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Dietary plant sterols and stanols from enrichment

Effects in an experimental model of colon cancer and
intake in the Finnish population

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ACADEMIC DISSERTATION

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To my family

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ORIGINAL PUBLICATIONS

Marttinen, Maija. Dietary plant sterols and stanols from enrichment: Effects in an experimental model of colon cancer and intake in the Finnish population [dissertation]. Helsinki, University of Helsinki. 2014.

ABSTRACT

Plant sterols and stanols (collectively named as phytosterols) are plant-derived dietary compounds. The intake of natural phytosterols from a habitual Western diet varies between 150 to 400 mg/d. Phytosterols are added to functional foods for their serum cholesterol-lowering effect and their intake increases greatly when phytosterol enriched functional foods are consumed. Phytosterols reduce the absorption of dietary and biliary cholesterol from the intestine, leading to increased concentrations of cholesterol excreted in the feces. Increased fecal cholesterol may act as a carcinogen in the intestinal lumen.

The focus of this thesis was to study the effect of plant sterols and stanols on the tumor formation in the *ApcMin* mouse, an experimental model of colon cancer. The *ApcMin* carries an inherited mutation in the *Apc* tumor-suppressor gene, which eventually leads to the development of adenomas in the intestine. Mice were fed a control diet or a 0.8% (w/w) plant sterol/ stanol diet. Commercial foods enriched with plant sterols or stanols were used to compose the experimental diets. The calculated daily intake of phytosterols was equivalent to 5 g/d for a man when adjusted for energy consumption. The impact of phytosterol feeding on cell signaling pathways involved in intestinal tumorigenesis and the changes in sterol metabolism were studied in the intestinal mucosa of *ApcMin* mouse. The final part of this work assesses the intake of phytosterols from enrichment among Finnish men and women from the FINDIET 2007 Survey.

Both plant sterols and plant stanols increased the number of adenomas in the small intestine of *ApcMin* mice. Plant stanol feeding increased the number of intestinal tumors in both genders, whereas plant sterol feeding increased the number of tumors more pronouncedly in female mice. Wnt- β -catenin and Egfr signaling were up-regulated in the intestinal mucosa of plant stanol fed *ApcMin* mice when compared with control mice. Plant sterol and stanol feeding increased fecal cholesterol

concentration that positively associated with the number of intestinal adenomas. Significant changes in the composition of intestinal sterols were observed after plant sterol and stanol feeding. It appears that plant sterol feeding affects genders differently, and estrogen signaling may play a role in intestinal tumorigenesis in female *ApcMin* mice after plant sterol feeding. Overall, the mice responded in a different manner to plant sterol and plant stanol feeding in intestinal sterol handling, cell signaling, and tumor development.

Finally, this thesis shows that according to the FINDIET 2007 Survey the intake of phytosterols from enrichment can go beyond the advised intake; for 20% of those using phytosterol enriched products the intake of phytosterols was more than 3 g/d. The consistency or duration of the consumption of phytosterol enriched products was not determined in this work. The results show that phytosterols at high intakes are harmful in the intestine of tumor-prone mice. Whether phytosterols from enrichment affect human intestinal health warrants for further research.

PREFACE

This study was carried out at the Department of Food and Environmental Sciences, Division of Nutrition, University of Helsinki. The work was financially supported by the Finnish Graduate School on Applied Bioscience (ABS), the Yrjö Jahnesson Foundation, the Jenny and Antti Wihuri Foundation and the Finnish Food Research Foundation. This work would not have been possible without these contributors and their support is sincerely acknowledged.

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Boston, April 2014

Maija Marttinen

LIST OF ORIGINAL PUBLICATIONS

- I Marttinen M, Päivärinta E, Storvik M, Huikko L, Luoma-Halkola H, Piironen V, Pajari AM, Mutanen M. Plant stanols induce intestinal tumor formation by up-regulating Wnt and EGFR signaling in *ApcMin* mice. *J Nutr Biochem*. 2013;24(1):343-52.
- II Marttinen M, Pajari A-M, Päivärinta E, Storvik M, Marttinen P, Nurmi T, Niku M, Piironen V, Mutanen M, Plant sterol feeding induces tumor formation and alters sterol metabolism in the intestine of *ApcMin* mice. *Nutr Cancer*. 2014;66:259-69.
- III Marttinen M, Kosola M, Ovaskainen M-L, Mutanen M, Männistö S. Plant sterol and stanol intake in Finland: a comparison between users and non-users of plant sterol and plant stanol enriched foods. *Eur J Clin Nutr*. 2014, in press. doi: 10.1038/ejcn.2014.3.

