer the question which factors contribute to the large inter-individual differences in fasting plasma oxyphytosterol concentrations. We have been able to exclude some parameters that seemed most logical to consider. In general, this search is complicated by the fact that information regarding oxy(phyto)sterol metabolism is scarce; not much is known about normal plasma concentrations in healthy subjects and in specific diseases, what the consequences are of increased concentrations, and whether plasma concentrations reflect tissue concentrations. To summarize, this study shows that plasma oxyphytosterol concentrations are linked to oxLDL concentrations, that concentrations remain relatively stable over time and that oxidation of plant sterols – in contrast to oxidation of LDL – might not be iron-mediated. Thus, many questions regarding oxyphytosterol metabolism still remain that deserve to be answered in future studies.

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Chapter 5

Postprandial plasma oxyphytosterol concentrations after consumption of plant sterol or stanol enriched mixed meals in healthy subjects

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Conditionally accepted

Abstract

Epidemiological studies have reported inconsistent results on the relationship between increased plant sterol concentrations with cardiovascular risk, which might be related to the formation of oxyphytosterols (plant sterol oxidation products) from plant sterols. However, determinants of oxyphytosterol formation and metabolism are largely unknown. It is known, however, that serum plant sterol concentrations increase after daily consumption of plant sterol enriched products, while concentrations decrease after plant stanol consumption. Still, we have earlier reported that fasting oxyphytosterol concentrations did not increase after consuming a plant sterol- or a plant stanol enriched margarine (3.0 g/d of plant sterols or stanols) for 4 weeks. Since humans are in a non-fasting state for most part of the day, we have now investigated effects on oxyphytosterol concentrations during the postprandial state. For this, subjects consumed a shake (50 g of fat, 12 g of protein, 67 g of carbohydrates), containing no, or 3.0 g of plant sterols or plant stanols. Blood samples were taken up to 8 hours and after 4 hours subjects received a second shake (without plant sterols or plant stanols). Serum oxyphytosterol concentrations were determined in BHT-enriched EDTA plasma via GC-MS/MS. 7 β -OH-campesterol and 7 β -OH-sitosterol concentrations were significantly higher after consumption of a mixed meal enriched with plant sterol esters compared to the control and plant stanol ester meal. These increases were seen only after consumption of the second shake, illustrative for a second meal effect. Non-oxidized campesterol and sitosterol concentrations also increased after plant sterol consumption, in parallel with 7 β -OH concentrations and again only after the second meal. Apparently, plant sterols and oxyphytosterols follow the same second meal effect as described for dietary cholesterol. However, the question remains whether the increase in oxyphytosterols in the postprandial phase is due to absorption or endogenous formation.

Introduction

A daily consumption of 2-3 gram plant sterols or plant stanols reduces fasting serum low-density lipoprotein cholesterol (LDL-C) concentrations up to 10% [1]. At the same time, however, serum plant sterol concentrations increase after daily consumption of plant sterol enriched products, while concentrations decrease after consumption of plant stanol enriched products [2]. We have previously reported increases of 36-50% in serum concentrations of the various plant sterols [3], which is in line with a recent meta-analysis that reported average increases of 31% (95% CI: 26-37%) and 37% (95% CI: 29-45%) in sitosterol and campesterol concentrations after consumption of plant sterol enriched products at an average dose of 1.6 g/d [4]. Although these concentrations are only marginal as compared to those observed in phytosterolemic subjects, the potential impact of these elevated circulating plant sterol concentrations on cardiovascular risk is strongly debated, as epidemiological studies have reported inconsistent results regarding the potential association of increased plant sterol concentrations and CVD risk [5, 6]. The controversy around the potential atherogenicity of plant sterols might relate to the question whether plant sterols are oxidized or not. We and others have previously shown that in transgenic mouse models plant sterol enriched diets lower atherosclerotic lesion development [7-9] while enrichment with oxidized plant sterols (oxyphytosterols) resulted in larger and more severe atherosclerotic lesions [10]. Data regarding origin, metabolism, pathophysiological effects and effects of specific diets on oxyphytosterol metabolism in humans are however scarce. Oxyphytosterols have been identified in serum of healthy and phytosterolemic subjects, and in aortic tissue of patients undergoing elective aortic valve replacement [11]. Previously, we have reported the effect of consuming a plant sterol or a plant stanol enriched margarine (3.0 g/d of plant sterols or stanols) for 4 weeks on fasting oxyphytosterol concentrations. No effects on fasting oxyphytosterol concentrations were observed after plant sterol consumption, while 7 β -OH-campesterol concentrations – and when standardized for cholesterol also 7-keto-campesterol levels – were reduced after plant stanol consumption. Since humans are in a non-fasting state for the major part of the day, we have now examined the 8-hour postprandial oxyphytosterol response after consuming plant sterol or plant stanol ester enriched margarine as part of a mixed meal.

Materials and Methods

Subjects

Samples were derived from a subgroup (N=10) randomly selected from a, double-blind placebo-controlled cross-over intervention study with 43 healthy subjects. Subject characteristics of the complete study population have previously been reported into detail when effects of plant sterol and plant stanol consumption on fasting lipoprotein and oxyphytosterol concentrations were reported [3]. The 10 healthy subjects (3 males and

7 females) were 44 ± 19 years old, had a body mass index (BMI) of 24.3 ± 2.8 kg/m², a total cholesterol concentration of 5.4 ± 0.8 mmol/L and a triglyceride concentration of 1.1 ± 0.4 mmol/L. Statistical analyses showed no differences between this subgroup and the remaining 33 subjects regarding baseline characteristics and fasting responses after plant sterol or plant stanol consumption. All participants gave their written informed consent before entering the study. The medical ethical committee of the Maastricht University Medical Centre+ (MUMC+) approved the protocol and the trial is registered at clinicaltrials.gov as NCT01559428.

Diet and Design

Subjects participated in a 20 weeks intervention study, which consisted of three intervention periods of 4 weeks, separated by washout periods of 4 weeks. Subjects consumed 20 gram of test margarine on a daily basis, which provided no, or 3.0 gram of plant sterols or stanols per day. At the end of each of the three intervention periods, a postprandial test was performed (**Figure 5.1**). For this, an intravenous cannula was placed and a fasting blood sample was collected. Subjects consumed a shake containing no, or 3.0 gram of plant sterols or plant stanols provided as their fatty acid esters, depending on the preceding 4-week intervention period. Subsequent blood samples were drawn up to 8 hours and subjects received a second shake (without cholesterol, plant sterol esters or plant stanol esters) after 4 hours. The compositions of the shakes are shown in **Table 5.1**. Subjects were not allowed to eat anything else, but were allowed to drink water (± 500 mL) throughout the entire test day.

Figure 5.1. Outline of the postprandial meal test

Table 5.1. Composition of the breakfast shakes enriched with plant sterol or plant stanol esters and the lunch shake

	Breakfast shake; control period	Breakfast shake; sterol period	Breakfast shake; stanol period	Lunch shake
Energy (MJ)	3.15	3.15	3.14	3.16
Fat (energy%)	59.6	59.6	59.5	60.1
SAFA*	24.7	24.7	27.8	23.4
MUFA	20.9	20.9	18.8	22.2
PUFA	10.1	10.1	9.1	12.2
Protein (energy%)	6.2	6.2	6.2	4.2
CHO (energy%)	35.2	35.2	35.3	36.4
Cholesterol (mg)	250.2	250.2	250.2	17.3
Plant sterols (g)	-	3.0	-	-
Plant stanols (g)	-	-	3.0	-
Oxyphytosterol (mg)	0.10	0.68	0.11	0.10
Σ oxycampesterol (mg)	0.04	0.22	0.05	0.04
Σ oxysitosterol (mg)	0.06	0.46	0.06	0.06
7β-OH-campesterol (mg)	0.01	0.06	0.00	0.01
7β-OH-sitosterol (mg)	0.02	0.23	0.01	0.02
7-keto-campesterol (mg)	0.03	0.11	0.05	0.03
7-keto-sitosterol (mg)	0.03	0.11	0.05	0.03

* SAFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, CHO: carbohydrates

Blood sampling and analyses

A clotting tube and an EDTA tube (Becton, Dickinson and Company, Franklin Lakes, NY, USA) were sampled at each time point. Serum was obtained from the clotting tube by low-speed centrifugation at 1300x g for 15 min at room temperature, at least half an hour after venipuncture and was used for the analysis of plant sterol concentrations. Plasma was obtained by low-speed centrifugation of the EDTA tube at 1300x g for 15 min at 4 °C and was used for the analysis of plasma oxyphytosterol concentrations. To avoid auto-oxidation, 10 µl BHT (25 mg/mL ethanol) was added per 1 mL of EDTA plasma, immediately after centrifugation. All serum and plasma samples were snap frozen in liquid nitrogen and stored at -80 °C until analysis.

Sitosterol and campesterol concentrations were analysed using gas chromatography – flame ion detection (GC-FID) as described previously [12] and concentrations are expressed as mg/dL. 7β-Hydroxy(OH)-campesterol, 7β-OH-sitosterol, 7-keto-campesterol and 7-keto-sitosterol were analysed by gas chromatography-mass spectroscopy (GC-MS/MS) according to the procedure as described by Husche et al [13] with minor modifications related to the use of a GC-MS triplequad in stead of a GC-MS singlequad and are expressed as ng/mL.

Statistics

All data are presented as means ± standard deviations (SD). Differences in baseline concentrations between the test meals were statistically evaluated using univariate analysis of variance (ANOVA) with diet as fixed factor and subject as random factor. Changes from baseline were analysed using linear mixed models with diet and time as within-subject fixed factors, and with diet-time interaction. If the interaction term was not statistically significant, it was omitted from the model. If the interaction term, factor time or factor diet was significant, Bonferroni's correction for multiple comparisons was used. Results were considered to be statistically significant if $p < 0.05$. All statistical analyses were performed using SPSS 20.0 for Mac Os X (SPSS Inc., Chicago, IL, USA).

Results

Fasting plasma oxyphytosterol concentrations at the start of the postprandial test day (after consuming plant sterol enriched, plant stanol enriched or placebo margarines for 4 weeks) were not different (**Table 5.2**). The postprandial responses in plasma oxyphytosterol concentrations are presented in **Figure 5.2**. There was a significant diet*time interaction for 7β-OH-campesterol concentrations during the postprandial period ($p < 0.05$). Following Bonferroni's correction for multiple comparisons, concentrations were significantly higher after consumption of a mixed meal enriched with plant sterol esters compared with the control meal at T240 ($p < 0.01$), T360 ($p < 0.001$) and T480 ($p < 0.001$). Moreover, concentrations were higher after the plant sterol enriched meal compared with the plant stanol enriched meal

at T360 ($p<0.001$) and T480 ($p<0.01$). Also for postprandial 7 β -OH-sitosterol concentrations, a significant diet*time interaction ($p<0.05$) was found. Following Bonferroni's correction, concentrations were higher after consumption of a mixed meal enriched with plant sterol esters compared with the plant stanol meal at T360 ($p<0.01$) and T480 ($p<0.05$). These data indicate that 7 β -OH-campesterol and 7 β -OH-sitosterol concentrations were only increased after the second meal (i.e. lunch without added plant sterols/stanols and cholesterol). For postprandial 7-keto-campesterol concentrations, there was only a significant time effect. Concentrations were increased at T360 and T480 compared with T0 ($p<0.05$), compared with T60 ($p<0.01$) and compared with T240 ($p<0.001$). Postprandial 7-keto-sitosterol concentrations were increased at T480 compared with T240 ($p<0.01$).

Table 5.2. Fasting plant sterol and oxyphytosterol concentrations after consumption of plant sterol enriched, plant stanol enriched or placebo margarines (n=10)

	Control period	Plant sterol period	Plant stanol period
Campesterol (mg/dL)	0.56 $\pm$ 0.21	0.89 $\pm$ 0.26 ¹³	0.35 $\pm$ 0.11
Sitosterol (mg/dL)	0.32 $\pm$ 0.11	0.41 $\pm$ 0.11 ^{2,3}	0.22 $\pm$ 0.06
7 β -OH-campesterol (ng/mL)	0.39 $\pm$ 0.15	0.37 $\pm$ 0.08	0.33 $\pm$ 0.08
7 β -OH-sitosterol (ng/mL)	0.57 $\pm$ 0.25	0.50 $\pm$ 0.14	0.51 $\pm$ 0.20
7-keto-OH-campesterol (ng/mL)	0.94 $\pm$ 0.26	0.96 $\pm$ 0.17	0.91 $\pm$ 0.11
7-keto-OH-sitosterol (ng/mL)	2.35 $\pm$ 0.67	2.76 $\pm$ 0.82	2.87 $\pm$ 0.76

significantly different compared with control period ¹($p<0.001$), ²($p<0.01$)

significantly different compared with plant stanol period ³($p<0.001$)

Besides oxyphytosterol concentrations, we also quantified non-oxidized plant sterol concentrations since these are the potential substrates to be oxidized. Fasting serum campesterol and sitosterol concentrations were higher after the plant sterol intervention compared with the plant stanol and control intervention (**Table 5.2**). The postprandial responses in serum campesterol and sitosterol concentrations are presented in **Figure 5.3**. Serum campesterol concentrations were increased after a mixed meal enriched with plant sterol esters compared with plant stanol esters (diet effect; $p<0.05$). Postprandial serum campesterol concentrations were also significant for the factor time; concentrations were higher at T360 and T480 compared with T60 ($p<0.05$ and $p<0.01$, respectively). Changes in sitosterol concentration were significant for diet (sterol > stanol, $p<0.01$) and for time (T480 > T60, $p<0.01$). In line with the oxyphytosterol concentrations, serum plant sterol concentrations were particularly increased after the second meal. Postprandial sitosterol and campesterol concentrations were comparable after a mixed meal enriched with plant stanol esters compared with the control intervention.

Figure 5.2. Plasma concentrations of (A) 7β-OH-campesterol, (B) 7β-OH-sitosterol, (C) 7-keto-campesterol and (D) 7-keto-sitosterol after consumption of a shake containing no, or 3.0 gram of plant sterols or plant stanols. Data are presented as mean $\pm$ SEM.

A: significantly different from control period * ($p < 0.01$), ** ($p < 0.001$), significantly different from stanol period # ($p < 0.01$), ## ($p < 0.001$)

B: significantly different from stanol period * ($p < 0.05$), ** ($p < 0.01$)

C: significant time effect, T360 > T0 and T480 > T0 * ($p < 0.01$)

D: significant time effect, T480 > T240 * ($p < 0.01$)

Figure 5.3. Serum concentrations of (A) campesterol and (B) sitosterol after consumption of a shake containing no, or 3.0 gram of plant sterols or plant stanols. Data are presented as mean $\pm$ SEM.

A: Significant diet effect, sterol > stanol # ($p < 0.05$), significant time effect, T360 > T60 * ($p < 0.05$) and T480 > T60 ** ($p < 0.01$)

B: Significant diet effect, sterol > stanol # ($p < 0.01$), significant time effect, T480 > T60 * ($p < 0.01$)

Discussion

Earlier, we have found that daily consumption of a plant sterol enriched diet increased fasting serum plant sterol concentrations, but did not elevate plasma oxyphytosterol concentrations. Here, we show that consumption of a mixed meal enriched with plant sterol esters increased postprandial serum plant sterol and plasma oxyphytosterol concentrations as compared to a mixed meal or a mixed meal enriched with plant stanol esters. Interestingly, these higher plant sterol and oxyphytosterol concentrations were particularly present after consumption of a second meal.

Plant sterols share structural similarities to cholesterol and also have the typical double bond between C5-C6 in the steroid nucleus. Therefore, plant sterols are – analogue to cholesterol – prone to reactive oxidative species (ROS)-mediated oxidation [14]. Plant stanols lack this double bond and can therefore not be oxidized by ROS. Since plant sterol oxidation products are present in foods [15, 16], circulating plant sterol oxidation products can either result from dietary absorption or from endogenous oxidation of non-oxidized plant sterols. Oxyphytosterols have been identified in foods such as heated vegetable oils, infant milk formulas, parenteral nutrition, French fries, potato chips and coffee beans [17-20]. However, the main sources of dietary oxyphytosterols are plant sterol enriched products and a recommended daily consumption of 20-25 gram of spread can provide up to 1.7 mg oxyphytosterols [21]. We here show that postprandial oxyphytosterol concentrations increase after consumption of plant sterol enriched margarine. However, given the experimental design, we cannot distinguish between oxyphytosterols originating from dietary absorption and from endogenous oxidation. Therefore, we can only speculate on their origin.

The postprandial patterns of non-oxidized plant sterol concentrations are suggestive for a second meal response as already reported in 1998 for dietary cholesterol [22-24], which states that cholesterol from the first meal is secreted via intestinally derived chylomicrons during subsequent meal consumptions. This might explain why most studies so far did not report increased postprandial plant sterol concentrations after plant sterol consumption. In two studies, blood sampling may have been too late after consumption of the meals [25, 26], while in another 4-hour study a second meal was not included [27]. Plasma 7 β -OH-phytosterol concentrations demonstrated a comparable second meal response, since peak concentrations were seen two hours after consumption of the second meal. This suggests that oxyphytosterols present in the shake were absorbed in the enterocytes after the first meal (breakfast) and released after the second meal (lunch). Alternatively, one can speculate that plant sterols in the shake were absorbed after the first meal followed by endogenous oxidation and appearance in the circulation. It could also be possible that plant sterols undergo enterohepatic recycling, implying that they are absorbed after the first meal, oxidized in the liver and then secreted back into the intestinal lumen via bile, after which absorption of oxidized plant sterols might take place.