Contribution of the author to Studies I-III

- I-II Maija Marttinen planned the study together with the other authors. She designed the experimental diets and carried out the animal and laboratory experiments. She had the main responsibility for the analyses and interpreting the results. She was the main author of the papers.
- III Maija Marttinen planned the study together with the other authors. She had the main responsibility for the analyses and interpreting the results. She was the main author of the paper.

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ABBREVIATIONS

ABCG5/8	ATP-binding cassette (ABC) transporters G5/8
ABCA1	ATP-binding cassette (ABC) transporter A1
ACAT	acetyl-CoA:cholesterol acyltransferase
AKT	protein kinase B
<i>APC</i>	human adenomatous polyposis coli gene
<i>Apc</i>	murine adenomatous polyposis coli gene
APC	adenomatous polyposis coli protein
CHD	coronary heart disease
CRC	colorectal cancer
CYP27	sterol 27-hydroxylase
DMH	1,2-dimethylhydrazine
EGFR	epidermal growth factor receptor
ER	estrogen receptor
ERK	extracellular signaling regulated kinase
FAP	familial polyposis coli
GSK3 β	glycogen synthase kinase 3 β
HDL	high-density lipoprotein
HMGCR	3-hydroxy-3-methylglutaryl-CoA-reductase
LDL	low-density lipoprotein
LDL-C	LDL-cholesterol
LXR	liver X receptor
MAPK	mitogen-activated protein kinase
Min	multiple intestinal neoplasia
MNU	methylnitrosourea
NPC1L1	Niemann-Pick C1 like 1
SREBP-2	sterol regulatory-element binding protein 2
TNF	tumour necrosis factor

1 INTRODUCTION

It has been well-established that elevated serum cholesterol level is associated with an increased risk for coronary heart disease (CHD). A 1-mmol/l reduction in serum total cholesterol is associated with a 24.5% reduction in CHD mortality and 17.5% in all-cause mortality (Gould et al. 2007). Several studies have shown that plant sterols and stanols (collectively referred to as phytosterols in this thesis) can efficiently lower serum low-density lipoprotein (LDL) level by reducing the absorption of cholesterol from the intestine. This characteristic of these plant derived compounds has tempted the food industry to enrich food products with plant sterols and plant stanols. These enriched food products are known to customers also as functional foods. The first commercial phytosterol enriched food was the plant stanol enriched Benecol® margarine (Raisio Group, Raisio, Finland) that was launched onto the market in 1995 in Finland. It is estimated that a customary intake of 2.5 g/d of phytosterols lowers serum LDL-cholesterol (LDL-C) concentration up to 10%, and only marginal additional effect is achieved with greater doses (Katan et al. 2003). The European Food Safety Authority (EFSA) has approved health claims for plant sterol and plant stanol enriched products to lower serum LDL-C (European Commission Regulation 384/2010).

Whereas phytosterol could lower CHD risk by reducing serum LDL-C concentration, the effect of phytosterols on colon cancer is not well understood. Although plant sterols have been shown consistently to have positive effects in colon cancer cells *in vitro* (Awad et al. 1996, Awad et al. 1998, Choi et al. 2003, Baskar et al. 2010), some controversy exists in the evidence provided by animal studies (Raicht et al. 1980, Quilliot et al. 2001, Jia et al. 2006). A Dutch cohort study on cancer (Normén et al. 2001) showed that a high intake of plant sterols did not reduce the risk of colon or rectal cancers but actually found a positive association between high intake of sitostanol and risk of rectal cancer in men. Although plant sterols and stanols have been widely studied and they are generally considered safe, studies on the long-term consumption of plant sterols and stanols in humans are lacking. The safety evaluation studies of plant sterols are described in more detail in the literature review of the thesis.

The risk of developing colorectal cancer increases with age. Mutation in the adenomatous polyposis coli (*APC*) tumor suppressor gene is required in developing hereditary colon cancer (familial adenomatous polyposis, FAP), and is also found in the majority of sporadic colorectal tumors (Powell et al. 1992). The incidence of *APC* mutations in the colon increases at middle-age (Luebeck and Moolgavkar 2002) and often at the same time consumption of phytosterol enriched products begins. Therefore the tumor prone *ApcMin* mouse (*Adenomatous polyposis coli*, Multiple intestinal neoplasia) serves a reasonable approach to study the effect of plant sterols and stanols from enrichment on intestinal tumor formation. No previous studies have reported the effect of plant sterols or stanols in the tumor prone *ApcMin* mouse, which is a widely used mouse model to study the impact of diet on colon carcinogenesis.

To study the effect of phytosterol from enrichment in *ApcMin* mice, commercial food products enriched with plant sterols or plant stanols were added to the experimental diets. This work elucidates the effects of phytosterol feeding on intestinal tumor formation and cellular mechanisms related to intestinal tumorigenesis in the *ApcMin* mouse. The effects of plant sterol and plant stanol feeding on sterol metabolism in the mouse small intestine were also examined. Finally, the intake of phytosterols from natural and enriched food sources was evaluated among the Finnish men and women from the national FINDIET 2007 Survey. This was done to assess if Finnish consumers using phytosterol enriched foods followed the label information on recommended phytosterol intake set by authorities.

2 REVIEW OF LITERATURE

2.1 Plant sterols and stanols

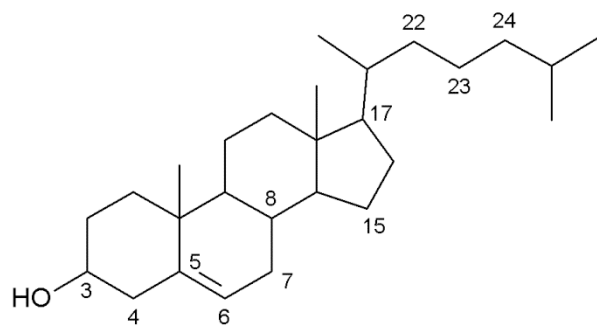
2.1.1 Structure

Sterols are essential molecules in cellular structures in animals and plants. Plant sterols are structurally similar to cholesterol found in animals. The term “plant sterols” is commonly used to indicate a group of plant derived sterols that are composed of a tetracyclic steroid ring with a side chain attached to C-17 (Figure 1). Plant stanols are saturated forms of plant sterols with no double bonds in the ring structure. Over 40 plant sterols have been identified in the nature, the major plant sterol being β -sitosterol followed by campesterol and stigmasterol (Law 2000). Saturated plant sterols such as sitostanol and campestanol are less abundant. In the present thesis, the term phytosterols is used to refer to both plant sterols and stanols, and specific compound names are applied when necessary.

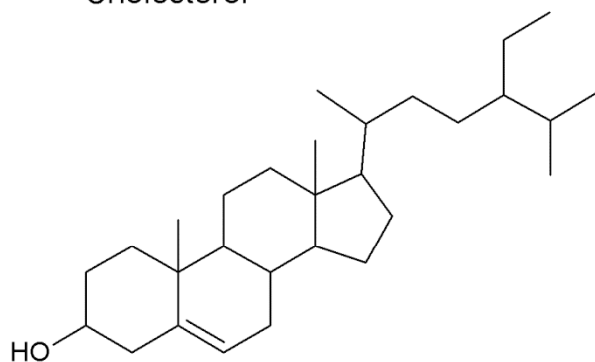
2.1.2 Food sources and dietary intake

Good natural sources of phytosterols are whole grains, vegetable, vegetable oils, nuts, and fruit, and the intake varies from 150 to 400 mg/ day in a typical Western diet (Normén et al. 2001, Katan et al. 2003, Escurriol et al. 2009). According to the FINDIET 1997 Survey the mean intake of phytosterols from natural sources was 305 mg/d for men and 237 mg/d for women in Finland (Valsta et al. 2004).