In the plant sterol enriched margarine, sitosterol concentrations were higher than those of campesterol. Also, concentrations of sitosterol oxidation products were higher than those of campesterol oxidation products. In humans, it has consistently been shown that non-oxidized campesterol is better absorbed than sitosterol, resulting in higher fasting serum concentrations [4, 28]. This is also reflected in our postprandial data, where postprandial increases in serum campesterol are higher than those of sitosterol after consumption of the plant sterol enriched shake. Postprandial oxycampesterol responses were also larger than oxysitosterol responses (peak change of 0.06 vs. 0.03 ng/mL). This would suggest that – analogue to non-oxidized plant sterols – campesterol oxidation products are better absorbed (or less efficiently excreted) than sitosterol oxidation products. Rodent models have been used to measure lymphatic recovery to estimate oxyphytosterol absorption rates and indeed reported that campesterol oxidation products were better absorbed than sitosterol oxidation products [29, 30]. However, when fasting plasma oxyphytosterol concentrations are compared, absolute plasma 7 β -OH-sitosterol concentrations are higher than those of 7 β -OH-campesterol (0.57 vs. 0.39 ng/mL, in control period). This is clearly different from the non-oxidized sterols, where campesterol concentrations are higher than sitosterol concentrations (0.56 vs. 0.32 mg/dL, in control period). This might suggest that sitosterol is preferentially oxidized (either in enterocytes or in the circulation) and that (part of) oxyphytosterol concentrations originate from endogenous oxidation of circulating plant sterols. However, we have reported previously that after plant sterol consumption fasting oxyphytosterol concentrations were not increased despite increased plant sterol concentrations (+36-50%), suggesting that the availability of substrate does not influence oxidation rates of serum plant sterol concentrations. Another argument for endogenous oxidation relates to oxyphytosterol differences between subjects [31], which might be attributed to different absorption rates or differences in endogenous oxidation of circulating plant sterols. The latter is probably more plausible, since other oxidative stress markers were also higher in subjects with increased oxyphytosterol concentrations.

In a previous study, fasting 7 β -OH-campesterol concentrations and – when standardized for cholesterol – also 7-keto-campesterol levels oxyphytosterols were reduced after plant stanol consumption [3]. We hypothesized that oxyphytosterol absorption – analogue to non-oxidized plant sterol absorption – is inhibited after consumption of plant stanol enriched products. Postprandial oxyphytosterol concentrations were not changed after plant stanol consumption, suggesting that postprandial oxyphytosterol absorption is not inhibited and oxyphytosterol concentrations in the postprandial phase might originate from endogenous formation. However, postprandial non-oxidized plant sterol concentrations were also not changed, while plant stanol consumption inhibits dietary absorption of plant sterols and reduces fasting plant sterol concentrations. However, this does not exclude that postprandial oxyphytosterols could still originate from dietary absorption. Also, levels of plant sterols and oxyphytosterol in the plant stanol enriched margarine may have been too low to observe clear changes. To provide definite answers to the question whether

plasma oxyphytosterols result from absorption or from endogenous oxidation of circulating plant sterols, studies using labelled non-oxidized plant sterols and subsequent enrichment in labelled plasma oxyphytosterols should be performed. In addition, postprandial plant sterol and oxyphytosterol curves demonstrate that future studies demand longer-term postprandial follow-up periods and inclusion of multiple meals or incorporation of labelled (oxy)phytosterols to assess origin. It would also be interesting to assess whether single or multiple daily intakes of plant sterol enriched products would lead to different changes in circulating oxyphytosterol concentrations.

In conclusion, while we previously reported that four weeks plant sterol enriched margarine consumption did not change fasting oxyphytosterol concentrations, we show here for the first time that postprandial oxyphytosterol concentrations increase after consumption of a mixed meal enriched with plant sterol esters. Data regarding the origin of these oxyphytosterols, i.e. absorption from diet, endogenous formation as well as further oxyphytosterol metabolism in humans is missing and further research is needed to understand the possible relationship between (postprandial) plant sterol and oxyphytosterol concentrations and CVD risk.

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Chapter 6

Effects of a plant sterol or stanol enriched mixed meal on postprandial lipid and glucose metabolism in healthy subjects

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Submitted

Abstract

The low-density lipoprotein cholesterol (LDL-C) lowering effect of plant sterols and stanols has unequivocally been established in numerous studies whereas more recently, a possible triacylglycerol (TAG) lowering effect also received attention. A potential TAG lowering effect of plant sterol and plant stanol consumption is interesting not only in a fasting state, but particularly when present in a postprandial state, since elevated postprandial TAG concentrations are emerging as an important cardiovascular disease (CVD) risk factor. The objective of this study was to examine the 8-hour postprandial response after consuming plant sterol or plant stanol ester enriched margarine as part of a mixed meal.

This postprandial study was part of a randomised, double-blind, cross-over study in which 42 healthy subjects (18-70 years) consumed for 4 weeks a plant sterol enriched (3 g/d of plant sterols), a plant stanol enriched (3 g/d of plant stanols), and a control margarine separated by wash-out periods of 4 weeks. At the end of each intervention period, subjects underwent a meal test in which they consumed a shake enriched with either 3 g of plant sterol esters (sterol period), 3g of plant stanol esters (stanol period) or no addition (control period). Blood sampling was performed up to 8-hours and subjects received a second shake without plant sterols or stanols after 4 hours. This prolonged protocol enabled us to evaluate effects on the 2nd meal response as well. To evaluate postprandial responses, incremental areas under the curves (iAUC) were calculated. The TAG and apoB48 iAUC of the total (0-8 hours) and 1st meal (0-4 hours) responses were comparable between the three test meals. However, the TAG and apoB48 iAUC after the second meal responses (4-8 hours) were higher in the stanol period compared to the sterol period. This 2nd meal response after plant stanol consumption was more pronounced with increasing age and might be related to a change in intestinal TAG absorption or a delay in lipoprotein clearance. This study demonstrates an age-dependent increase in postprandial TAG and apoB48 responses after plant stanol ester consumption. Further validation of these results is warranted, particularly since the effect was most pronounced at a higher age, i.e. the target population of plant sterol and plant stanol consumption.

Introduction

It has unequivocally been established that a daily consumption of plant sterol- and plant stanol enriched products reduces fasting serum low-density lipoprotein cholesterol (LDL-C) concentrations [1]. Functional foods enriched with plant sterols and plant stanols, as part of a healthy diet, have therefore been advocated in the management of mild hypercholesterolemia [2, 3]. Except for LDL-C, more recent studies have also suggested that plant sterols and plant stanols lower serum triacylglycerol (TAG) concentrations [4, 5]. Prospective and case-control studies have identified high fasting serum TAG concentrations as an independent risk marker for CVD, which are routinely measured to assess CVD risk [6]. However, most people are in a non-fasting state for most part of the day and non-fasting serum TAG concentrations are an emerging marker contributing to CVD risk due to the postprandial atherogenicity of TAG-rich (remnant) lipoprotein particles [6, 7]. Indeed, independent of other risk markers, non-fasting TAG concentrations estimated CVD risk more precisely than fasting TAG concentrations did [8, 9]. Besides non-fasting TAG concentrations, remnant cholesterol (cholesterol present in TAG-rich particles during the fasting and non-fasting state) may be an important CVD risk marker [3, 10], illustrating the importance of including postprandial lipoprotein metabolism to assess CVD risk. Therefore, evaluation of the effects of plant sterol and plant stanol consumption on postprandial lipid metabolism is warranted. So far, only three studies have addressed postprandial lipid and lipoprotein responses after plant stanol ester intake [11-13], while there are no reports on postprandial responses after plant sterol ester intake. Although these three studies have their limitations with respect to assessing postprandial responses, they do suggest that chronic or acute consumption of plant stanol esters does not affect postprandial lipid and lipoprotein concentrations. This is remarkable, since it is generally acknowledged that lipoprotein metabolism is affected by plant stanol and sterol ester consumption in fasting state through inhibition of intestinal cholesterol absorption [14]. The mechanism underlying the reduction in fasting serum TAGs is not known, but was suggested to be due to a reduced intestinal fatty acid absorption, at least in mice [15]. This illustrates that there is a need for a more detailed knowledge regarding postprandial effects of plant sterol and stanol ester consumption on lipoprotein metabolism. Therefore, the objective of the present study was to examine the 8-hour postprandial response after consuming plant sterol or plant stanol ester enriched margarines as part of a shake. Since the appearance of dietary cholesterol in the circulation is delayed [16-18], we decided to provide a second mixed meal after 4 hours. This postprandial meal test was performed after completing a 4-week intervention period during which subjects consumed plant sterol or plant stanol ester enriched margarine on a daily basis.

Subjects and Methods

Subjects

Details of the study have been described previously, when the effects of the intervention on fasting lipoprotein and oxyphytosterol concentrations were reported [19]. Briefly, subjects were recruited in Maastricht and met the following criteria: 18-70 years of age; body mass index (BMI) between 20-30 kg/m²; no active cardiovascular disease or severe medical period during the past 5 years that might interfere with the study and no use of lipid-lowering medication or a medically prescribed diet. In addition, serum total cholesterol concentrations were <7.8 mmol/L; serum TAG concentrations <3.0 mmol/L and plasma glucose concentrations <6.1 mmol/L, as determined during two screening visits. All participants gave their written informed consent before entering the study. The medical ethical committee of the Maastricht University Medical Centre+ (MUMC+) had approved the protocol and the trial is registered at clinicaltrials.gov as NCT01559428.

Diet and Design

The complete 20 weeks intervention study was a randomised placebo-controlled crossover trial, which consisted of three intervention periods of 4 weeks, separated by washout periods of 4 weeks [19]. Subjects replaced their own spread with the test margarine of which 20 gram had to be consumed on a daily basis. The margarines provided no, or 3.0 gram of plant sterols or stanols per day. At the end of each of the three intervention periods a postprandial test was carried out. Subjects were asked not to perform any strenuous exercise, not to consume alcohol, and to refrain from high-fat foods on the day prior to the postprandial test days. Subjects arrived at the university by public transport or by car after a 12-hour overnight fast. An intravenous cannula was placed in an antecubital vein and a fasting blood sample was collected (T=0 min). Subjects were then asked to consume the test meal within 10 minutes. The test meal was provided as a shake and contained no, or 3.0 gram of plant sterols or stanols provided as their fatty acid esters, similar to the intervention of the preceding 4-week intervention period. This breakfast shake contained 250 mg cholesterol and in addition 100.000 IU oil-soluble vitamin A (retinyl palmitate). Subsequent blood samples were drawn after 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420 and 480 minutes. Following the blood sample after 240 minutes (approximately around lunch time), subjects received a second meal, which consisted again of a shake. This lunch shake contained no cholesterol, no plant sterols or stanols and no vitamin A. The composition of the two shakes is shown in **Table 6.1**. Subjects were not allowed to eat anything else, but were allowed to drink water (± 500 mL) throughout the entire test day.

Table 6.1. Composition of the breakfast shakes enriched with plant sterol or stanol esters and the lunch shake

	Breakfast shake; sterol/stanol period	Breakfast shake; control period	Lunch shake
Energy (MJ)	3.15	3.14	3.16
Fat (en%)	59.6	59.5	60.1
SAFA (en%)	24.7	27.8	23.4
MUFA (en%)	20.9	18.8	22.2
PUFA (en%)	10.1	9.1	12.2
Protein (en%)	6.2	6.2	4.2
Carbohydrate (en%)	35.2	35.3	36.4
Cholesterol (mg)	250.2	250.2	17.3
Plant sterol or stanol esters (g)	3.0	-	-
Retinyl palmitate (IU)	100.000	100.000	-

Values are means $\pm$ SD. All subjects (n=43) received the three breakfast shakes in random order.

¹SAFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, CHO: carbohydrates

Blood sampling and analyses

A clotting tube and a sodium fluoride (NaF) tube (Becton, Dickinson and Company, Franklin Lakes, NY, USA) were sampled at each time point. Serum was obtained from the clotting tube by low-speed centrifugation at 1300x g for 15 min at room temperature, at least half an hour after venipuncture and was used for the analysis of TAG, apolipoprotein (apo) B48 and insulin concentrations. Plasma was obtained by low-speed centrifugation of the NaF tube at 1300x g for 15 min at 4 °C and was used for the analysis of plasma glucose and retinyl palmitate (RP) concentrations. All serum and plasma samples were snap frozen in liquid nitrogen and stored at -80 °C until analysis.

Plasma glucose concentrations (Roche Diagnostic Systems, Hoffmann-La Roche) and serum TAG concentrations (GPO Trinder; Sigma-Aldrich) with correction for free glycerol were measured at baseline and after 15, 30, 45, 60 and 90 minutes, and at hourly intervals up to 8 hours. Serum insulin concentrations were measured at baseline and after 30, 45, 60 and 90 minutes and at hourly intervals up to 8 hours with a human insulin-specific RIA kit (Linco Research). Serum apoB48 concentrations were measured at baseline and after 30 minutes and at hourly intervals up to 8 hours with an ELISA kit (Shibayagi). Retinyl palmitate concentrations were measured at baseline and after 15, 30, 45, 60 and 90 minutes and at hourly intervals up to 8 hours with high-performance liquid chromatography in a randomly selected subgroup of 13 subjects (4 men, 9 women). All samples from one subject were analysed within one run at the end of the study.

Statistics

All data are presented as means $\pm$ standard deviations (SD). To evaluate the overall postprandial responses, the incremental areas under the curve (iAUC) were calculated using the trapezoidal rule. We assessed the total postprandial response (0-8 hours), as well as the first meal response (0-4 hours) and second meal response (4-8 hours). Maximal changes were calculated by subtracting fasting concentrations (T=0) from the maximal concentrations. Differences in fasting concentrations, iAUC and maximal changes between the test meals were statistically evaluated by repeated-measures analysis of variance (ANOVA) with diet as within subject factor. We also evaluated whether the observed postprandial changes were dependent on gender, body mass index (BMI) or age. There were, however, no gender or BMI effects present. The interaction term diet*age was significant for TAG iAUC (total and 2nd meal responses), for glucose iAUC (total response), but not for apoB48 iAUC. This indicated that dietary effects were influenced by age and diet effects were therefore further evaluated per age category (category I: 18-35y (n=17), category II: 36-52y (n=11) and category III: 53-69y (n=14)). Results were considered to be statistically significant if $p < 0.05$. All statistical analyses were performed using SPSS 18.0 for Mac Os X and adjusted for Bonferroni's correction for multiple comparisons when appropriate (SPSS Inc., Chicago, IL, USA).

Results

The study was completed by 43 subjects [19]. However, for one woman it was not possible to insert the cannula and results of the present postprandial study therefore refer to 42 subjects (Table 6.2).

Table 6.2. Characteristics of the subjects

	All subjects 18-69y (n=42)	Age category I 18-35y (n=17)	Age category II 36-52y (n=11)	Age category III 53-69y (n=14)
Age (y)	42 $\pm$ 18	23 $\pm$ 4	46 $\pm$ 4	62 $\pm$ 4
Male / female (n)	17 / 25	5 / 12	4 / 7	8 / 6
Weight (kg)	73.5 $\pm$ 11.3	73.0 $\pm$ 11.4	68.5 $\pm$ 12.6	78.2 $\pm$ 8.8
Height (m)	1.72 $\pm$ 0.09	1.74 $\pm$ 0.10	1.69 $\pm$ 0.11	1.73 $\pm$ 0.06
BMI (kg/m ²)	24.7 $\pm$ 2.8	24.1 $\pm$ 2.3	23.9 $\pm$ 3.1	26.1 $\pm$ 2.7
Glucose (mmol/L)	5.26 $\pm$ 0.55	4.82 $\pm$ 0.23	5.42 $\pm$ 0.36 ¹	5.66 $\pm$ 0.58 ¹
TCH (mmol/L)	5.73 $\pm$ 1.13	5.09 $\pm$ 0.95	5.62 $\pm$ 1.15	6.60 $\pm$ 0.74 ^{1,3}
HDL-C (mmol/L)	1.69 $\pm$ 0.38	1.64 $\pm$ 0.36	1.81 $\pm$ 0.39	1.66 $\pm$ 0.39
TC/HDL ratio	3.52 $\pm$ 1.03	3.22 $\pm$ 0.90	3.16 $\pm$ 0.70	4.19 $\pm$ 1.11 ^{2,3}
TAG (mmol/L)	1.13 $\pm$ 0.46	1.15 $\pm$ 0.50	1.13 $\pm$ 0.49	1.09 $\pm$ 0.41

Values are means $\pm$ SD

Significantly different compared with age category I ¹($p < 0.01$), ²($p < 0.05$)

Significantly different compared with age category II ³($p < 0.05$)

Postprandial lipemia

Fasting serum TAG and apoB48 concentrations were not different after the three intervention periods. The postprandial responses in serum TAG concentrations are presented in **Figure 6.1** and **Table 6.3**. Serum TAG concentrations gradually increased after consumption of the first meal to peak at 3-4 hours. A second peak was observed already one hour after consumption of the second meal (after 5 hours). The TAG iAUC of the total TAG response ($iAUC^T$) and after the 1st meal ($iAUC^1$) and 2nd meal ($iAUC^2$) were comparable between the test meals in the first age category (18-35 y). In the second age category (36-52 y), $iAUC^T$ and $iAUC^1$ were comparable between the test meals, but there was a trend for a higher $iAUC^2$ in the stanol period as compared with the sterol period (difference of 46.8 ± 58.8 mmol/L/min; $p=0.07$). In the third age category (53-69 y), $iAUC^T$ and $iAUC^1$ were again comparable between the test meals, but there was a significantly higher $iAUC^2$ in the stanol period as compared with the sterol period (63.1 ± 53.0 mmol/L/min; $p<0.01$) and the control period (43.2 ± 52.4 mmol/L/min; $p<0.05$).

Age was positively correlated with $iAUC^T$ in all three intervention periods (control: $r=0.47$; $p<0.01$, sterol: $r=0.44$; $p<0.01$, stanol: $r=0.56$; $p<0.01$), while the correlation between age and $iAUC^2$ was only significant in the stanol period ($r=0.57$; $p<0.01$). The maximal TAG (maxTAG) concentration increased with age in all three periods (control: $r=0.49$; $p<0.01$, sterol: $r=0.36$; $p<0.05$, stanol: $r=0.45$; $p<0.01$). In addition, there was a trend towards a higher maxTAG after stanol consumption compared with sterol consumption in age category II (0.60 ± 0.74 mmol/L; $p=0.07$).