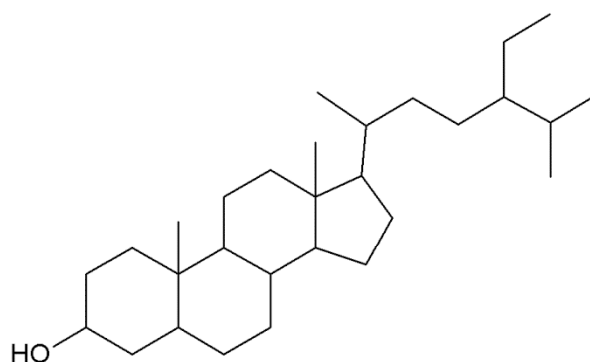
For two decades, functional foods enriched with plant sterol and stanol esters have been marketed for their beneficial effect on lowering plasma low-density lipoprotein cholesterol (LDL-C). Phytosterol intake may notably increase when plant sterol and stanol enriched functional foods are consumed. Simulation studies have suggested that the potential daily intake of phytosterols could exceed 8 grams when phytosterol enriched products were virtually replaced with conventional food products in the diet (Raulio et al. 2001, De Jong et al. 2004, Kuhlmann et al. 2005).



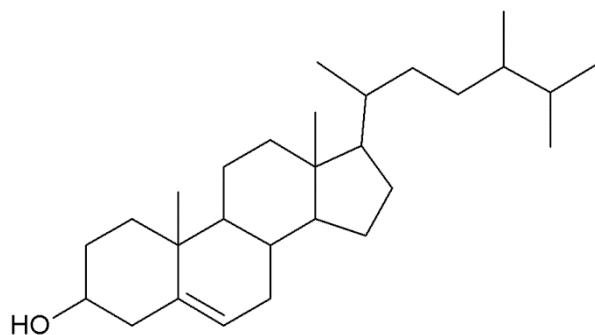
Cholesterol



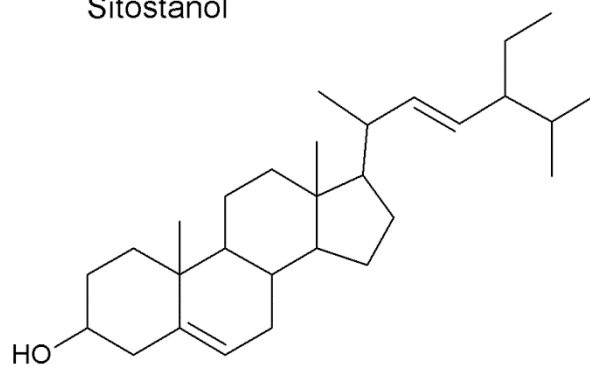
Sitosterol



Sitostanol



Campesterol



Stigmasterol

Figure 1. Chemical structures of cholesterol and some phytosterols.

The European Food Safety Authority (EFSA) has approved a health claim stating that *"Plant sterols and stanols lower blood cholesterol. High cholesterol is a risk factor in the development of coronary heart disease."* (European Commission Regulation No 384/2010). The approved food matrices regulated by EFSA (Regulation (EC) No 376/2010) include yellow fat spreads, dairy products, mayonnaise, and salad dressings. Moreover the phytosterol enriched product should clearly state in the label that the products are intended exclusively for people who want to lower blood LDL-C levels, and they are not appropriate for pregnant and breastfeeding women and children under the age of five (Regulation (EC) No 608/2004). Intakes above 3g/d should not be recommended on the basis of the current scientific evidence on plant sterols and stanols and their lowering effect on blood carotenoid levels (EFSA 2008). Phytosterol enriched products should be advised to be consumed as a part of a balanced diet that includes fruit and vegetables to provide adequate carotene intake (Regulation (EC) No 608/2004). Recently EFSA concluded that plant sterol and stanol esters at an intake of 3.0 g/d (range 2.6-3.4 g/d) lower blood LDL-cholesterol by 11.3% at similar efficacy when used for at least for two to three weeks (EFSA 2012).

In the USA, Food and Drug Administration (FDA) authorized a health claim that plant sterol and stanol esters reduce the risk of CHD, and the US National Cholesterol Education Program recommend a daily intake of 2 g of plant sterols and stanols to treat high cholesterol levels (NCEP 2002). The advised daily dose of phytosterols according to manufacturers is around 2 g and often a daily dose of 2 g can be achieved using a portion of youghurt drink.