The postprandial responses in serum apoB48 concentrations are presented in **Figure 2** and **Table 4**. Interestingly, serum apoB48 concentrations displayed a much earlier peak (60 minutes-3 hours) after the first meal as compared with the TAG response (3-4 hours). Another clear difference is that the time-to-peak after the second meal was delayed from the younger to the older age categories (i.e. from 5 hours to 6 hours after meal ingestion), which was not seen in the postprandial TAG response. ApoB48 $iAUC^T$ and $iAUC^1$ were comparable between the three test meals in all age categories. The apoB48 $iAUC^2$ was comparable in the first two age categories, but in age category III the $iAUC^2$ after stanol consumption was increased as compared with the sterol period (67.1 ± 77.0 mg/L/min; $p<0.05$) and tended to be higher $iAUC^2$ after stanol consumption compared with the control period (43.1 ± 64.5 mg/L/min; $p=0.08$).

Age was positively correlated with $iAUC^T$ in the stanol period ($r=0.47$; $p<0.01$) and with $iAUC^2$ in the control and stanol period ($r=0.32$; $p<0.05$ and $r=0.43$; $p<0.01$, respectively). The maximal apoB48 (maxApoB48) concentration correlated with age in the stanol period ($r=0.43$; $p<0.01$), but not in the sterol and control period. In addition, there was a trend towards a higher maxApoB48 after stanol consumption compared with the control period in age category III (2.9 ± 4.2 mg/L; $p=0.07$).

Retinyl palmitate was incorporated only in the breakfast shake and appearance in the circulation provides information about the lipid content of the chylomicron fraction,

whereas apoB48 provides information about the number of particles [20, 21]. Postprandial plasma retinyl palmitate concentrations (shown in **Figure 6.3, panel A**) were determined in 13 subjects and the curve closely resembled the serum TAG curve, showing a first meal peak between 3-4 hours and a second meal peak one hour after consuming the lunch shake. Due to the smaller sample size ($n=13$ instead of $n=42$), no statistical analyses in age subgroups were performed on the retinyl palmitate data. Changes in postprandial TAG concentrations always consist of a combined effect of changes in chylomicron and very low-density lipoprotein (VLDL) particle numbers and composition.

Figure 6.1. Serum triglyceride concentrations in (A) age category I: 18-35 years, (B) age category II: 36-52 years, (C) age category III: 53-69 years after consumption of a mixed meal containing no, or 3.0 gram of plant sterols or plant stanols. Inserts show second meal response (4-8 hours). Data are presented as mean $\pm$ SEM.

B: trend different iAUC² between stanol and sterol period $^*(p=0.07)$

C: significantly different iAUC² between stanol and control period $^{\#}(p<0.05)$ and stanol and sterol period $^{**}(p<0.01)$

To visualize the postprandial lipid response of the first 4 hours, we plotted retinyl palmitate concentrations as ratio over serum apoB48 concentrations (**Figure 6.3, panel B**), which gives information about the amount of TAG derived from the diet as compared to chylomicron particles in the circulation and specifically provides information about the lipid content of chylomicrons, e.g. their size [22]. Figures 2A-C show increases in apoB48 concentrations up to 60 minutes, after which concentrations remain relatively stable up to 4 hours, indicating that production rate of chylomicrons equals their clearance rate.

Table 6.3. Area under the curves (AUCs) and maximal increases in triglyceride concentrations after consumption of the test meals

Control period			Sterol period			Stanol period			
	18-35 y	36-52 y	53-69 y	18-35 y	36-52 y	53-69 y	18-35 y	36-52 y	53-69 y
iAUC ^T (mmol/L/min)	181.6 ± 90.2	226.5 ± 120.0	325.5 ± 171.8	186.6 ± 135.9	202.9 ± 116.7	387.1 ± 212.6	163.4 ± 80.3	308.5 ± 191.2	405.1 ± 211.2
iAUC ¹ (mmol/L/min)	60.9 ± 28.2	68.4 ± 32.5	85.5 ± 53.3	64.8 ± 44.8	62.9 ± 39.2	110.0 ± 57.2	55.1 ± 25.3	81.1 ± 62.1	100.9 ± 48.1
iAUC ² (mmol/L/min)	58.9 ± 54.8	67.3 ± 60.3	102.4 ± 49.3	61.3 ± 44.0	45.7 ± 43.6	82.5 ± 55.5	48.9 ± 34.5	92.6 ± 69.5 ^a	145.6 ± 72.2 ^{b,c}
MaxTAG (mmol/L/min)	0.93 ± 0.38	1.19 ± 0.55	1.62 ± 0.71	1.08 ± 0.54	1.13 ± 0.61	1.71 ± 0.72	0.97 ± 0.44	1.74 ± 0.98 ^a	1.85 ± 0.92

^a stanol > sterol p=0.07
^b stanol > sterol p <0.01
^c stanol > control p<0.05

Figure 6.2. Serum apoB48 concentrations in (A) age category I: 18-35 years, (B) age category II: 36-52 years, (C) age category III: 53-69 years after consumption of a mixed meal containing no, or 3.0 gram of plant sterols or plant stanols. Inserts show second meal response (4-8 hours). Data are presented as mean $\pm$ SEM. C: significantly different iAUC² between stanol and sterol period * ($p < 0.05$) and trend different iAUC² between stanol and control period # ($p = 0.08$)

This observation combined with Figure 3B, shows that TAG-poor chylomicrons are produced during the first hour, after which the dietary TAG content of chylomicrons (e.g. size) increases up to 4 hours. No diet effects were observed for RP/ApoB48 ratios. Due to the smaller sample size, no statistical analyses in age subgroups were performed.

Table 6.4. Area under the curves (AUCs) and maximal increases in apolipoprotein B48 concentrations after consumption of the test meals

	Control period				Sterol period				Stanol period		
	18-35 y	36-52 y	53-69 y	18-35 y	36-52 y	53-69 y	18-35 y	36-52 y	53-69 y	36-52 y	53-69 y
iAUC [†]	2716.5 ±	3360.3 ±	3506.1 ±	2754.7 ±	2551.3 ±	3833.9 ±	2508.5 ±	3262.9 ±	4311.3 ±	1927	
(mg/L/min)	1643	1664	2108	1874	1387	2495	1222	1637			
iAUC [‡]	1050.7 ±	1210.0 ±	1063.4 ±	1065.6 ±	967.5 ±	1124.5 ±	895.4 ±	1081.9 ±	1293.0 ±	600	
(mg/L/min)	470	547	651	507		668		717			
iAUC [§]	910.8 ±	1148.0 ±	1569.5 ±	1059.1 ±	796.9 ±	1328.7 ±	1050.9 ±	1188.1 ±	2000.0 ±		
(mg/L/min)		747	978	706		1151	789	626	1030 ^{a,b}		
maxApoB48	11.3 ± 6.4	13.8 ± 5.2	15.1 ± 7.7	12.3 ± 7.1	10.8 ± 5.8	16.2 ± 9.4	11.1 ± 5.2	14.3 ± 7.8	18.0 ± 6.7 ^c		
(mg/L)											

^a stanol > sterol p<0.05

^b stanol > control p=0.08

^c stanol > control p=0.07

Postprandial glycaemia

Fasting glucose and insulin concentrations were comparable between the three test days. Postprandial plasma glucose concentrations increased during the first 30 min after consumption of the first meal, returned to baseline after 60 min and remained below baseline till consumption of the second meal (**Figure 6.4**). Plasma glucose concentrations were maximally increased one hour after consumption of the second meal. Glucose iAUC^T was higher in the stanol period compared with the sterol period in age category I (229.4 ± 108.1 vs. 168.5 ± 106.4 mmol/L/min; $p < 0.05$; data not shown). Postprandial glucose responses did not differ between the test meals in age category II and III. Serum insulin concentrations were maximally increased 30 min after first meal consumption and returned to baseline concentrations after 180 min. A second (smaller) peak was observed after

Figure 6.3. (A) Plasma retinyl palmitate concentrations and (B) ratio retinyl palmitate/apoB48 ($n=13$) after consumption of a mixed meal containing no, or 3.0 gram of plant sterols or plant stanols. Insert shows second meal response (4-8 hours). Data are presented as mean $\pm$ SEM.

consumption of the second meal. No diet effects were observed for postprandial insulin responses in all subjects or in the different age categories.

Figure 6.4. (A) Plasma glucose and (B) insulin concentrations ($n=42$) after consumption of a mixed meal containing no, or 3.0 gram of plant sterols or plant stanols. Data are presented as mean $\pm$ SEM.

Discussion

In this randomised controlled trial, we found an increased TAG and apoB48 second meal response in subjects aged between 53 and 69 years after consuming a mixed meal enriched with plant stanol esters as compared to a mixed meal with or without plant sterol esters. The LDL-C lowering effect of plant sterol and plant stanols is well known, but recent studies have suggested that plant sterol and plant stanol esters also reduce fasting TAG concentrations [1, 5] by a so far unknown mechanism. Although fasting and postprandial TAG concentrations are positively associated with CVD risk [7-9], only three studies so far have addressed the postprandial effects of foods enriched with plant stanol esters on lipid and lipoprotein metabolism. Relas et al. performed two postprandial mixed meal studies enriched with plant stanol esters: a 24-hour study with 3.0g of plant stanol esters after a 2-week intervention period and an acute study with 1.0g of plant stanol esters [12, 13]. In both studies, the addition of plant stanol esters to a high-fat breakfast did not affect postprandial cholesterol and TAG concentrations in serum or in lipoprotein fractions. Gylling et al. provided a low-fat breakfast containing 4.5g of plant stanol esters after a 10-week intervention period (daily intake 8.8g of plant stanol esters) [11]. Unfortunately, changes in serum TAG and apoB48 concentrations were not determined in that study. The different postprandial study designs complicates the comparison to our study, especially because we found that plant stanol esters affect the second meal TAG and apoB48 response by using a sequential meal protocol with regular blood sampling. We demonstrated that the iAUC for TAG and apoB48 responses were similar after the first meal, while these responses were increased after consumption of the second meal in the plant stanol period. This effect was stronger with increasing age and more evident compared to the sterol period than to the control period. The second meal response predominantly addresses a potential change in TAG clearance [23, 24] and suggests that postprandial TAG and chylomicron particle clearance are less efficient after plant stanol consumption. LPL may play a crucial role in this respect. After plant stanol consumption, plasma plant sterol concentrations decrease [25], which may offer an explanation for the delay in TAG clearance. Theoretically, LPL activity might be decreased by reduced plant sterol concentrations through several mechanisms. LPL activity is regulated at the level of gene transcription by peroxisome proliferator activated receptor (PPAR)- α and $-\gamma$. Although studies on the effects of plant sterols and stanols on PPAR is scarce, a study performed in mice reported reduced plasma and hepatic TAG concentrations in response to plant sterol consumption with an unexpected reduction in intestinal PPAR α mRNA expression and no change in hepatic PPAR α mRNA expression [15]. Plat et al. found in peripheral mononuclear blood cells from metabolic syndrome patients after plant stanol consumption, no change in PPAR α or liver X receptor (LXR) expression, although serum TAG concentrations were reduced [26]. Future studies are needed to elucidate whether changes in local PPAR expression in for example adipose and/or skeletal muscle might play a role in LPL modulation after plant sterol or stanol consumption. Also, other modulators of LPL activity, such as apoCII and apoCIII could have been affected by a change in plant sterol

concentrations and would definitely be interesting for future research. Another explanation for the second meal effect after plant stanol ester consumption might be found in changed oxyphytosterol concentrations (oxidized plant sterols). We have already reported that plant stanol consumption reduced fasting oxyphytosterol concentrations [19]. Oxysterols (oxidized cholesterol) are natural ligands of LXR, which in turn induce LPL expression. There are some suggestions that oxyphytosterols also activate LXR, which could lead to an induction of LPL expression. If so, a positive effect of oxyphytosterols on LPL activity might be lost after plant stanol consumption, which could have resulted in decreased TAG clearance.

The increase in TAG concentrations after consumption of a second meal seems to contradict recent findings of reduced fasting TAGs concentrations after plant stanol consumption [4, 5]. However, reductions in fasting TAG concentrations were only observed in subjects with elevated fasting TAG concentrations and there is no data describing the effect of plant sterol and plant stanol consumption on postprandial responses in these populations. In the present study in apparently healthy, slightly hypercholesterolemic, normotriglyceridemic subjects, no effects of plant sterol and stanol esters on fasting TAGs were found [19], while postprandial TAG and apoB48 responses were increased in the plant stanol ester group. For now, it remains speculative whether the same observation on postprandial TAGs would have been found in a population with elevated fasting TAGs. It should however be noted that the observed plant stanol effect after consumption of a second meal was only present in older subjects, which may form a relatively large part of the target population for the consumption of plant sterol and plant stanol enriched products i.e. subjects with mild hypercholesterolemia (consensus paper). As reported before, non-fasting TAG concentrations are an emerging CVD risk marker and these findings need to be confirmed and evaluated into more detail.

This study also addresses the difference in conclusions that could be made after single meal consumption or after including a second meal, which highlights the metabolic consequences of sequential meal consumption in general. Unfortunately, different postprandial lipoprotein fractions were not collected in this study, but the addition of RP to the first breakfast meal gives us some opportunity to speculate concerning the postprandial lipemic response. After absorption, RP associates with chylomicrons and their remnants until taken up - without being re-secreted - by the liver, providing information regarding the lipid content of chylomicrons [21, 27]. In contrast, apoB48 concentrations provide information regarding the number of chylomicron particles [20]. Even though RP has several drawbacks, i.e. mainly accurate in early postprandial period (0-9h) [21], it provides clear evidence for a second meal effect. The fact that a RP peak is seen one hour after consumption of the lunch shake (while no RP was present in the lunch shake), indicates that this second meal induced chylomicron appearance containing fat from the previous breakfast shake. This illustrates the importance of choosing the appropriate study design to address a certain research question, especially when investigating nutritional effects on postprandial lipid responses. The postprandial response after the first meal shows a discrepancy between TAG and apoB48 concentrations,

i.e. an earlier rise and decrease to baseline in apoB48 concentrations as compared to TAG concentrations. By calculating ratios of dietary TAG concentrations to apoB48 concentrations, we have proposed that TAG-enrichment of chylomicrons continues to increase up to 4 hours without a concomitant increase in apoB48 concentrations after the first hour. This suggests that more TAG-rich chylomicrons are produced and our proposed ratios are in line with literature, which states that dietary lipid transport following a meal is mainly through increased chylomicron particle size with a relatively lesser increase in particle numbers, i.e. an increase in lipid content per chylomicron [28].

In conclusion, this study demonstrates an age-dependent increase in the postprandial TAG and apoB48 response after plant stanol ester consumption as part of a mixed meal, compared to a control meal and to a meal enriched in plant sterol esters. Further research is warranted to validate these results. Also, the mechanism by which plant sterols and plant stanols exert different effects on postprandial lipemic responses needs to be investigated, whether this observation maintains in subjects with elevated fasting TAGs, and if these effects have health consequences. In addition, our findings highlight the impact of sequential meal consumption as it provides more detailed information on postprandial lipid and lipoprotein responses.

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Chapter 7

Effects of vitamin E or lipoic acid supplementation on fasting oxyphytosterol concentrations in subjects with impaired glucose tolerance or type II diabetes

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Abstract

Consumption of plant sterol enriched products increases serum plant sterol concentrations. Plant sterols can be oxidized and form oxyphytosterols (plant sterol oxidation products). We have previously shown that fasting oxyphytosterol concentrations are not increased when healthy subjects consumed a plant sterol or a plant stanol enriched margarine (3.0 g/d of plant sterols or stanols) for 4 weeks. Now we have examined the effects of 4 weeks vitamin E or lipoic acid supplementation on fasting plasma oxyphytosterol concentrations in subjects with impaired glucose tolerance (IGT) or type II diabetes. For this, 20 subjects with IGT or type II diabetes consumed for 4 weeks in a random order placebo capsules, vitamin E capsules (804 mg/d) or lipoic acid capsules (600 mg/d). Plasma oxyphytosterol and oxysterol concentrations were determined in butylated hydroxytoluene (BHT)-enriched EDTA plasma via GC-MS. In addition, markers reflecting oxidative stress (oxidized LDL levels and MDA concentrations) and antioxidant capacity (TEAC assay, uric acid concentrations and GSH/GSSG) were also measured.

Oxyphytosterol concentrations were higher in subjects with IGT or type II diabetes as compared to concentrations in healthy subjects. Despite these higher values, plasma oxyphytosterol and oxysterol were not reduced after consumption of vitamin E or lipoic acid for 4 weeks. In addition, none of the other markers reflecting oxidative stress and anti-oxidative capacity were changed after consuming high-doses of these two antioxidants for 4 weeks. In conclusion, we demonstrated that the higher oxyphytosterol concentrations in subjects with impaired glucose tolerance or type II diabetes are not reduced by vitamin E and lipoic acid supplementation.

Introduction

Consumption of plant sterol enriched products reduces fasting serum low-density lipoprotein cholesterol (LDL-C) concentrations. However, as shown by a recent meta-analysis, at the same time serum concentrations of sitosterol increases by 31% [CI: 26-37%] and those of campesterol by 37% [CI: 29-45] at recommended intakes of 1.6 g/d [1]. Whether these increased plant sterol concentrations have an impact on CVD risk has been subject of debate for many years now, as epidemiological studies have reported contradictory findings [2, 3]. Previously, we have postulated that this controversy might relate to the fact whether plant sterols are oxidized or not [4]. Cholesterol and plant sterols share structural similarities and can therefore both be oxidized to form either oxysterols (cholesterol oxidation products) or oxysterols (plant sterol oxidation products) [5]. While oxysterol concentrations have been characterised as an oxidative stress marker [6], information regarding oxysterol metabolism and their health effects in humans is scarce. In fact, there is a clear lack of knowledge regarding absorption, endogenous formation via (ROS-mediated) oxidation of circulating plant sterols, and potential effects of other dietary components on circulating serum oxysterol concentrations. We have previously shown that increased dietary plant sterol consumption did not change fasting oxysterol concentrations, despite elevated fasting plant sterol concentrations. However, postprandial oxysterol concentrations increased after consumption of a high-fat meal enriched with plant sterol esters [7]. Information of dietary antioxidant supplementation on circulating oxysterol concentrations in metabolically stressed subjects would expand our knowledge on oxysterol metabolism in humans. Therefore, the objective of this study was to examine changes in plasma oxysterol concentrations after 4 weeks vitamin E or lipoic acid supplementation in subjects with impaired glucose tolerance or diabetes type II (DMII), who are characterized by increased oxidative stress. These two antioxidants were chosen since vitamin E is a typical fat-soluble antioxidant, while lipoic acid is a fat- and water-soluble antioxidant and both vitamin E and lipoic have been shown to reduce oxidative markers in type II diabetics.

Subjects and Methods

Subjects

The study population consisted of 20 men and women with impaired glucose tolerance (IGT) or type II diabetes. They were recruited via announcements in local newspapers in Maastricht and surroundings between October 2012 and March 2013. Subjects were invited for two screening visits if they met the following criteria: aged between 18 and 75 years, BMI between 20 and 35 kg/m², no active cardiovascular disease or severe medical condition that might interfere with the study and no use of insulin or lipid-lowering medication. During screening visits, weight and height were measured and a fasting blood sample was

drawn to determine lipid and lipoprotein concentrations. The diagnosis of type II diabetes or IGT was based on the use of oral hypoglycemic agents or an oral glucose tolerance test. Only subjects without diagnosed diabetes underwent an oral glucose tolerance test to verify potential presence of impaired glucose tolerance. For this, subject consumed 82.5 gram of dextrose monohydrate dissolved in 250 mL of tap water and plasma glucose concentrations were measured after 2 hours with an value between 7.8 -11.1 mmol/L defined as having IGT [8]. Twenty subjects of which 8 subjects with type II diabetes and 12 subjects with IGT were enrolled and completed the study. Baseline characteristics are shown in **Table 7.1**. All participants gave written informed consent before entering the study. The protocol was approved by the medical ethical committee of the Maastricht University Medical Centre+ (MUMC+) and the trial is registered at clinicaltrials.gov as NCT01984567.

Table 7.1. Baseline characteristics

Age (years)	63.1 ± 5.8
Male / Female (n)	16 / 4
Weight (kg)	90.1 ± 13.6
Height (m)	1.75 ± 0.1
BMI (kg/m ²)	29.5 ± 3.7
Total cholesterol (mmol/L)	5.3 ± 0.9

Diets and design

The study had a randomised placebo-controlled cross-over design and consisted of three intervention periods of 4 weeks, separated by washout periods of 4 weeks. Subjects were allocated to the intervention periods in a randomised order, based upon a computer-generated table with random numbers. During each period, subjects were asked to consume daily either three placebo capsules, three capsules containing R-vitamin E (268 mg three times; 804 mg a day) or three capsules containing R-lipoic acid (200 mg three times; 600 mg a day). They were instructed to consume one capsule during each meal with a glass of water. Subjects visited the university at the start of each period, and again after 3 and 4 weeks of intervention. They were provided with at least the amount of capsules needed for 4 weeks and excess capsules were returned at the end of each intervention period. Subjects completed a validated food frequency questionnaire (FFQ) at the end of each intervention periods and a dietician checked these questionnaires to calculate energy and nutrient intakes over the previous 4 weeks using the Dutch food composition table. Subjects were asked not to change their habitual diet, level of physical exercise or use of alcohol throughout the three intervention periods in the study.

Blood sampling

Blood was sampled after an overnight fast at the start of each intervention period and after 3 and 4 weeks of intervention. The same person performed all venipunctures at approximately the same time of the day at the same day of the week.

A clotting tube (Becton, Dickinson and Company, Franklin Lakes, NY, USA) was sampled at each occasion and serum was obtained by low-speed centrifugation at 1300 g for 15 min at room temperature, at least half an hour after venipuncture. Serum was stored at -80 °C and used for analysis of lipid and (apo)lipoprotein concentrations, hsCRP concentrations, iron status parameters (total iron, ferritin and transferrin), copper status parameters (i.e. copper and ceruloplasmin), inflammatory markers and cellular adhesion molecules. EDTA tubes (Becton, Dickinson and Company, Franklin Lakes, NY, USA) were sampled at the end of each period in weeks 3 and 4 and plasma was obtained by low-speed centrifugation at 1300 g for 15 min at 4 °C. To avoid auto-oxidation, 10 ml butylated hydroxytoluene (BHT; 25 mg/mL ethanol) was added per 1 mL of EDTA plasma, immediately after centrifugation and then stored at -80 °C. BHT-enriched plasma was used for analysis of oxyphytosterol, oxysterol, oxidized LDL and malondialdehyde (MDA) concentrations. Fresh whole EDTA blood was sampled in week 4 and was used for the isolation of red blood cells (RBCs) as described previously [9]. BHT was added to the RBC and PBMC fraction, stored at -80 °C and used for the analysis of oxyphytosterol and oxysterol concentrations. Heparin tubes (Becton, Dickinson and Company, Franklin Lakes, NY, USA) were also sampled in week 4 and plasma was obtained by low-speed centrifugation at 1300 g for 15 min at 4 °C. Heparin plasma was used for the analyses of the trolox equivalent antioxidant capacity (TEAC) assay and uric acid concentrations. For this, 10% TCA was added to the plasma (1:1) followed by centrifugation (13.000 rpm, 5 min at 4 °C). Whole heparin blood was sampled in week 4 and 1.3% 5-Sulfosalicylic Acid (SSA) in 10 mM HCL was added for the analysis of GSH/GSSG. Fresh whole heparin blood was used for the analysis of HbA1c concentrations. An Acid Citrate Dextrose (ACD) tube was sampled at the end of each period and was used for the isolation of blood platelets. BHT was added to the platelets fraction, stored at -80 °C and used for the analysis of oxyphytosterol and oxysterol concentrations. A NaF tube was sampled at the end of each period and plasma was obtained by low-speed centrifugation at 1300 g for 15 min at 4 °C. NaF plasma was stored at -80 °C and was used for the analysis of glucose concentrations.

Analyses

Total cholesterol (CHOD-PAP method; Roche Diagnostic, Mannheim, Germany), high-density lipoprotein cholesterol (HDL-C) (CHOD-PAP method; Roche Diagnostics, Mannheim, Germany) after precipitation of apoB-containing lipoproteins with phosphotungstic acid and magnesium ions, and triacylglycerol (TAG) concentrations, with correction for free glycerol, were analysed in serum enzymatically (GPO-Trinder; Sigma-Aldrich Corp., St. Louis, MO,

USA). LDL-C concentrations were calculated according to the Friedewald equation [10]. Apolipoprotein concentrations (apoA1 and apoB100) were analysed using highly sensitive immunoturbidimetric assays (Horiba ABX, Montpellier, France). Serum hs-CRP concentrations (Kamiya Biomedical Company, Seattle, USA) and glucose concentrations (Roche Diagnostics Systems, Hoffmann-La Roche Ltd., Basel, Switzerland) were also analysed enzymatically. All samples from one subject were analysed within one run at the end of the study. Glycated haemoglobin (HbA1c) was measured by high-pressure liquid chromatography (HPLC) (Bio-Rad variant II, Bio-Rad Laboratories, Inc.). Sitosterol and campesterol concentrations were analysed using liquid chromatography-flame ion detection (LC-FID) as described previously [11]. Concentrations are expressed as mg/dL and as 10^2 μ mol/mmol total cholesterol. Oxyphytosterol concentrations (7 α -hydroxy(OH)-campesterol, 7 α -OH-sitosterol, 7 β -OH-campesterol, 7 β -OH-sitosterol, 7-keto-campesterol and 7-keto-sitosterol) and oxysterol concentrations (7 α -OH-cholesterol, 7 β -OH-cholesterol and 7-keto-cholesterol) in plasma as well as in RBC 's, PBMC's and platelets were analysed by gas-liquid chromatography-mass spectroscopy (GC-MS) according to the procedure as described by Husche et al [12] and expressed as ng/mL, ng/mg or as pg/mg. Oxysterol and oxyphytosterol concentrations in plasma were also standardized for cholesterol concentrations and expressed as nmol/mmol cholesterol. Oxidized LDL (oxLDL) was measured by chemiluminescent ELISA by determining the content of oxidized phospholipids per particle of apoB100 (oxidized phospholipids:apoB100 ratio) as described elsewhere [13]. Iron and copper status parameters were analysed as previously described [29]. MDA concentrations (oxidative stress marker), trolox equivalent, GSH/GSSG and uric acid concentrations (antioxidative markers) were analysed as previously described [14, 15]. Plasma inflammatory markers (IL-6 and TNF α), cellular adhesion molecules (E-selectin, P-selectin, soluble (s) ICAM-1, sICAM-3 and sVCAM-1), thrombomodulin and serum amyloid A (SAA) were measured with a commercially available Multi Spot ELISA kit (Meso Scale Discovery). Fibroblast Growth Factor 21 (FGF-21) and 19 (FGF-19) concentrations were measured with a single spot ELISA kit (R&D systems). All analyses were performed in samples from week 4, except for lipid, (apo)lipoprotein and hsCRP concentrations which were measured in weeks 3 and 4 and averaged for data analysis.

Statistics

Effects of vitamin E and lipoic acid supplementation were evaluated by a univariate analysis of variance (ANOVA) with subject number as a random factor. When dietary effects reached statistical significance ($p < 0.05$), diets were compared pairwise using a Bonferroni's correction. Oxyphytosterol and oxysterol levels in RBC, PBMC and blood platelet fraction were not normally distributed and were therefore reported as medians with ranges and differences between treatments were evaluated by a Friedman's test. Oxyphytosterol concentrations in healthy subjects were measured in our previous study using exactly the

same method [4] and compared to those of subjects with IGT or type II diabetes by an unpaired Student's T-test. All statistical analyses were performed using SPSS 20.0 for Mac Os X (SPSS Inc., Chicago, IL, USA).

Results

Average daily intakes of energy, fibre, and cholesterol, and the proportions of energy from protein, carbohydrates, total fat, saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids, and habitual vitamin E intake as calculated from the FFQ's did not differ between the three intervention periods (**Table 7.2**).

Table 7.2. Daily dietary intake during the study

	Control period	Vitamin E period	Lipoic acid period
Energy (MJ)	8.3 ± 2.8	8.3 ± 2.3	8.3 ± 2.3
Fat (energy%)	38.2 ± 7.4	37.0 ± 6.5	36.7 ± 6.7
SAFA ^a	11.8 ± 3.1	11.6 ± 2.3	12.1 ± 2.9
MUFA ^a	13.6 ± 2.9	13.4 ± 3.2	12.8 ± 3.2
PUFA ^a	9.0 ± 3.8	8.4 ± 2.6	8.3 ± 2.9
Protein (energy%)	16.4 ± 4.1	16.4 ± 3.4	16.1 ± 2.9
CHO ^a (energy%)	41.0 ± 5.7	42.3 ± 6.4	42.8 ± 6.1
Alcohol (energy%)	1.9 ± 2.7	1.8 ± 2.3	1.7 ± 2.4
Fibre (g/day)	23.4 ± 10.0	24.1 ± 5.6	24.2 ± 6.0
Cholesterol (mg/day)	204 ± 66	201 ± 56	196 ± 64
Vitamin E (mg/day) ^b	13.9 ± 7.4	13.2 ± 5.3	13.1 ± 5.5

Values are means ± S.D. All subjects (n=20) received the three dietary periods in random order.

^a SAFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, CHO: carbohydrates

^b dietary vitamin E intake without capsule supplementation

Fasting oxyphytosterol concentrations after the control period in subjects with IGT or type II diabetes were compared with those from a previous study with healthy subjects during the control period [4]. Oxyphytosterol concentrations were comparable between subjects with IGT and type II diabetes. **Figure 7.1** shows that 7 α -OH-campesterol and 7 α -OH-sitosterol were significantly higher in subjects with IGT or type II diabetes compared with healthy subjects ($p < 0.001$). 7 β -OH-campesterol concentrations were comparable between the

two populations, while 7 β -OH-sitosterol concentrations were higher in subjects with IGT or type II diabetes compared with healthy subjects ($p<0.001$). Concentrations of 7-keto-campesterol and 7-keto-sitosterol were also significantly higher in subjects with IGT or type II diabetes compared with healthy subjects ($p<0.001$). Thus, these findings suggest that IGT and diabetic patients had increased oxidative stress.

Figure 7.1. Fasting plasma oxyphytosterol concentrations after a control period in healthy subjects and subjects with IGT or type II diabetes (* $p<0.001$)

Fasting oxyphytosterol and oxysterol levels in plasma, RBC's, and platelets in subjects with IGT or type II diabetes are shown in **Table 7.3**. In PBMC's, oxyphytosterol levels were below the detection limit. Plasma 7 α -OH-, 7 β -OH- and 7-keto-cholesterol, -campesterol and -sitosterol concentrations were comparable in the vitamin E, liponic acid and control periods. Cholesterol-standardized oxyphytosterol and oxysterol levels showed similar results. In RBC's, oxysterol concentrations were comparable during the vitamin E, liponic acid and control periods. For the oxyphytosterols, 7-keto-campesterol median levels were lower in the vitamin E period as compared to the control period (37 [23-87] pg/mg and 51 [31-331] pg/mg respectively; $p<0.05$), while the other oxyphytosterol isoforms were comparable at the end of the three periods. In blood platelets, 7 α -OH-, 7 β -OH- and 7-keto-cholesterol, -campesterol and -sitosterol levels were comparable in the vitamin E, liponic acid and control periods. In the PBMC fraction, 7 β -OH-cholesterol median levels were higher in the vitamin E period compared to the control period (223 [2-9925] pg/mg and 174 [22-16997] pg/mg respectively; $p<0.05$) while the other cholesterol oxidation levels were comparable in the vitamin E, liponic acid and control periods.

Serum total cholesterol concentrations were 0.26 ± 0.35 mmol/L higher at the end of the vitamin E period (4.9%; $p<0.05$) as compared with the liponic acid period and 0.23 ± 0.44 mmol/l (4.2%; $p<0.05$) higher as compared with the control period. This was also reflected in the total cholesterol/HDL cholesterol ratio, which was increased in the vitamin E period by 0.27 ± 0.40 (5.6%; $p<0.01$) compared with the control period, while there was a trend towards reduction in the liponic acid period (-4.4%; $p=0.06$). Also serum apoB100 concentrations were higher in the vitamin E period compared with the liponic acid ($0.05 \pm$

0.08 g/L; $p<0.05$) and control periods (0.07 ± 0.10 g/L $p<0.01$). Finally, apoA1 concentrations were also increased in the vitamin E period compared with lipoic acid (0.06 ± 0.08 g/L; $p<0.01$) and control periods (0.05 ± 0.09 g/L; $p<0.05$). No differences were found in serum TAG and HDL-C concentrations between the three periods.

Table 7.4 shows markers reflecting oxidative stress and antioxidative capacity. Oxidized LDL levels and MDA concentrations, which are both markers of oxidative stress, were comparable at the end of the three intervention periods.

In addition, parameters reflecting antioxidative capacity did not differ between the vitamin E, lipoic acid and control periods. Also plasma cytokines, cellular adhesion molecules, and iron and copper status were comparable between the three periods (**Table 7.5**).

Plasma glucose, HbA1c, FGF-19, FGF-21, serum plant sterols and lathosterol and cholestanol concentrations were comparable between the vitamin E, lipoic acid and control periods (**Table 7.6**).

Correlations

In the control period, positive correlations were found between plasma 7 α -OH-campesterol and 7 α -OH-sitosterol concentrations ($r=0.845$; $p<0.001$), between 7 β -OH-cholesterol and 7 β -OH-sitosterol concentrations ($r=0.625$; $p<0.01$), and between 7-keto-campesterol and 7-keto-sitosterol concentrations ($r=0.902$; $p<0.001$). Although plasma concentrations between the different oxyphytosterol isoforms also correlated significantly, correlations were less strong than between similar isoforms. Also in RBC's and platelets, oxycampesterol and oxysitosterol levels were correlated, although stronger correlations were seen in platelets compared with RBC's (**Table 7.7**). Plasma and RBC oxyphytosterol levels, plasma and platelet oxyphytosterol levels and RBC and platelets oxyphytosterol levels did not correlate.

In plasma 7 α -OH-cholesterol and 7 α -OH-campesterol concentrations were significantly correlated ($r=0.535$; $p<0.02$), whereas plasma 7 α -OH-cholesterol and 7 α -OH-sitosterol concentrations were not ($r=0.288$; $p=0.232$). 7 β -OH-cholesterol and 7 β -OH-campesterol concentrations correlated significantly ($r=0.502$; $p<0.05$) and there was a trend towards a correlation between 7 β -OH-cholesterol and 7 β -OH-sitosterol ($r=0.407$; $p=0.084$). There were no correlations between 7-keto-cholesterol concentrations and 7-keto-campesterol or between 7-keto-cholesterol and 7-keto-sitosterol concentrations. Oxidized LDL levels only correlated with 7-keto-cholesterol concentrations.