2.1.3 Effects of phytosterols on serum cholesterol and phytosterols

Already in the 1950's phytosterols were observed to lower blood cholesterol level and reduce cholesterol absorption from the intestine (Peterson 1958). Since then the effect of plant sterols and stanols on blood LDL levels has been verified in many randomized control trials which have been summarized in many systematic meta-analyses. The effect of phytosterols on serum LDL-C is achieved within few weeks, and the effect has been shown to remain stable (Miettinen et al. 1995). Katan and

coworkers (Katan et al. 2003) concluded in their meta-analysis of 41 randomized trials that a daily intake of 2.5 g of plant sterols or stanols lowers serum LDL-C level up to 10%, and intakes above this have only little additional effect. In the meta-analysis conducted few years later by Abumweis and coworkers (Abumweis et al. 2008), 59 randomized placebo controlled clinical trials were included. A greater decrease in LDL-C levels was observed in subjects with higher baseline levels compared with those with lower baseline LDL-C levels. Furthermore, a dose-response effect was found and the maximum effect on LDL-C was found with daily doses greater than 2.5 g of phytosterols. The dose-response effect of phytosterol intake was confirmed in a meta-analysis by Demonty et al. (Demonty et al. 2009). Overall, in 84 randomized trials that were included in their analysis, the mean daily dose of 2.15 g of phytosterols resulted in 8.8% pooled LDL-C reduction.

Results from meta-analysis comparing the effect of plant sterols and plant stanols separately indicate that they do not differ in the LDL-C lowering effect within the intake range of 0.6-2.5 g/d (Talati et al. 2010). The efficacy of plant sterols and plant stanols may differ at high doses (Musa-Veloso et al. 2011). Recently, two studies on the effect of high plant stanol intake have been conducted. Mensink and co-workers (Mensink et al. 2010) found a dose-response relationship between plant stanol intake and LDL-C reduction, and an intake of 9 g/d lowered LDL-C by 17% compared to control. Similarly, Gylling and her co-workers (Gylling et al. 2010a) showed a 17.4% reduction in LDL-C at an intake of 8.8 g/d of plant stanols compared to control. In contrast, a linear dose-response was not observed with plant sterols by Davidson et al. (Davidson et al. 2001). In the meta-analysis performed by Musa-Veloso et al., intakes above 2 g/d of plant stanol, but not plant sterol, were associated with additional and dose-dependent reductions in LDL-C (Musa-Veloso et al. 2011). The number of high-dose studies in this meta-analysis was, however, limited.

Dietary plant sterols are poorly absorbed from the intestine. In comparison to dietary cholesterol of which 35-70% is absorbed (De Jong et al. 2003), 5% of β -sitosterol, 15% of campesterol and less than 1% of plant stanols is absorbed (Law 2000). The serum phytosterol concentration is partly regulated by intestinal absorption and partly by excretion in bile back to the intestine (Miettinen et al. 2000). In the normal population, serum plant sterol concentrations vary between 3

and 21 μM and plant stanol concentrations between 0.05 and 0.3 μM when phytosterol enriched foods are not consumed (Gylling and Miettinen 2010). Customary use of phytosterol enriched products increases serum plant sterol concentrations but plant stanol concentrations are increased only slightly (Fransen et al. 2007, Kratz et al. 2007, Gylling et al. 2010a). Furthermore, plant stanol supplementation decreases serum plant sterol concentrations (Kratz et al. 2007, Gylling et al. 2010a).

2.1.4 Phytosterols and CHD

In 2010, EFSA approved a health claim for plant sterol and stanol ester enriched products to lower serum LDL-C as part of a healthy diet (European Commission Regulation 2010/384). Data from drug studies suggest that a 10% reduction in LDL-C levels could reduce the incidence of ischaemic heart disease by 12% to 20% over 5 years; however no trials have tested the effects of phytosterols on coronary heart disease incidence. Dietary phytosterols has been found to reduce atherosclerosis plaque formation for instance in apo E -deficient mice when consumed along with high cholesterol diet (Xu et al. 2008).

Elevated concentrations of serum plant sterols are found in subjects with sitosterolemia (phytosterolemia), a rare genetic disorder where the sterol transporters ABCG5/8 are affected (Lee et al. 2001). In these patients, serum and tissue plant sterol concentrations are 10-25 higher than in unaffected individuals, whereas serum cholesterol concentrations are normal or moderately increased (Lee et al. 2001). Since sitosterolemic patients suffer from premature atherosclerosis and coronary artery disease, high serum phytosterol concentration has been suggested as a risk factor of atherosclerosis. Even in normal subjects, elevated concentrations of plasma plant sterols have been associated with increased risk for coronary events (Assmann et al. 2006). The variation in serum phytosterol levels in general population is caused by genetic variants in ABCG transporters (Teupser et al. 2010). The polymorphisms of ABCG8 that are associated with elevated serum phytosterol concentrations have been found to be associated with an increased risk of coronary artery disease (Teupser et al. 2010). A systematic literature review and meta-analysis

based on 17 studies found, however, no association between serum phytosterol levels and risk for cardiovascular diseases (CVD) (Genser et al. 2012). Studies included in the meta-analysis reported inconsistent findings on moderately elevated levels of serum sitosterol and campesterol and CVD risk, but the effects of long-term consumption of plant sterol enriched foods have not been assessed.

2.1.5 Effects of phytosterols on cholesterol metabolism in the intestine

The intracellular free cholesterol pool is tightly regulated by a network of proteins and transcription factors that respond to cellular free cholesterol content. When free cholesterol concentration drops below a threshold level, the sterol regulatory-element binding protein 2 (SREBP-2) transcription factor is activated and translocated from the endoplasmic reticulum via Golgi to nucleus where its target genes are regulated (Brown and Goldstein 1997). The targets of SREBP-2 transcribe proteins that induce cholesterol synthesis (e.g. 3-hydroxy-3-methylglutaryl-CoA-reductase, HMGCR; farnesyl diphosphate synthase, and squalene synthase), and increase transport of cholesterol into the cell via LDL-receptor (Horton et al. 2002). In contrast, when cellular free cholesterol concentration is increased, the expression of proteins that regulate cholesterol efflux is activated through an orphan nuclear receptor LXR mediated transcription of target genes such as ATP-binding cassette (ABC) transporters ABCG5, ABCG8 and ABCA1 (Ory 2004).

The balance in whole-body cholesterol pool is regulated by absorption of dietary and biliary cholesterol, excretion of cholesterol into the bile and *de novo* synthesis of cholesterol. Disruption of processes regulating whole-body cholesterol homeostasis influences circulating cholesterol levels, and therefore treatment that targets LDL-C levels have been developed. Whereas cholesterol-lowering statins inhibit the synthesis of cholesterol, phytosterols primarily lower serum LDL-cholesterol level by reducing the absorption of dietary and biliary cholesterol in the small intestine. Several mechanisms have been suggested how plant sterols and stanols reduce cholesterol absorption (Figure 2). In the intestinal lumen, phytosterols compete with cholesterol for incorporation into mixed micelles (Ostlund et al. 1999, Nissinen et al. 2002, Mel'nikov et al. 2004). Since phytosterols are more hydrophobic than

cholesterol they displace cholesterol from the micelles reducing the absorption of cholesterol.

In addition to replacing cholesterol from micelles, phytosterols may reduce cholesterol absorption within the enterocyte. Plat et al. (Plat et al. 2000) demonstrated that reduction in absorbed cholesterol did not require simultaneous consumption of dietary cholesterol and plant stanols since plant stanols in one daily dose had a similar effect on serum LDL-C level as several doses of plant stanols during the day. This finding suggested that phytosterols could regulate cholesterol absorption in long-term. To date, several studies have shown that this long-term effect may be related to the regulation of cholesterol metabolism in the enterocyte. These mechanisms include the transport of cholesterol in and out of the enterocyte. The intestinal absorption of free cholesterol and phytosterols is mediated by the Niemann-Pick C1 like 1 (NPC1L1) transporter protein found on the brush border of enterocytes (Davis and Altmann 2009, Jia et al. 2011). NPC1L1 may contribute to the selective transport of cholesterol and phytosterols into the enterocyte (Jia et al. 2011) and certain plant sterols have been found to down-regulate NPC1L1 expression in cultured intestinal epithelial cells (Jesch et al. 2009).