Table 7.3. Effect of vitamin E and lipoic acid supplementation on oxyphytosterol and oxysterol concentrations in plasma, RBC's and platelets

	Control period	Vitamin E period	Lipoic acid period
Plasma (ng/mL)			
7 α -OH-cholesterol	48.83 $\pm$ 32.10	50.33 $\pm$ 31.13	52.97 $\pm$ 33.08
7 α -OH-campesterol	0.20 $\pm$ 0.09	0.22 $\pm$ 0.12	0.18 $\pm$ 0.10
7 α -OH-sitosterol	0.46 $\pm$ 0.23	0.51 $\pm$ 0.27	0.53 $\pm$ 0.21
7 β -OH-cholesterol	8.82 $\pm$ 4.03	8.95 $\pm$ 4.07	8.32 $\pm$ 4.03
7 β -OH-campesterol	0.33 $\pm$ 0.17	0.35 $\pm$ 0.27	0.35 $\pm$ 0.26
7 β -OH-sitosterol	3.73 $\pm$ 1.63	4.05 $\pm$ 1.61	4.51 $\pm$ 2.21
7-keto-cholesterol	23.53 $\pm$ 10.36	26.44 $\pm$ 12.90	21.74 $\pm$ 7.76
7-keto-campesterol	2.71 $\pm$ 1.20	2.59 $\pm$ 1.15	2.81 $\pm$ 1.39
7-keto-sitosterol	6.50 $\pm$ 3.07	7.21 $\pm$ 3.82	7.70 $\pm$ 3.23
Sum oxysterol	80.64 $\pm$ 37.90	88.93 $\pm$ 40.54	83.03 $\pm$ 39.0
Sum oxyphytosterol	13.28 $\pm$ 5.22	14.93 $\pm$ 5.65	16.32 $\pm$ 6.61
RBC's (oxyphytosterols: pg/mg, oxysterol: ng/mg)			
7 α -OH-cholesterol	1.0 (0.3-24.8)	0.4 (0.2-1.0)	0.4 (0.2-19.4)
7 α -OH-campesterol	2.5 (1.6-76.0)	2.3 (0.7-3.8)	6.2 (0.85-74.6)
7 α -OH-sitosterol	8.8 (4.0-80.1)	7.2 (2.1-11.0)	6.8 (3.4-81.4)
7 β -OH-cholesterol	1.4 (0.2-35.3)	0.4 (0.2-1.3)	0.4 (0.1-28.2)
7 β -OH-campesterol	2.6 (0-76.8)	1.5 (0-3.6)	1.9 (0.1-109.0)
7 β -OH-sitosterol	20.7 (11.5-122.7)	18.5 (4.5-24.8)	18.4 (3.7-127.0)
7-keto-cholesterol	3.3 (0.8-95.9)	1.3 (0.7-3.9)	1.1 (0.8-115.7)
7-keto-campesterol	51.0 (31.4-330.7)	36.7 (23.4-86.6) ^a	40.2 (23.7-301.7)
7-keto-sitosterol	91.3 (65.8-333.0)	89.0 (45.5-157.1)	81.7 (43.1-282.6)

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Sum oxysterol	5.6 (1.6-156.0)	2.1 (1.1-6.1)	2.0 (1.3-161.4)
Sum oxysterol	180.3 (24.9-992.2)	156.4 (76.4-271.3)	153.0 (75.3-885.1)
Platelets (pg/mg)			
7 α -OH-cholesterol	72.6 (23.1-229.0)	86.2 (31.9-504.7)	107.6 (7.0-400.1)
7 α -OH-campesterol	3.6 (0.9-18.2)	5.0 (1.8-26.2)	2.6 (1.1-13.8)
7 α -OH-sitosterol	16.2 (5.2-63.4)	19.0 (5.2-98.4)	7.0 (5.1-65.3)
7 β -OH-cholesterol	81.3 (24.6-328.5)	115.0 (33.9-536.4)	104.0 (27.7-371.4)
7 β -OH-campesterol	2.6 (1.2-15.0)	2.4 (0-23.2)	3.0 (0.5-15.1)
7 β -OH-sitosterol	30.6 (4.1-101.0)	30.8 (4.6-145.8)	16.4 (2.8-122.5)
7-keto-cholesterol	345.4 (19.0-1068.6)	376.5 (36.9-1185.2)	234.1 (61.8-2110.9)
7-keto-campesterol	254.0 (71.2-757.0)	253.4 (63.5-1260.9)	112.0 (69.8-884.2)
7-keto-sitosterol	444.9 (152.8-1227.8)	469.8 (156.0-2075.6)	233.4 (155.2-1618.9)
Sum oxysterol	560.4 (66.7-1472.5)	642.0 (102.6-2104.7)	465.1 (112.7-2833.8)
Sum oxysterol	752.5 (245.9-2169.4)	779.6 (234.8-3564.5)	357.5 (248.6-2670.8)
PBMC's (pg/mg)			
7 α -OH-cholesterol	291.5 (57.0-6840.1)	462.5 (64.6-5201.5)	226.5 (63.3-1895.2)
7 β -OH-cholesterol	173.5 (21.6-16997.1)	223.4 (1.81-9924.9) ^a	178.4 (18.8-9287.7)
7-keto-cholesterol	184.1 (97.3-665.8)	183.3 (49.3-1013.6)	191.5 (64.7-81761.8)
Sum oxysterol	999.4 (282.1-24078.0)	1146.6 (1.81-15470.9)	1116.5 (401.6-82869.8)

Values are means $\pm$ SD or median (ranges) (n=20)

^a Significantly different compared with control period (p<0.05)

Table 7.4. Effect of vitamin E and lipoic acid supplementation on oxidative and antioxidative parameters

	Control period	Vitamin E period	Lipoic acid period
OxLDL (oxidized phospholipids:apoB100 ratio)	0.05 ± 0.07	0.04 ± 0.06	0.04 ± 0.06
MDA (μmol/L)	2.2 ± 0.4	2.1 ± 0.4	2.0 ± 0.3
Trolox equivalent (μmol/L)	489.5 ± 73.7	479.3 ± 77.8	493.6 ± 90.1
GSH/GSSG	49.4 ± 30.8	44.7 ± 22.8	50.0 ± 27.2
Uric acid (μmol/L)	312.4 ± 59.8	313.5 ± 59.4	321.3 ± 70.4

Values are means ± SD (n=20)

Table 7.5. Effect of vitamin E and lipoic acid supplementation on plasma inflammatory markers, cellular adhesion molecules and iron and copper status parameters

	Control period	Vitamin E period	Lipoic acid period
Inflammation			
CRP (g/L)	2.75 ± 2.49	2.33 ± 1.60	3.58 ± 4.78
IL-6 (ng/mL)	1.18 ± 0.45	1.21 ± 0.52	1.18 ± 0.47
TNFα (ng/mL)	3.65 ± 1.04	3.67 ± 1.02	3.79 ± 0.95
Thrombomodulin (ng/mL)	5.11 ± 1.38	5.04 ± 1.40	5.05 ± 1.34
SAA (mg/L)	8.88 ± 11.40	6.05 ± 4.56	8.47 ± 5.30
Cellular adhesion			
E-selectin (ng/mL)	27.00 ± 9.67	30.01 ± 12.93	29.74 ± 13.17
P-selectin (ng/mL)	65.54 ± 18.65	68.30 ± 22.18	69.79 ± 24.74
sICAM-1 (ng/mL)	267.75 ± 55.85	269.55 ± 60.37	277.40 ± 65.36
sICAM-3 (ng/mL)	0.58 ± 0.15	0.62 ± 0.17	0.60 ± 0.17
VCAM-1 (ng/mL)	428.45 ± 117.41	426.00 ± 119.21	423.55 ± 98.91
Iron and copper status			
Total iron (μmol/l)	14.48 ± 4.32	15.72 ± 4.83	15.69 ± 5.83
Ferritin (μg/L)	140.95 ± 199.80	139.48 ± 166.61	147.48 ± 174.53
Transferrin (g/L)	2.74 ± 0.46	2.70 ± 0.37	2.68 ± 0.38
Transferrin saturation (%)	21.96 ± 8.02	24.08 ± 9.22	24.10 ± 10.22
Copper (μmol/L)	12.24 ± 1.64	11.93 ± 1.61	12.02 ± 1.51
Ceruloplasmin (g/L)	0.25 ± 0.05	0.24 ± 0.02	0.24 ± 0.03

Values are means ± SD (n=20)

Table 7.6. Effect of vitamin E and lipoic acid supplementation on plasma glucose, serum lipid and lipoprotein concentrations and serum plant sterol, lathosterol and cholestanol concentrations

	Control period	Vitamin E period	Lipoic acid period
Glucose (mmol/L)	7.26 ± 1.64	7.29 ± 1.68	7.16 ± 1.47
HbA1c (%)	6.18 ± 0.86	6.19 ± 0.84	6.22 ± 0.85
FGF-19 (pg/mL)	46 ± 45	49 ± 47	50 ± 54
FGF-21 (pg/mL)	400 ± 263	486 ± 278	437 ± 227
Total cholesterol (mmol/L)	5.28 ± 0.97	5.52 ± 0.90 ^{1,3}	5.28 ± 0.96
LDL cholesterol (mmol/L)	3.30 ± 0.89	3.44 ± 0.90 ³	3.21 ± 0.86
HDL cholesterol (mmol/L)	1.34 ± 0.27	1.30 ± 0.25	1.30 ± 0.25
Total cholesterol/HDL	4.12 ± 0.90	4.38 ± 1.01 ^{2,5}	4.18 ± 0.89
Triacylglycerol (mmol/L)	1.62 ± 0.64	1.72 ± 0.65	1.73 ± 0.66
ApoB100 (g/L)	1.04 ± 0.19	1.11 ± 0.20 ^{2,3}	1.06 ± 0.19
ApoA1 (g/L)	1.41 ± 0.22	1.45 ± 0.20 ^{1,4}	1.40 ± 0.22
Sitosterol*	1.40 ± 0.83	1.48 ± 0.84	1.40 ± 0.72
Campesterol*	2.03 ± 1.11	2.08 ± 1.17	2.00 ± 1.12
Lathosterol*	1.64 ± 0.62	1.60 ± 0.49	1.54 ± 0.53
Cholestanol*	2.07 ± 0.37	2.05 ± 0.35	2.04 ± 0.34

Values are means ± SD and *sterols are expressed as 10² × μmol/mmol cholesterol.

Significantly different compared with control period: ¹(*p*<0.05), ²(*p*<0.01)

Significantly different compared with lipoic acid period ³(*p*<0.05), ⁴(*p*<0.01), ⁵(*p*=0.06)

Table 7.7. Oxyphytosterol correlations within plasma and within tissue (RBC's & platelets) in control period

Parameter	Parameter	Compartment	Correlation*	p-value
7 α -OH-campesterol	7 α -OH-sitosterol	Plasma	0.845	<0.001
7 β -OH-campesterol	7 β -OH-sitosterol	Plasma	0.625	<0.01
7-keto-campesterol	7-keto-sitosterol	Plasma	0.902	<0.001
7 α -OH-campesterol	7 β -OH-campesterol	Plasma	0.442	0.058
	7-keto-campesterol	Plasma	-0.408	0.083
7 β -OH-campesterol	7-keto-campesterol	Plasma	-0.071	0.773
7 α -OH-sitosterol	7 β -OH-sitosterol	Plasma	0.678	<0.01
	7-keto-sitosterol	Plasma	-0.067	0.785
7 β -OH-sitosterol	7-keto-sitosterol	Plasma	0.538	<0.015
7 α -OH-campesterol	7 β -OH-campesterol	RBC	0.922	<0.001
	7-keto-campesterol	RBC	0.390	0.110
7 β -OH-campesterol	7-keto-campesterol	RBC	0.420	0.080
7 α -OH-sitosterol	7 β -OH-sitosterol	RBC	0.798	<0.001
	7-keto-sitosterol	RBC	0.440	0.07
7 β -OH-sitosterol	7-keto-sitosterol	RBC	0.340	0.152
7 α -OH-campesterol	7 β -OH-campesterol	Platelets	0.577	<0.01
	7-keto-campesterol	Platelets	0.746	<0.001
7 β -OH-campesterol	7-keto-campesterol	Platelets	0.653	<0.01
7 α -OH-sitosterol	7 β -OH-sitosterol	Platelets	0.809	<0.001
	7-keto-sitosterol	Platelets	0.741	<0.001
7 β -OH-sitosterol	7-keto-sitosterol	Platelets	0.794	<0.001

* correlations in plasma concentrations were determined with Pearson's correlation and correlations in RBC and platelet levels were determined with Spearman's rho correlation

Discussion

This study demonstrates that circulating oxyphytosterol as well as oxysterol concentrations are not lowered by vitamin E or lipoic acid supplementation in metabolically stressed subjects with IGT or type II diabetes.

There is a clear lack of knowledge which factors affect oxyphytosterol concentrations in plasma and tissues. We have earlier shown that consumption of plant sterol enriched products did not increase fasting plasma oxyphytosterol concentrations, despite an increase in fasting serum non-oxidized plant sterol concentrations [4]. Now, we have evaluated whether plasma oxyphytosterol concentrations are reduced after antioxidant supplementation. However, circulating oxyphytosterol concentrations were not reduced after consumption of vitamin E or lipoic acid for 4 weeks. In addition, none of the other oxidative stress markers were changed and parameters reflecting anti-oxidative capacity did also not change after consuming these antioxidants for 4 weeks. This was rather unexpected and the question is why these antioxidants did not have an effect. Potential factors that could have affected the outcome are the population, the dose and the duration of the intervention period. The present study was performed in a population which consisted of subjects with IGT or type II diabetes, a population that is generally recognized for their increased oxidative stress [16]. This allowed us to compare oxyphytosterol concentrations between healthy subjects from our earlier study and those in our subjects with increased oxidative stress. Oxidative stress plays an important role in the initiation and progression of type II diabetes and these subjects have increased oxidized LDL-C and oxysterol concentrations [17]. We here demonstrate for the first time that not only oxysterol, but also plasma oxyphytosterol concentrations are higher in subjects with IGT or type II diabetes as compared with healthy subjects. Circulating oxycampesterol concentrations were 122% higher and oxysitosterol concentrations were 83% higher, while plant sterol concentrations were comparable between the two populations. Almost all oxyphytosterol isoforms were higher in IGT and diabetic subjects, except 7 β -OH-campesterol concentrations, which were comparable. Regarding the dose and the duration, both vitamin E and lipoic acid have been shown to reduce oxidative stress markers in various populations in both short-term (<6 weeks) and in longer-term studies [18, 19]. For example, vitamin E consumption (400 mg/d) for 3 months reduced MDA concentrations in a study with 80 type 2 diabetic patients showed [20]. In patients with carotid atherosclerosis 6 weeks of vitamin E (900 mg/d) supplementation reduced 7 β -OH-cholesterol levels [21]. Plasma MDA and oxysterol concentrations were also measured in our study, but were not changed after high-dose vitamin E supplementation. As reviewed by Pazdro et al. most studies have demonstrated convincingly that vitamin E protects against lipid peroxidation in diabetic subjects, while effects on protein and DNA oxidation are less clear [22]. Intravenous lipoic acid therapy (600 mg/d) reduced MDA and prostaglandin concentrations already after two weeks in 13 obese subjects with impaired glucose tolerance [23], while 8 weeks oral lipoic acid supplementation (1200 mg/d) in obese subjects failed to reduce oxLDL and prostaglandin

concentrations [24]. This may suggest that intravenous lipoic acid supplementation is more effective than oral supplementation in human subjects [24], which might explain the lack of effect in our study. However, Marangon et al. used a comparable oral dose of lipoic acid, e.g. 600 mg/d and demonstrated a decrease in urinary isoprostane concentrations and a reduction in LDL oxidative susceptibility in 31 healthy subjects [25]. Their intervention period lasted 2 months and it might be postulated that our intervention period of 4 weeks was too short to induce changes in oxidative stress markers. This overview illustrates that there is no clear explanation for the lack of effect when considering population, dose and study duration.

Besides evaluating effects on oxidative parameters we also measured a panel of markers reflecting antioxidative capacity. Previous studies showed an increase in endogenous antioxidant enzyme activities after vitamin E consumption (400 mg/d) in type II diabetic subjects [20, 26]. In addition, lipoic acid increased intracellular glutathione concentrations in a variety of cell types and tissues [27, 28]. However, we were not able to demonstrate an improvement in these anti-oxidative markers in plasma concentrations of subjects with IGT or type II diabetes after vitamin E or lipoic acid consumption.

Whether increased oxyphytosterol concentrations are potentially atherogenic remains to be determined. If so, it would be of interest to further characterize oxyphytosterol concentrations in different (diseased) population and assess which dietary interventions might be successful in reducing plant sterol oxidation products. In our earlier study with healthy control subjects we tried to assess whether oxidation of plant sterols might be iron or copper-mediated [29]. In that study, oxLDL concentrations correlated positively with three different iron status markers, while no correlations were observed between any of the three iron status markers and oxyphytosterol concentrations, suggesting that oxidation of plant sterols might not be iron-mediated. Copper status markers did not correlate with oxLDL or with oxyphytosterol concentrations. Iron status markers and copper status markers were again determined in the current study. Serum concentrations in the IGT and diabetic population were lower as compared to healthy subjects. The observation that iron and copper concentrations are lower while oxyphytosterol concentrations are higher in IGT and diabetic patients may suggest that oxidation of plant sterols is not regulated at all via iron or copper-mediated oxidation. However, iron status parameters were also not correlated with oxysterol concentrations. This suggests that the relation between oxysterol and iron or copper-mediated oxidation is not as straightforward, even though it is generally assumed that cholesterol oxidation is (partly) regulated via iron or copper-mediated oxidation [30].

An important question is not only whether plasma oxyphytosterol concentrations are changed after vitamin E or lipoic acid, but also what happens with oxyphytosterols inside tissues. Schött et al. recently determined oxyphytosterol concentrations in plasma and aortic valve cusps in patients undergoing elective aortic valve replacement due to severe aortic stenosis [31]. Oxyphytosterol isoforms correlated significantly within plasma and

within tissue [aortic valve cusps], while a correlation between plasma and aortic valve cusp tissue was lacking. These results are (partly) in agreement with our study, since we also observed no relation between plasma and blood cell oxyphytosterol concentrations, while the different oxyphytosterol isoforms within the various cells types did correlate. We therefore suggest that oxidation of plant sterols might occur locally within tissues and does not originate from uptake via plasma, which might explain the lack of correlation between plasma and tissue concentrations. However, vitamin E and lipoic acid failed to reduced oxyphytosterol and oxysterol values in RBC's and platelet fractions.

Rather unexpected findings in this study were increased serum lipid and (apo)lipoprotein concentrations in the vitamin E period compared with lipoic acid and control periods. This effect was consistent since cholesterol (total and LDL-C) and its carrier (apoB100) were increased. ApoA1 concentrations were increased in the vitamin E period, even though HDL-C concentrations were comparable in all three period. This cholesterol-raising effect of vitamin E was unanticipated and has only been described a few times before [19, 32]. Daily intake of vitamin E capsules cannot explain the observed effect, since they only provided minor amounts of linoleic and oleic acid. Changes in dietary intake are also highly unlikely, since food frequency questionnaires showed comparable dietary intake in all three periods. For now, we have no obvious explanation for this highly unexpected effect of vitamin E supplementation.