Phytosterols may also regulate the efflux of cholesterol from enterocytes. In 2002, Plat and co-workers demonstrated that plant stanol supplementation increased the expression of ABCA1 transporter in CaCo-2 cells, a model for intestinal epithelial cells (Plat and Mensink 2002). At that time, the authors suggested that ABCA1 mediated the efflux of cholesterol into the intestinal lumen; however, ABCA1 transporter is currently supposed to localize on the basolateral membrane of enterocytes (Ohama et al. 2002) and transport sterols to HDL (Murthy et al. 2002).

The export of sterols back into the intestinal lumen is mediated by heterodimeric transporters ABCG5 and ABCG8 localized on the apical membrane of enterocytes (Tachibana et al. 2007). These transporters are also expressed on the canalicular membrane of hepatocytes, regulating the removal of cholesterol and phytosterols into the bile (Graf et al. 2003). Mutations that lead to functional defects in ABCG5/8 transporters are found in individuals with sitosterolemia, where the absorption of phytosterols is increased (Lee et al. 2001). Findings on whether phytosterols reduce

cholesterol absorption by regulating ABCG5/8 transporters are conflicting. Although phytosterols have been demonstrated to act as potent activators of LXR in CaCo2 human intestinal cell line (Plat et al. 2005), plant sterol or stanol feeding did not activate the known LXR target genes, such as ABCG5/8 in the mouse intestine (Field et al. 2004, Calpe-Berdiel et al. 2005, Plösch et al. 2006). Reduction in the expression levels of intestinal ABCG5/8 after plant sterol feeding to mice has also been reported (Brufau et al. 2011). Recently, plant sterols were found to reduce the activity of CYP27 enzyme that converts sterols into 27OH metabolites that are potent activators of LXR (Brauner et al. 2012). Consequently, plant sterols reduced LXR activation and ABCA1 expression with no change in ABCG8 expression in CaCo-2 cells. The authors concluded that in enterocytes plant sterols reduce cholesterol absorption by upregulating ABCA1 pathway.

Furthermore, plant sterols have been shown to inhibit the activity of acetyl-CoA:cholesterol acyltransferase-2 (ACAT-2) that esterifies free cholesterol to fatty acids in the enterocyte (Igel et al. 2003). As only esterified sterols are incorporated into chylomicrons, the absorption of cholesterol is thereby reduced (Fig. 2). Plant sterols and stanols themselves are poorly esterified by ACAT (de Jong et al. 2003), which in part also explains the poor absorption of these compounds.

In addition, β -sitosterol supplementation to CaCo-2 cells has been reported to down-regulate the activity of HMGCR, the key enzyme in cholesterol synthesis (Field et al. 1997). By contrast, sitosterol feeding to rats up-regulated HMGCR activity and receptor-mediated LDL binding with no change in cholesterol concentration in the intestinal mucosa (Nguyen et al. 2001). Similarly, plant sterol feeding to mice increased mRNA levels of Hmgcr in enterocytes (Brufau et al. 2011). It seems that compensatory mechanisms are activated to produce endogenous cholesterol when cholesterol absorption is reduced. Individual plant sterols may, however, have specific effects on cholesterol metabolism: whereas β -sitosterol did not displace cholesterol from the plasma membrane and activate ACAT, campesterol induced influx of membrane cholesterol and ACAT activity (Field et al. 1997).