In conclusion, we here show that circulating oxyphytosterol concentrations are not reduced by vitamin E and lipoic acid supplementation, while we previously demonstrated that oxyphytosterol concentration are not increased after plant sterol consumption. However, oxyphytosterol concentrations clearly differ between healthy subjects and subjects with impaired glucose tolerance or type II diabetes. The possible clinical implications of these differences in oxyphytosterol concentrations and whether these may have an impact on CVD risk in IGT or type II diabetic subjects remains to be determined.

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Chapter 8

General Discussion

Introduction

Functional foods enriched with plant sterols and plant stanols have been demonstrated to lower LDL-C concentrations in numerous intervention studies. Several meta-analyses have summarized this evidence and described the dose-response LDL-C lowering effect of plant sterols and plant stanols [1-3]. However, guidelines are not unanimous with respect to consumption of plant sterol and stanol enriched foods. A European Atherosclerosis Consensus (EAS) panel recommended the use of functional foods with plant sterols and stanols in patients with high cholesterol levels at intermediate and low global cardiovascular risk that do not qualify for pharmacotherapy. In addition, the panel concluded that plant sterol and stanols could be considered in (very) high-risk patients who do not reach their LDL-C targets on pharmacotherapy or in those with statin intolerance [4]. On the other hand, recently updated guidelines from the National Institute of Health and Clinical Excellence (NICE) advice against the use of plant sterols or stanols for the prevention of CVD in subjects treated for primary and secondary prevention or in subjects with diabetes (type I and II) [5]. This inconsistency in guidelines is partly explained by a lack of endpoint studies for plant sterol and plant stanol consumption and because there are uncertainties regarding health effects of increased plant sterol concentrations after plant sterol consumption, which is a hotly debated topic. In addition, plant sterols are susceptible to oxidation and can form plant sterol oxidation products (oxyphytosterols). Information regarding oxyphytosterol metabolism is very scarce. For example, it is not known 1) which factors affect circulating oxyphytosterol concentrations, 2) if and how these oxidation products are related to metabolic health parameters and 3) whether they are atherogenic as suggested earlier for oxysterol concentrations. Information regarding plant sterols and plant stanols in the postprandial phase is also limited and it is not known 1) what the effects are of a mixed meal rich in plant sterols and plant stanols on postprandial lipid and glucose responses and 2) whether postprandial oxyphytosterol concentrations are affected by plant sterol and plant stanol consumption.

Therefore, we aimed to investigate whether fasting oxyphytosterol concentrations are affected by dietary determinants, such as plant sterol and plant stanol consumption and antioxidant supplementation and whether oxyphytosterol concentrations differ between populations. In addition, we also evaluated effects of plant sterol and plant stanol consumption in the postprandial state, by assessing effects on lipid, lipoprotein and glucose responses and effects on postprandial oxyphytosterol concentrations.

Increased plant sterol concentration and CVD risk

Plant sterols and plant stanols are structurally related to cholesterol and have cellular functions in plants analogous to those of cholesterol in animals. The difference between plant sterols and cholesterol resides in the side-chain, while plant stanols are the saturated derivative of plant sterols. The main sources of plant sterols are vegetable oils, cereals, vegetables, fruits,

nuts and seeds, with a mean daily intake that ranges between 200 and 300 mg/d, but can be as high as 600 mg/d in vegetarians. Plant stanols are less abundant in nature, with a mean daily intake of 17-24 mg/d [6]. Plant sterols and plant sterols (together with cholesterol and other lipophilic substances) are incorporated into mixed micelles that serve as vehicles to deliver these products to the brush border membrane. Here they are taken up into the enterocyte by Niemann-Pick C1-Like1 (NPC1L1), which is a general sterol transporter protein. After uptake into enterocytes, plant sterol and stanols are excreted back from the intestinal mucosa into the intestinal lumen by the ATP-binding cassette (ABC) transporters ABCG5/8. Absorption rates are very low, i.e. 0.5-2% for plant sterols and 0.04-0.2% for plant stanols [7]. Once present in the circulation, plant sterols and plant stanols are cleared by the liver and rapidly secreted from the liver into bile by hepatic ABCG5/G8 transporters. Due to low absorption and high biliary secretion rates, circulating concentrations of plant sterols and plant stanols are low, with plant sterol concentrations ranging from 0.3-1.0 mg/dL and plant stanol concentrations ranging from 0.002-0.012 mg/dL [8, 9]. Consuming plant sterol enriched food products increases circulating plant sterol concentrations. A recent meta-analysis by Ras et al. included 41 studies with 2084 subjects and showed that an average plant sterol intake of 1.6 g/d resulted in 31.3% increase in sitosterol and 37.3% increase in campesterol concentrations [10]. Plant stanol enriched products increase plant stanol concentrations (increase of 0.012-0.028 mg/dL at an intake of 0.6 ± 0.4 g/d) and additionally reduce serum plant sterol concentrations with observed reductions ranging from 16-23% [11]. The (patho)-physiological relevance of increased plant sterol concentrations after plant sterol consumption has been subject of scientific debate for two different reasons. First, questions were raised regarding the safety of elevated serum plant sterol concentrations after the identification of phytosterolemia, a rare genetic disorder. Subjects suffering from phytosterolemia have a mutation in the ABCG5/8 genes, which causes a lower secretion rate of plant sterols and stanols from enterocytes into the intestinal lumen and from hepatocytes into the bile ducts. Subjects have very high plant sterol concentrations and often have an early onset of atherosclerosis, while serum cholesterol concentrations are not always elevated [12]. However, recent evidence reports phytosterolemic patients with high plant sterol concentrations, but without any signs of atherosclerotic disease [13]. This observation suggests that the relation between extremely high plant sterol concentrations and CVD risk is not as straightforward as once thought, but rather more complicated. This might be explained by the presence of an additional second trigger that together with elevated plant sterols results in premature atherosclerosis. A second reason why increased plant sterol concentrations have been implicated as a CVD risk factor relates to epidemiological studies that demonstrated positive associations between plant sterol concentrations and CVD risk [14, 15]. However, this relation is again not as straightforward, since there are also studies that show negative associations and studies that do not report any association between plant sterol concentrations and CVD risk [16-18]. Although speculations have been raised,

such as use of different study designs and adjustments for potential confounding variables, the reason for this controversy in epidemiological studies is not known.

Fasting oxysterol and oxysterol concentrations

Due to the presence of a double bond between C5-C6, cholesterol and plant sterols can be oxidized resulting in the formation of oxysterols and oxysterols. Since plant stanols lack this double bond in their ring structure, they cannot be oxidized [19]. Cholesterol oxidation products have been studied more intensively than plant sterol oxidation products. This relates to the fact that cholesterol is associated with development of atherosclerosis, is endogenously produced and plasma (oxy)cholesterol concentrations are higher than (oxy) phytosterol concentrations [20]. Brooks et al. first identified cholesterol oxidation products in human atheromatous plaques in 1966 [21], while plant sterol oxidation products were identified in 1983 in plasma of patients with Waldenström's macroglobulaemia [22]. In 1999, Grandgirard et al. measured oxysterols in healthy subjects with a concentration of 0.3 µg/mL [23]. Hereafter, oxysterols have been determined in plasma of phytosterolemic patients, where a concentration of 4.7 µg/mL (sitosterol oxidation products) was determined, representing 1.4% of circulating sitosterol concentrations [24]. A few years later, oxysterols were again measured in healthy subjects, but now with a lower limit of detection, in a range of 0.005-0.057 µg/mL, which is a 100 times lower than the first measurement in 1999. Since then, oxysterols have been determined by several research groups worldwide and due to the variability in measuring techniques combined with very low circulating concentrations, large variations were present in oxysterol concentrations [25-27]. During recent years, methods for the analysis of oxysterols have improved and oxysterol concentrations between 0.1 and 4.9 ng/mL are reported in healthy subjects [26-28], which are still 10-100 times lower than plasma concentrations found for oxysterol [29]. **Figure 8.1** demonstrates cholesterol and oxysterol concentrations (A) and plant sterol and oxysterol concentrations (B) in our population of healthy subjects in a control period. In this population, 0.006% of cholesterol was oxidized, while 0.06% of plant sterols were present as oxysterols, which is a factor 10 higher.

Figure 8.1. (Oxy)cholesterol (panel A) and (oxy)phytosterol concentrations (panel B) in healthy subjects in a control period (means $\pm$ SD). Units differ for non-oxidized and oxidized sterols (mg/dL vs. ng/mL), while the scales differ between (oxy)cholesterol (0-300 mg/dL and 0-300 ng/mL) and (oxy)phytosterol concentrations (0-6 mg/dL and 0-6 ng/mL).

Table 8.1 shows the sum of oxyphytosterol concentrations in healthy subjects, of whom subjects were identified as low or high oxidizers, and in subjects with impaired glucose tolerance or type II diabetes. This data shows a clear distinction between these populations and this is the first time that this comparison is made, since oxyphytosterol concentrations were previously only determined in healthy subjects and in phytosterolemic patients. Importantly, it was also measured with the same method in the same lab, which is important regarding the above-mentioned variation in measuring assays between laboratories.

Table 8.1. Oxyphytosterol concentrations in healthy subjects and subjects with impaired glucose tolerance (IGT) or type II diabetes (DMII)

	Sum OPS (ng/mL) ^a
Low oxidizers (n=3)	3.6 $\pm$ 0.01
Healthy subjects (n=37)	4.7 $\pm$ 1.1
High oxidizers (n=3)	6.4 $\pm$ 2.2
IGT and DMII subjects (n=20)	13.9 $\pm$ 5.3

Values are expressed as means $\pm$ SD

^a Sum OPS consists of 7 α -OH-campesterol, 7 α -OH-sitosterol, 7 β -OH-campesterol, 7 β -OH-sitosterol, 7-keto-campesterol and 7-keto-sitosterol

This difference between healthy and diabetic subjects with respect to oxyphytosterol concentrations is also reflected in oxysterol concentrations. Arca et al. measured 7 β -OH-cholesterol and 7-keto-cholesterol in healthy subjects and in familial combined hyperlipidemic (FCHL) patients as a marker of oxidative stress [30]. They already established that these concentrations differ significantly from each other in these two populations. Since we measured oxyphytosterol concentration in our type II diabetic subjects, we were able to compare this data (**Table 8.2**) demonstrating that 7 β -OH-cholesterol differed markedly between the populations, while this distinction was less clear for 7-keto-cholesterol

concentrations. Data shown in Table 8.1 and Table 8.2 suggest that there is a strong shared oxidative component in oxysterol and oxysterol concentrations.

Table 8.2. Oxysterol concentrations in healthy and subjects with IGT or DMII and in familial combined hyperlipidemic (FCHL) patients

	7 β -OH-cholesterol (ng/mL)	7-keto-cholesterol (ng/mL)
Healthy subjects (n=54) ^a	4.7 $\pm$ 1.3	12.2 $\pm$ 7.0
DMII subjects (n=20)	8.3 $\pm$ 4.0	23.5 $\pm$ 10.4
FCHL patients (n=45)	17.7 $\pm$ 13.8	25.5 $\pm$ 15.2

Values are expressed as means $\pm$ SD

^a Data from healthy subjects and FCHL patients were derived from Arca et al. [30] and data from DMII subjects are derived from this thesis.

Dietary interventions and fasting oxysterol concentrations

We were able to establish that oxysterol concentrations are clearly different between subjects but it would be of interest to investigate whether oxysterol concentrations can be influenced by nutritional interventions. For this reason, we performed our first intervention study as described in **chapter 3**, to evaluate whether oxysterol concentrations would change after plant sterol and plant stanol consumption. As expected, campesterol concentrations increased by 50% and sitosterol concentrations increased by 36% after consumption of plant sterol enriched margarine, while reductions of 38% and 35% in campesterol and sitosterol concentrations were found after consumption of plant stanol enriched margarine for 4 weeks. These changes are in line with literature, as recently described in a meta-analysis by Ras et al. [10]. However, despite these increases in plant sterol concentrations, circulating oxysterol concentrations did not change after plant sterol consumption, while reductions in 7 β -OH-campesterol concentrations and – when standardized for cholesterol – also 7-keto-campesterol concentrations were seen after plant stanol consumption. These data suggest that circulating oxysterol concentrations are influenced by additional factors besides the presence of substrate, i.e. non-oxidized plant sterols in plasma. On the other hand, a study by Husche et al. reported a significant 87% increase in 7 β -OH-sitosterol concentrations in healthy subjects after consumption of 3.0 g/d plant sterol enriched margarine for 4 weeks [26]. Both studies used the same margarine and plasma samples were also determined in the same laboratory. While our study was a double blind randomised cross-over trial, Husche's study had a smaller sample-size and lacked a control group, which might explain (part of) this apparent discrepancy. However, both studies demonstrated that increases in serum plant sterol concentrations after plant sterol consumption does not correlate with changes in plasma oxysterol concentrations. In addition, serum plant sterol concentrations and circulating oxysterol concentrations also did not correlate in the control period.

As discussed previously, controversies exist regarding increased plant sterol concentrations and an increased CVD risk in epidemiological studies. A potential answer explaining this controversy might be found in increased oxyphytosterol concentrations. For now, our data suggests that an increase in serum plant sterol concentrations does not change circulating oxyphytosterol concentrations and might therefore not explain discrepancies in epidemiological studies. However, to definitely answer this question, oxyphytosterol concentrations should be measured in epidemiological studies that report contradictory results on the association between plant sterol concentrations and CVD risk. As mentioned before, serum plant sterol concentrations and circulating oxyphytosterol concentrations do not correlate cross-sectional nor after plant sterol consumption. On the other hand, oxyphytosterol concentrations correlated well between different periods in the same subjects, indicating that plasma oxyphytosterol concentrations seem to be consistent within subjects under different dietary circumstances. This resulted in the identification of 'low and high oxidizers' as described in **chapter 4**, who differed with respect to their oxyphytosterol concentrations. To assess whether these differences also extend to other oxidized compounds, oxysterol and oxidized LDL (oxLDL) concentrations were determined and these oxidative stress markers also differed between low and high oxidizers, highlighting that there is an oxidative component that determines circulating oxyphytosterol concentrations in healthy human subjects. In vivo oxidation is often catalysed by iron ions, which can generate hydroxyl radicals from hydrogen peroxide. We demonstrated a positive correlation between iron stores and oxLDL concentrations, while plasma oxyphytosterol concentration were not correlated with iron status markers. This suggests that different pathways are responsible for oxidation of plant sterols and LDL particles. On the other hand, oxysterol concentrations were positively associated with serum ferritin concentrations in a large cohort of Finnish men [31], while we were not able to show correlations with iron status markers and oxysterol concentrations in 20 subjects with impaired glucose tolerance or type II diabetes (**chapter 7**). To investigate the role of oxidative stress on plasma oxyphytosterol concentrations, we investigated whether plasma oxyphytosterol concentrations might be changed after antioxidant supplementation. For cholesterol oxidation products, antioxidant supplementation has been shown to reduce cholesterol oxidation products in previous studies. Szuchman et al. showed that patients with diabetes and hypercholesterolemia have higher oxysterol concentrations than control subjects [32]. In addition, consumption of pomegranate juice (high in antioxidants) suppressed the capacity of diabetic blood to oxidize an exogenous oxidative stress marker and the ratio oxysterol/total cholesterol was reduced by 93%. A study performed in patients with carotid atherosclerosis showed reductions in 7 β -OH-cholesterol after 6 weeks of vitamin E (900 mg/d) supplementation [33]. In **chapter 7**, we described the first study that evaluated potential changes in oxyphytosterol concentrations after antioxidant supplementation. The study was performed with 20 subjects with impaired glucose tolerance of type 2 diabetes who received daily vitamin E (804 mg) and lipoic acid (600 mg) capsules for 4 weeks in a randomised cross-over trial.

We showed that oxyphytosterol concentrations were not changed after 4 weeks high-dose antioxidant supplementation. Besides comparable oxyphytosterol concentrations in the vitamin E, lipoic acid and control period, other oxidation parameters such as oxysterol, oxLDL and MDA levels were also not changed after vitamin E or lipoic acid supplementation. To summarize the two described intervention studies: we attempted to change plasma oxyphytosterol concentrations by providing more substrate, e.g. increased plant sterol concentrations and enabling a greater anti-oxidative capacity, e.g. by supplementing of vitamin E and lipoic acid. Except for small reductions in 7 β -OH-campesterol and 7-keto-campesterol after plant stanol consumption, fasting oxyphytosterol concentrations were not changed, at least not by the nutritional means described in this thesis.

Oxyphytosterol concentrations in serum versus tissue

Oxyphytosterol concentrations are mainly determined in plasma, while information regarding tissue concentrations is scarcer. Oxysterols have been analysed into more detail since certain oxysterols (mainly side-chain oxidation products) carry out important functions within the body such as ligands for the nuclear receptors liver X receptor (LXR) α and LXR β and as intermediates in bile acid biosynthesis [34]. ROS-mediated oxysterols are suggested to be associated with initiation and progression of atherosclerosis 7 β -OH-cholesterol and 7-keto-cholesterol concentrations have been found in atherosclerotic plaques [35]. Staprans et al. showed that dietary α -epoxy cholesterol is incorporated into chylomicrons (CM) and CM-remnants and then transferred to LDL and HDL implying that transportation of oxysterols to different tissues within the body occurs through lipoproteins [36]. The addition of oxyphytosterols to the diet on tissue level has also been investigated. Oxyphytosterols were fed to hamsters at different concentrations and the same oxyphytosterols isoforms were measured in heart, kidney, liver and aortic tissue. A higher oxyphytosterol content in the diet resulting in a dose-dependent oxyphytosterol increase in the different tissues, while very low dietary oxyphytosterols resulted in hardly detectable plasma and tissue levels, implying that small amounts of oxyphytosterols can probably be eliminated fast by the animals. Oxyphytosterol concentrations were lower in liver tissue compared to kidney and heart tissue, which might reflect a higher metabolic rate in this organ [37]. The provided diets contained more oxysterols than oxycampesters and sitosterol oxidation products were consistently higher in plasma and tissue than campesterol oxidation products. To assess the effect of oxysterol or oxyphytosterol consumption on atherosclerotic lesion formation, female LDL receptor^{+/-} mice were fed a control diet, a high-fat oxysterol diet or a high-fat oxyphytosterol diet. The addition of oxysterol and oxyphytosterols to an atherogenic high-fat diet resulted in a higher proportion of severe lesions and in addition, oxyphytosterol levels were the highest in the groups receiving the oxyphytosterol diet, indicating that diet-derived oxyphytosterols are incorporated into lesions. Unfortunately, plasma and tissue oxyphytosterol concentrations

could not be compared, since plasma oxyphytosterol concentrations were not measured in this study [38]. In patients undergoing elective aortic valve replacement due to severe aortic stenosis, oxyphytosterol concentrations were measured in plasma and in tissue (aortic valve cusps). While there was a strong correlation between different oxyphytosterol isoforms within plasma and within tissue, a correlation between plasma and aortic tissue was lacking [39]. This data is in agreement with our study in type II diabetic subjects, where we measured oxyphytosterol concentrations in red blood cells and in platelets and also did not report a correlation between plasma and tissue concentrations. However, a study performed in male apoE^{-/-} mice did report significant correlations between oxyphytosterol concentrations in plasma and aortic tissue [40]. In this study, mice received daily intraperitoneal injections over 4 weeks with cholesterol, sitosterol, 7 β -OH-cholesterol, 7 β -OH-sitosterol or a control vehicle. Compared to the control period, daily injections of cholesterol did not result in increased serum or aortic cholesterol concentrations, while injections of 7 β -OH-cholesterol increased 7 β -OH-cholesterol concentrations in serum but not in aortic tissue. On the other hand, sitosterol and 7 β -OH-sitosterol concentrations both increased in serum and in the arterial wall after injection of sitosterol and 7 β -OH-sitosterol, respectively. An explanation why this study reports a correlation between plasma and tissue concentrations, whereas previous studies were not able to show this correlation, might be explained by the method of intraperitoneal injections. Injecting a substance results in high plasma concentrations, which probably will lead to an incorporation of the injected substance into tissues, causing plasma and tissue concentrations to correlate, which might explain the lack of correlation in the patients that underwent elective aortic valve replacement and in our type II diabetic subjects.

Postprandial oxyphytosterol responses after plant sterol and plant stanol consumption

Besides fasting oxyphytosterol concentrations, we were also able to measure postprandial oxyphytosterol concentrations after consumption of a mixed meal enriched with plant sterol and plant stanol esters. We already established that fasting oxyphytosterol concentrations did not change after plant sterol consumption. In **chapter 5**, we assessed postprandial oxyphytosterol concentrations and demonstrated that postprandial oxyphytosterol concentrations are increased after plant sterol consumption. Serum (non-oxidized) campesterol and sitosterol concentrations increased in parallel with 7 β -OH-campesterol and 7 β -OH-sitosterol after plant sterol consumption, while 7-keto-phytosterol concentrations only changed over time without a plant sterol effect. The question whether postprandial plant sterol oxidation products are formed endogenously or absorbed from the diet is difficult to answer, since the consumed margarine contained plant sterols as well as oxyphytosterols. Therefore, we can only speculate on the origin of postprandial oxyphytosterol concentrations. Linseisen et al. measured postprandial oxysterol concentrations in five healthy subjects, which consumed a meal rich in cholesterol and in

cholesterol oxidation products [41]. Analogue to our observation regarding postprandial oxyphytosterol concentrations, oxysterol concentrations increased in parallel with non-oxidized cholesterol concentrations and the authors concluded that absorption patterns of non-oxidized and oxidized cholesterol are comparable with differences in the bioavailability of single oxysterol isoforms. Another indication that circulating oxyphytosterol concentration predominantly origin from dietary absorption is presented in the study by Weingärtner et al, who reported that mice injected with non-oxidized cholesterol or sitosterol did not show an increase in plasma oxysterols or oxyphytosterols concentrations, suggesting that there was no endogenous formation, at least not in the circulation [40]. Tomoyori et al. suggested that campesterol oxidation products have a higher absorption rate than sitosterol oxidation products in rats [42]. If this holds also true in the human situation, then these higher absorption rates are reflected in postprandial concentrations but not in fasting oxyphytosterol concentrations, since fasting oxysterol concentrations are higher than oxysterol concentrations. For non-oxidized plant sterols, it has been shown that campesterol is better absorbed than sitosterol and this is reflected in both fasting as well as postprandial concentrations [7]. In addition, the postprandial pattern of plant sterols and oxyphytosterols resembles the second meal effect as described for cholesterol, which states that cholesterol from a first meal is secreted during subsequent meal consumptions [43-45]. Based on our postprandial data and this possible second meal effect, we suggest that plasma oxyphytosterol concentrations predominantly originate from dietary absorption. The question remains how to explain the difference between fasting oxysterol and oxysterol concentrations. If indeed absorption rates of campesterol oxidation products are higher as suggested from animal data, then clearance rates probably differ between campesterol and sitosterol oxidation products. To provide a definite answer to the question whether plasma plant sterol oxidation products result from endogenous formation or dietary absorption, future studies should be performed where labelled non-oxidized plant sterols are consumed and subsequent enrichment in labelled plasma oxyphytosterols are measured in plasma.

Postprandial metabolic responses after plant sterol and plant stanol consumption

The LDL-lowering effect of plant sterols and plant stanols has been demonstrated in numerous studies. However, recent studies have suggested that plant sterols and plant stanols also lower fasting triacylglycerol (TAG) concentrations, especially in individuals with elevated baseline TAG concentrations [46, 47]. Although high fasting TAG concentrations are independent risk marker for CVD [48], non-fasting serum TAG concentrations are emerging as an important CVD risk marker due to the postprandial atherogenicity of TAG-rich (remnant) lipoprotein particles [49, 50]. Most people are in the non-fasting state or postprandial state for most part of the day and in **chapter 6**, we examined the 8-hour postprandial response after consuming plant sterol or plant stanol ester enriched margarines as part of a shake.

We demonstrated a similar response in TAG and apolipoprotein (apo) B48 concentrations after the first meal, while these responses were increased after consumption of the second meal in the plant stanol period. This effect was stronger with increasing age and more evident compared to the sterol period than to the control period. Previous studies suggest that chronic or acute plant stanol consumption does not affect postprandial lipid and lipoprotein concentrations [51-53]. Although these studies have some limitations, they also differ from our study design since we used a sequential meal protocol with regular blood sampling. As described by Jackson, a sequential two-meal protocol provides a more physiological challenge to detect subtle changes in TAG metabolism, especially after the second meal consumption when the clearance cascade is stressed [54]. Since the observed plant stanol effect was only evident after consumption of the second meal, this suggests that the activity of enzymes involved in processing of TAG-rich lipoproteins (TRLs) or the rate of clearance of TRL remnants by receptor-mediated processes was affected. In general, the increase of the postprandial TAG concentrations correlates positively with age and our observed effect was more prominent with an increase in age [55]. The most important determinant of postprandial TRL accumulation is the capacity of lipoprotein lipase (LPL) to hydrolyse TAG, thus to convert very low-density lipoproteins (VLDL) and chylomicrons into remnants. A decrease in LPL activity would probably be the most likely explanation for the observed plant stanol effect. Possibilities might be found in modulators of LPL activity; such as changed local peroxisome proliferator activated receptor (PPAR)- α and $-\gamma$ expression in adipose and/or skeletal muscle or increased serum apoCIII concentrations after plant stanol consumption. Increased levels of apoCIII are associated with an impaired catabolism of TRLs and reducing apoCIII concentrations has gained much attention as a potential target to correct hypertriglyceridemia in subjects with type II diabetes or insulin resistance [56]. As mentioned before, recent studies reported reduced fasting TAG concentrations after plant stanol consumption, while we report an increase in postprandial TAG concentrations after consumption of a second meal. However, reductions in fasting TAG concentrations were only observed in subjects with elevated fasting TAG concentrations while our population consisted of healthy subjects and as expected, we were not able to demonstrate a reduction in fasting TAG concentrations in this population. There are no studies available that investigated postprandial responses after plant sterol and plant stanol consumption in subjects with higher baseline TAG concentrations. However, Bozetto et al. investigated whether ezetimibe, a cholesterol-lowering drug that acts through a reduction in cholesterol absorption – analogue to plant sterols and plant stanols – affects postprandial TRLs in subjects with type II diabetes. They reported that a 6-week treatment with ezetimibe and simvastatin, compared to simvastatin alone, reduced fasting and postprandial TAG and apoB48 concentrations [57]. The reduced postprandial responses mainly reflected the chronic action of ezetimibe to reduce cholesterol absorption, since fasting TAG and apoB48 concentrations were also reduced. Plant sterols and plant stanols also reduce cholesterol absorption – although to a lesser extent than ezetimibe – but this was not reflected in the

postprandial responses. However, as indicated, our population consisted of healthy subjects while the ezetimibe study was performed in type II diabetic subjects. It remains to be determined how postprandial responses are changed after consumption of plant sterols and plant stanols in subjects with elevated TAG concentrations or in subjects with type II diabetes.

Conclusion and future directions

This thesis describes that fasting oxyphytosterol concentrations differ between low and high oxidizers and between healthy and diabetic subjects. Whether these differences in oxyphytosterol concentrations have an impact on CVD risk remains to be determined in future studies. Fasting oxyphytosterol concentrations were not increased after daily consumption of a plant sterol enriched margarine for a period of 4 weeks, while plant stanol consumption reduced 7 β -OH-campesterol concentrations and – when standardized for cholesterol – also 7-keto-campesterol levels. In addition, high-dose antioxidant supplementation (804 mg/d vitamin E and 600 mg/d lipoic acid) for a period of 4 weeks did not change plasma or blood cell oxyphytosterol concentrations in type II diabetic subjects. In contrast to fasting oxyphytosterol concentrations, postprandial oxyphytosterol concentrations did increase after plant sterol consumption. Against our expectations, postprandial TAG and apoB48 responses were increased after consumption of a mixed meal rich in plant stanols, which might be explained by a reduced postprandial LPL activity although underlying mechanisms remain to be elucidated.

Future directions regarding oxyphytosterols

Future studies should be carried out to assess whether oxyphytosterols causes metabolic disturbances and if they are atherogenic as suggested earlier for oxysterol concentrations. In addition, relationships between plasma vs. tissue concentrations of oxyphytosterols should be further evaluated, which may also provide insight into the site of oxidation. Furthermore, oxyphytosterol concentrations have only been determined in healthy subjects, in subjects with impaired glucose tolerance or type II diabetes and in phytosterolemic patients. Characterization of oxyphytosterols in other populations, such as patients with atherosclerotic disease or neurodegenerative diseases would expand our knowledge on the role of oxyphytosterol concentrations in the metabolism of (diseased) populations. In addition, measurement of oxyphytosterols in phytosterolemic patients with and without atherosclerotic disease could provide more information on the potential atherogenicity of oxidized plant sterols. Whether oxyphytosterols are derived from endogenous formation or absorption from the diet can only be answered by a study in which labelled non-oxidized plant sterols are consumed and subsequent enrichment in labelled plasma oxyphytosterols is measured.

Future directions regarding postprandial lipid metabolism

Postprandial lipid responses were increased after consumption of a mixed meal rich in plant stanols. To provide more insight into postprandial responses after plant sterol and plant stanol consumption, kinetic studies should be performed in which lipoprotein production and clearance can be further evaluated. An increased postprandial lipid response was in contrast to our expectations since fasting TAG may be reduced after plant sterol and stanol consumption. However, this TAG-lowering effect was seen in subjects with elevated baseline TAG concentrations and it would be interesting to assess whether postprandial lipid responses are different in diabetic subjects or in subjects with high baseline TAG concentration compared with healthy subjects.

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Summary

Summary

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in many parts of the world. Risk factors to develop CVD include cigarette smoking, high blood pressure, diabetes mellitus, obesity and an unfavourable serum lipoprotein profile, such as elevated low-density lipoprotein cholesterol (LDL-C) concentrations. Dietary lifestyle changes are important to reduce CVD risk and in this context the development of functional foods that lower serum LDL-C concentrations has been groundbreaking. Plant sterols and plant stanols are lipid-like compounds that share structural similarities with cholesterol. They consist of a steroid nucleus with a double bond present at C5-C6. There are various plant sterols that differ only in the side-chain configuration. Plant stanols are the saturated derivative of plant sterols. Plant sterols and plant stanols have been incorporated in a variety of functional foods and consumption of approximately 2.5-3.0 g/d of plant sterols or plant stanols can lower serum LDL-C concentrations up to 12%. In addition, consuming plant sterol enriched products will increase serum plant sterol concentrations, while the consumption of plant stanols will decrease plant sterol concentrations. Plant sterols are susceptible to oxidation and can form oxyphytosterols, while plant stanols cannot be oxidized since they lack a double bond in their ring structure.

The aim of the studies described in this thesis was to investigate the effects of plant sterol and plant stanol consumption on lipoprotein metabolism and oxyphytosterol concentrations in the fasting and postprandial state and to evaluate the effects of antioxidant supplementation on plasma oxyphytosterol concentrations.

Chapter 2 is an overview of the available literature regarding various aspects of using plant sterols and plant stanols in the treatment of dyslipidaemia. Besides the well-known LDL-C lowering effect of plant sterols and plant stanols, this chapter also describes their beneficial effects on triacylglycerol (TAG) concentrations and provides an overview of arguments used in the ongoing debate whether increased plant sterol concentrations are associated with an increased CVD risk. In **chapter 3**, we investigated the effects of plant sterol and plant stanol consumption on fasting plasma oxyphytosterol concentrations in healthy human subjects. For this, 43 healthy subjects (18-70 years) participated in a randomised, double-blind, cross-over study and consumed for 4 weeks a plant sterol enriched (3.0 g/d of plant sterols), a plant stanol enriched (3.0 g/d of plant stanols), and a control margarine separated by wash-out periods of 4 weeks. Compared with control, LDL-C concentrations were reduced after plant sterol (-8.1%) and after plant stanol consumption (-7.8%), but oxyphytosterol concentrations were not changed after plant sterol consumption. However, 7 β -OH-campesterol concentrations were reduced after plant stanol consumption by 0.07 ng/mL (~14%) and by 0.07 ng/mL (~15%) compared with the control and sterol margarines. We concluded from this study that a daily consumption of a plant sterol enriched margarine for a period of 4 weeks does not increase fasting plasma oxyphytosterol concentrations,

while minor reductions were seen after plant stanol consumption. This study showed large but consistent differences in oxyphytosterol concentrations between individuals. In **chapter 4**, we tried to identify factors that might explain these inter-individual differences in plasma oxyphytosterol concentrations. This study demonstrated that plasma oxyphytosterol concentrations do not correlate with serum non-oxidized plant sterol concentrations and that plasma oxyphytosterol concentrations remained relatively stable over time. We identified six subjects with consistently low or high plasma oxyphytosterol concentrations during all three dietary interventions, which were also reflected in oxidized LDL (oxLDL) concentrations. Oxidative and anti-oxidative capacity markers, such as iron/copper status, α -tocopherol concentrations and TEAC values were measured but could not explain differences between the high and low plasma oxyphytosterol concentrations. Besides the effect of plant sterol and plant stanol consumption on fasting oxyphytosterol concentrations, we also evaluated postprandial plasma oxyphytosterol concentrations after consumption of a plant sterol or stanol enriched mixed meal (**chapter 5**). Humans are in a non-fasting state for most part of the day and to investigate effects on oxyphytosterol concentrations during the postprandial state, 10 healthy subjects consumed a shake (50 g of fat, 12 g of protein, 67 g of carbohydrates), containing no, or 3.0 g of plant sterols or plant stanols. Blood samples were taken up to 8 hours and after 4 hours subjects received a second shake (without plant sterols or plant stanols). This postprandial meal test was performed at the end of each of the three intervention periods in which subjects consumed 20 gram of margarine on a daily basis, providing them with no, or 3.0 gram of plant sterols or stanols per day. 7β -OH-campesterol and 7β -OH-sitosterol concentrations increased after consumption of a mixed meal enriched with plant sterol esters compared with a mixed meal or a mixed meal enriched with plant stanol esters. These higher oxyphytosterol concentrations were particularly present after consumption of a second meal. A comparable postprandial pattern was seen for non-oxidized campesterol and sitosterol concentrations, which also increased after a mixed meal enriched with plant sterol esters compared with a control or plant stanol meal. We speculated whether plasma oxyphytosterols originate from dietary absorption or endogenous formation and suggested that plasma oxyphytosterol concentrations most likely are derived from dietary absorption. However, a definite answer to this question can only be provided by a study in which labelled non-oxidized plant sterols are consumed and enrichment in labelled plasma oxyphytosterols is measured. Recent studies have suggested that consumption of plant sterol and plant stanol enriched products not only lower LDL-C concentration, but also reduce fasting TAG concentrations, especially in subjects with higher TAG concentrations. Since humans are in a non-fasting state for most part of the day and non-fasting serum TAG concentrations are emerging as an important CVD risk marker, we investigated the effects of plant sterol or plant stanol consumption on postprandial lipid and glucose and metabolism (**chapter 6**). For this, 42 healthy subjects underwent a meal test at the end of each intervention period, in which they consumed a shake containing no, or 3.0 g of plant sterols or plant stanols. Blood sampling was performed up to 8 hours and

subjects received a second shake without plant sterols or stanols after 4 hours. Incremental areas under the curves (iAUC) were calculated to evaluate the total (0-8 hours), first meal (0-4 hours) and second meal (4-8 hours) postprandial responses. TAG and apolipoprotein (apo) B48 of the total and first meal response were comparable between the three meals, while TAG and apoB48 responses after the second meal were higher after consumption of the plant stanol enriched shake compared with the control shake and the plant sterol enriched shake. This increased TAG and apoB48 response after the second meal was more pronounced with increasing age and statistically higher compared with the sterol shake than with the control shake. We suggested that consumption of plant stanols might have affected the clearance rate of TAG-rich lipoproteins (TRLs), but to provide more insight into postprandial responses, future studies should assess lipoprotein production and clearance after plant sterol and stanol consumption. As mentioned, we were not able to demonstrate a change in fasting oxyphytosterol concentrations in healthy subjects after consumption of a plant sterol or a plant stanol enriched margarine. In **chapter 7**, we describe the results of a human intervention study in subjects with impaired glucose tolerance (IGT) or type II diabetes, in which the effects of vitamin E or lipoic acid supplementation on fasting plasma oxyphytosterol concentrations are studied. Twenty subjects with IGT or type II diabetes consumed for 4 weeks placebo capsules, vitamin E capsules (804 mg/d) or lipoic acid capsules (600 mg/d) and oxyphytosterol concentrations were higher in subjects with IGT or type II diabetes as compared to concentrations in healthy subjects. However, fasting plasma oxyphytosterol were not reduced after consumption of vitamin E or lipoic acid for 4 weeks. In addition, other markers reflecting oxidative stress (oxysterol, MDA concentrations and oxidized LDL levels) and antioxidant capacity (TEAC assay, uric acid concentrations and GSH/GSSG) were also not changed after 4 weeks high-dose antioxidant supplementation.

In conclusion, fasting oxyphytosterol concentrations differ between healthy subjects and subjects with impaired glucose tolerance or type II diabetes. In healthy subjects, fasting oxyphytosterol concentrations are not increased after consumption of a plant sterol enriched margarine for 4 weeks, while a plant stanol enriched reduced 7 β -OH-campesterol concentrations. In addition, vitamin E or lipoic acid consumption did not change fasting oxyphytosterol concentrations in subjects with IGT or type II diabetes. Furthermore, postprandial plant sterol and oxyphytosterol concentrations increased after consumption of a mixed meal enriched with plant sterols compared to a mixed meal alone, or enriched with plant stanols. This increase was particularly evident after the second meal, which is indicative for a second meal response, which has also been described for cholesterol. Finally, postprandial TAG and apoB48 concentrations increased age-dependently after plant stanol consumption as part of a mixed meal, compared to a control meal and a meal rich in plant sterols.

Samenvatting

Samenvatting

Hart- en vaatziekten (HVZ) zijn wereldwijd de belangrijkste oorzaak van ziekte en sterfte. Risicofactoren om HVZ te ontwikkelen zijn roken, een hoge bloeddruk, suikerziekte, overgewicht en een ongunstig serum lipidenprofiel, zoals een verhoogd cholesterolgehalte in de laag-dichtheid lipoproteïnen (LDL-C). Het risico om HVZ te ontwikkelen kan verlaagd worden door het voedingspatroon te veranderen en functionele voedingsmiddelen, die het LDL-C gehalte verlagen, spelen hierbij een belangrijke rol. Plantensterolen en plantenstanolen zijn vetachtige stoffen, die lijken op cholesterol. Zowel plantensterolen als plantenstanolen worden verwerkt in functionele voedingsmiddelen en het LDL-C gehalte kan tot 12% worden verlaagd als er dagelijks 2.5-3.0 g/d plantensterolen of plantenstanolen worden gegeten. Daarnaast stijgt het gehalte plantensterolen in het bloed na het eten van plantensterolen, terwijl het gehalte plantensterolen in het bloed daalt na het eten van plantenstanolen. Omdat plantenstanolen een iets andere structuur hebben dan plantensterolen, kunnen de plantenstanolen niet oxideren.

Het doel van de studies die beschreven staan in dit proefschrift, was om het effect te onderzoeken van de consumptie van plantensterolen en plantenstanolen op de stofwisseling van lipoproteïnen en oxyphytosterolconcentraties in de gevaste staat en na de maaltijd. Daarnaast zijn de effecten van antioxidant suppletie op plasma oxyphytosterolconcentraties onderzocht.

In **hoofdstuk 2** wordt een overzicht weergegeven van de beschikbare literatuur over de verschillende aspecten van het gebruik van plantensterolen en plantenstanolen bij de behandeling van een ongunstig lipidenprofiel. Naast het bekende LDL-C- verlagend effect van plantensterolen en plantenstanolen, worden in dit hoofdstuk ook de gunstige effecten op het triacylglycerol (TAG)-gehalte beschreven en er wordt een overzicht gegeven van de argumenten die gebruikt worden in de discussie rondom verhoogde plantensterolconcentraties en een verhoogd risico op HVZ. In **hoofdstuk 3** onderzochten we de effecten van de consumptie van plantensterolen en plantenstanolen op de gevaste oxyphytosterolconcentraties in gezonde proefpersonen. Aan deze studie hebben 43 gezonde personen (18-70 jaar) deelgenomen en zij hebben gedurende 4 weken een plantensterol-verrijkte (3.0 g/d), een plantenstanol-verrijkte (3.0 g/d) en een controle margarine gegeten, gescheiden door een rustperiode van 4 weken. Ten opzichte van de controle margarine, was het LDL-C gehalte verlaagd na het eten van de plantensterol-verrijkte margarine (-8.1%) en na het eten van de plantenstanol-verrijkte margarine (-7.8%), maar de oxyphytosterolconcentraties waren niet veranderd na het eten van de plantensterol-verrijkte margarine. Daarentegen waren de 7 β -hydroxy(OH)-campesterolconcentraties verlaagd na het eten van de plantenstanol-verrijkte margarine; er was namelijk een verlaging van 0.07 ng/mL (~14%) vergeleken met de controle margarine en een verlaging van 0.07 ng/mL (15%) vergeleken met de plantensterol-verrijkte margarine. Uit deze studie konden we concluderen dat na een dagelijkse consumptie van plantensterol-

verrijkte margarine gedurende 4 weken, gevaste oxyphytosterolconcentraties niet toenemen, maar dat plasma oxyphytosterolconcentraties enigszins dalen na het eten van plantenstanol-verrijkte margarine. Deze studie toonde aan dat er grote, maar consistente verschillen bestaan in oxyphytosterolconcentraties tussen verschillende mensen. In **hoofdstuk 4** hebben we geprobeerd om factoren te vinden die deze verschillen tussen mensen in plasma oxyphytosterolconcentraties zouden kunnen verklaren. Deze studie toonde aan dat plasma oxyphytosterolconcentraties niet correleren met niet-geoxideerde plantensterolconcentraties en dat plasma oxyphytosterolconcentraties relatief stabiel zijn gedurende een langere periode. We identificeerden zes personen met consistente lage of hoge plasma oxyphytosterolconcentraties gedurende alle drie voedingsinterventies. Deze lage of hoge oxyphytosterolconcentraties waren ook terug te zien in de geoxideerde LDL (oxLDL) concentraties. Oxidatieve en antioxidatieve capaciteit markers, zoals ijzer/koperstatus, vitamine E-concentraties en TEAC waardes werden gemeten maar deze konden de verschillen tussen de hoge en lage plasma oxyphytosterolconcentraties niet verklaren. Naast het effect van de consumptie van plantensterolen en plantenstanolen op gevaste oxyphytosterolconcentraties, hebben wij ook de plasma oxyphytosterolconcentraties na de maaltijd gemeten na de consumptie van een gemengde maaltijd rijk aan plantensterolen of plantenstanolen (**hoofdstuk 5**). Tijdens het grootste gedeelte van de dag zijn mensen in een niet-gevaste staat en om het effect op de oxyphytosterolconcentraties na de maaltijd te onderzoeken, hebben 10 gezonden proefpersonen een shake gegeten (50 g vet, 12 g eiwit, 67 g koolhydraten), met of zonder 3.0 g plantensterolen of plantenstanolen. Gedurende deze 8-uur durende testdag werden bloedmonsters afgenomen en na 4 uur werd weer een shake gegeten, maar nu zonder toegevoegde plantensterolen of plantenstanolen. Deze testdag werd uitgevoerd aan het einde van elk van de drie interventieperioden waarbij proefpersonen dagelijks 20 gram margarine hebben gegeten (met of zonder 3.0 g plantensterolen of plantenstanolen). 7β -OH-campesterol- en 7β -OH-sitosterolconcentraties waren verhoogd na het eten van de plant sterol-verrijkte shake vergeleken met de plant stanol-verrijkte shake en de controle shake. Oxyphytosterolconcentraties waren vooral hoger na het eten van de tweede shake. De niet-geoxideerde campesterol- en sitosterolconcentraties vertoonden een vergelijkbaar patroon na de maaltijd, aangezien deze ook toenamen na het eten van de plant sterol-verrijkte shake vergeleken met de plant stanol-verrijkte shake en de controle shake. De vraag of plasma oxyphytosterolconcentraties afkomstig zijn uit de voeding of dat deze gevormd worden in het lichaam, kan alleen worden beantwoord door gelabelde niet-geoxideerde plantensterolen te eten en vervolgens een verrijking in gelabelde plasma oxyphytosterolen te meten in het bloed. Recente studies hebben gesuggereerd dat de het eten van producten verrijkt met plantensterolen en plantenstanolen niet alleen het LDL-C gehalte verlagen maar ook het TAG gehalte in het bloed kunnen verlagen, vooral bij personen met verhoogde TAG-concentraties. Aangezien mensen het grootste gedeelte van de dag in een niet-gevaste staat zijn en niet-gevaste TAG-concentraties een belangrijke risicofactor zijn om HVZ te ontwikkelen, onderzochten wij het

effect van het eten van plantensterolen en plantenstanolen op de vet- en glucosetofwisseling na de maaltijd (**hoofdstuk 6**). Om dit te onderzoeken, hebben 42 gezonden proefpersonen aan het einde van elk van de drie interventieperioden een shake gegeten (met of zonder 3.0 g plantensterolen of plantenstanolen). Bloedafnames werden afgenomen tot 8 uur na het eten van de shake en na 4 uur werd weer een shake gegeten, maar nu zonder toegevoegde plantensterolen of plantenstanolen. De oppervlakte onder de curves (iAUC) werden berekend om de responsen na de maaltijd te vergelijken tussen de interventies en dit werd gedaan voor de totale periode (0-8 uur), de periode na de eerste maaltijd (0-4 uur) en de periode na de tweede maaltijd (4-8 uur). De TAG en apolipoproteïne (apo)B48-concentraties na de totale en de eerste maaltijd responsen waren vergelijkbaar tussen de drie interventies, waarbij TAG en apoB48 responsen na de tweede maaltijd hoger waren na het eten van een plant stanol-verrijkte shake vergeleken met de plant sterol-verrijkte shake en de controle shake. Deze verhoogde TAG en apoB48 responsen na de tweede maaltijd waren meer uitgesproken met toenemende leeftijd en hoger in vergelijking met de plant sterol verrijkte-shake dan met de controle shake. We suggereerden dat de consumptie van plantenstanolen een effect zou kunnen gehad op de klaring van TAG-rijke lipoproteïnen (TRL), maar om meer inzicht te krijgen in de responsen na het eten van producten rijk aan plantensterolen en plantenstanolen, zal de productie en de klaring van lipoproteïnen verder moeten worden bestudeerd in toekomstige studies. Zoals al eerder hier beschreven, hebben wij geen verandering gevonden in gevaste oxyphytosterolconcentraties na het eten van een margarine rijk aan plantensterolen of plantenstanolen. In **hoofdstuk 7** beschrijven we de resultaten van een studie in personen met een verminderde glucosetolerantie (IGT) of met type II diabetes, waarbij de effecten van vitamine E of liponzuur suppletie op gevaste oxyphytosterolconcentraties worden bestudeerd. Twintig proefpersonen met IGT of diabetes type II hebben gedurende 4 weken elke dag placebo capsules, vitamine E capsules (804 mg/d) of liponzuur capsules (600 mg/d) ingenomen en oxyphytosterolconcentraties waren hoger bij proefpersonen met IGT of diabetes type II in vergelijking met gezonde proefpersonen. Echter, na de inname van vitamine E of liponzuur gedurende 4 weken was er geen verlaging in de gevaste oxyphytosterolconcentraties. Daarnaast waren andere markers die oxidatieve stress weergeven (oxycholesterol, MDA-concentraties en oxLDL) en antioxidant weergeven in het lichaam (TEAC assay , urinezuur en GSH/GSSG) ook niet veranderd na een hoge dosis antioxidant inname gedurende 4 weken.

In conclusie, gevaste oxyphytosterolconcentraties verschillen tussen gezonde proefpersonen en proefpersonen met een verminderde glucosetolerantie of diabetes type II. Het eten van een plant sterol-verrijkte margarine gedurende 4 weken zorgt niet voor een toename in gevaste oxyphytosterolconcentraties in gezonde proefpersonen, terwijl 7 β -OH-campesterolconcentraties verlaagd waren na het eten van een plant stanol-verrijkte margarine. Bovendien heeft de inname van vitamine E of liponzuur geen effect op gevaste oxyphytosterolconcentraties bij personen met IGT of diabetes type II. Daarnaast waren

plantensterol en oxyphytosterolconcentraties na de maaltijd verhoogd na het eten van een gemengde maaltijd verrijkt met plantensterolen in vergelijking met een gemengde maaltijd alleen, of een maaltijd verrijkt met plantenstanolen. Deze toename was vooral duidelijk te zien na het eten van een tweede maaltijd, wat duidt op een tweede maaltijd response zoals al eerder werd beschreven voor cholesterol. Tenslotte stijgen postprandiale TAG- en apoB48-concentraties na het eten van een plant stanol-verrijkte shake vergeleken met een plant sterol-verrijkte shake en een controle shake, een effect wat meer uitgesproken is met toenemende leeftijd.

Dankwoord

Dankwoord

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Curriculum Vitae

Curriculum Vitae

Sabine Baumgartner was born on December 25th 1985 in Heerlen, the Netherlands. She completed secondary school at the Sintermeerten College in Heerlen in 2004 and she started her study Health Sciences at Maastricht University in the same year. She performed internships at the department of Human Biology with an additional internship of 6 months at the Universidad de Navarra in Pamplona, Spain and she obtained her Master's degree for the European master of Metabolism and Nutrition in January 2009. After graduation, she worked as a research assistant at the department of Human Biology of Maastricht University under the supervision of prof. dr. ir. R.P. Mensink, where she conducted a human intervention trial investigating the effects of interesterified dietary fats in collaboration with King's College London. In September 2009, she was appointed as a PhD-student under supervision of prof. dr. ir. R.P. Mensink and prof. dr. J. Plat at the department of Human Biology of Maastricht University. She conducted several nutritional human intervention trials to investigate the effects of plant sterol and plant stanol consumption on fasting and postprandial oxyphytosterol concentrations and their effects on postprandial glucose and lipoprotein concentrations. In 2011, she was awarded with a best poster award at the European Atherosclerosis Congress (EAS) in Göteborg, Sweden and in 2014 she was awarded with the Foppe ten Hoor Young Investigator Award at the Nutritional Science Days (NSD) in Deurne, the Netherlands.

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Submitted manuscripts

Baumgartner S, Mensink RP, Plat J. Effects of a plant sterol or stanol enriched mixed meal on postprandial lipid and glucose metabolism in healthy subjects. Submitted

Baumgartner S, Mensink RP, Haenen G, Bast A, Binder CJ, Bekers O, Husche C, Lütjohann D, Plat J. Effects of vitamin E or lipoic acid supplementation on fasting oxyphytosterol concentrations in subjects with impaired glucose tolerance or type II diabetes. Submitted

Presentations

Baumgartner S, Mensink RP, Plat J. Effect of plant sterol and plant stanols on CVD risk: do oxyphytosterols play a role? Presented at The Dutch Nutritional Science Days (NSD) 2014 in Deurne, The Netherlands. Oral presentation

Awarded with the Foppe ten Hoor young investigator award

Baumgartner S, Mensink RP, Konings M, Schött H-F, Friedrichs S, Husche C, Lütjohann D, Plat J. Fasting vs. postprandial plasma oxyphytosterol concentrations: endogenous formation or uptake? Presented at the 4th ENOR symposium in Coimbra, Portugal, 2014. Oral presentation

Baumgartner S, Mensink RP, Plat J. Effects of a plant sterol- or stanol-enriched high-fat meal on postprandial lipid and glucose metabolism in healthy subjects. Presented at the European Atherosclerosis Society (EAS) Congress 2014 in Madrid, Spain. Poster presentation

Baumgartner S, Mensink RP, Plat J. Effects of a plant sterol- or stanol-enriched high-fat meal on postprandial lipid and glucose metabolism in healthy subjects. Presented at 43. Jahrestagung des Arbeitskreises "Klinischer Lipidstoffwechsel", Maikammer, Germany, 2013. Oral presentation

Baumgartner S, Mensink RP, Plat J. Effects of a plant sterol- or stanol-enriched high-fat meal on postprandial lipid and glucose metabolism in healthy subjects. Presented at The Dutch Nutritional Science Days (NSD) 2013 in Deurne, The Netherlands. Oral presentation

Baumgartner S, Mensink RP, den Hartog G, Lütjohann D, Plat J. Oxyphytosterol formation in humans; how to identify high vs. low oxidizers. Presented at the European Atherosclerosis Society (EAS) Congress 2013 in Lyon, France. Poster presentation

Baumgartner S, Mensink RP, den Hartog G, Lütjohann D, Plat J. Oxyphytosterol formation in humans; how to identify high vs. low oxidizers. Presented at the 2nd ENOR symposium in Dijon, France, 2012. Oral presentation

Baumgartner S, Mensink RP, Husche C, Lütjohann D, Plat J. Effects of plant sterol- or stanol-enriched margarine on serum oxyphytosterol concentrations in healthy subjects. The Dutch Nutritional Science Days (NSD) 2011 in Deurne, The Netherlands. Oral presentation

Baumgartner S, Mensink RP, Husche C, Lütjohann D, Plat J. Effects of plant sterol- or stanol-enriched margarine on serum oxyphytosterol concentrations in healthy subjects. Presented at the European Atherosclerosis Society (EAS) Congress 2011 in Gothenburg, Sweden. Poster presentation

This poster presentation was awarded with a best poster award.

Baumgartner S and Mensink RP. The acute effects of triacylglycerol structure of palmitic acid-rich fats on postprandial metabolism. Presented at The Dutch Nutritional Science Days (NSD) 2010 in Deurne, The Netherlands. Oral presentation

Baumgartner S, Mensink RP, Plat J. The influence of daily egg consumption on serum lipid concentrations and cardiovascular risk markers in humans. Presented at The Dutch Nutritional Science Days (NSD) 2009 in Deurne, The Netherlands. Oral presentation