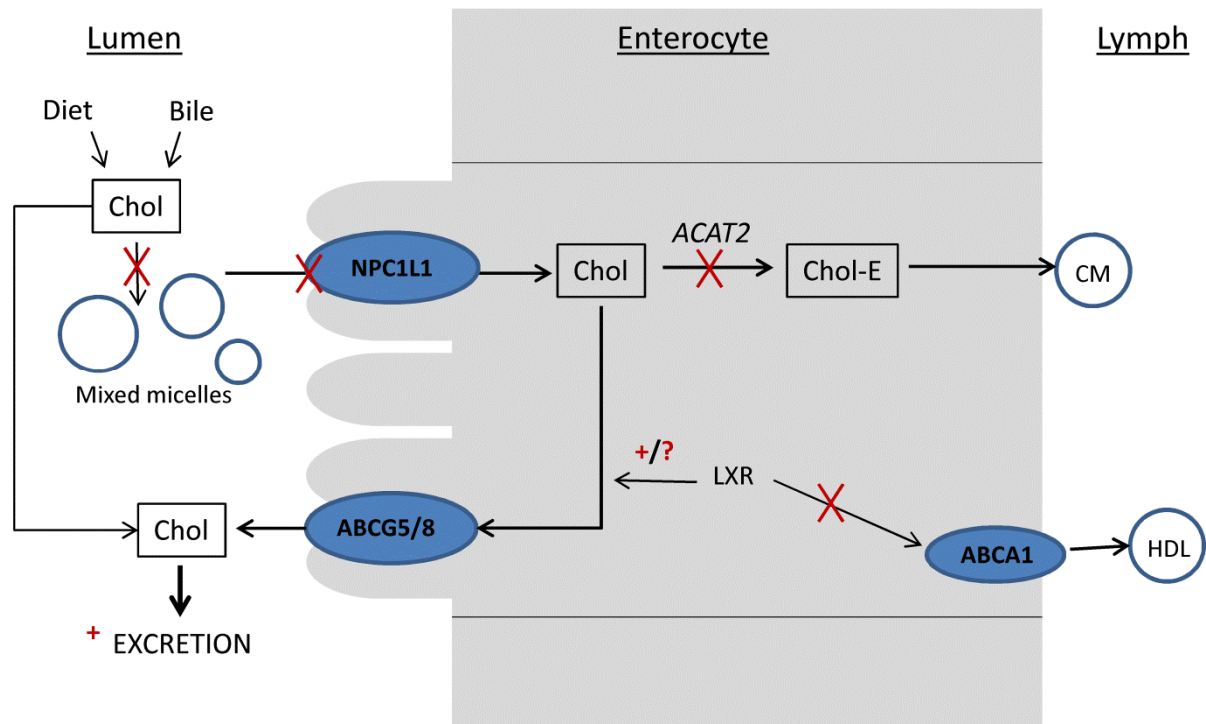


Figure 2. Mechanisms by which phytosterols possibly reduce cholesterol absorption.

Taken together, it is clear that plant sterols and plant stanols affect cellular cholesterol metabolism, but the exact mechanism how cholesterol absorption is reduced at the cellular level needs still to be discovered. Findings from *in vitro* and *in vivo* experiments are inconsistent when the effect of plant sterols/ stanols on cellular cholesterol homeostasis is studied. First, absorption of cholesterol by enterocytes *in vivo* and uptake of sterols by cultured cells are not comparable. Second, the effect of phytosterols on intestinal cholesterol metabolism in animals can be both direct and indirect. Direct actions of phytosterols include functioning as a signaling molecule as such, (Park and Carr 2013), acting as a ligand for transcription factors (Plat et al. 2005), or replacing cholesterol at the plasma membrane (Awad et al. 1996). Indirect mechanisms involve activated cellular mechanisms to compensate for reduced cholesterol absorption (Nguyen et al. 2001). When phytosterols are supplemented to cultured cells, phytosterols affect cellular metabolism more or less directly. In addition, a mixture of phytosterols may affect cholesterol metabolism differentially when compared with individual phytosterols.

2.1.6 Effects of phytosterols on cholesterol metabolism in the liver

The liver is a central player in the whole-body cholesterol homeostasis regulating the cholesterol levels in the circulation. In order to compensate for the reduction of absorbed cholesterol in circulating chylomicrons after phytosterol ingestion, hepatocytes upregulate the LDL receptor mediated uptake of cholesterol. As a consequence of reduced cholesterol absorption, blood LDL-C levels are reduced. However, decreased circulating cholesterol levels have also been observed after injection of phytosterols (Vanstone et al. 2001), and therefore phytosterols may affect LDL-C levels via mechanisms that do not involve intestinal cholesterol absorption.

In general, endogenous cholesterol synthesis is increased after phytosterol consumption, which seems to be a compensatory mechanism to regulate whole-body homeostasis when cholesterol absorption is reduced. This has been observed both in humans and animals after phytosterol consumption (Vanhanen et al. 1993) (Moghadasian et al. 2001, Mensink et al. 2002, Batta et al. 2005, Harding et al. 2010). In addition, phytosterol feeding increases plant sterol and stanol concentrations in the liver (Awad et al. 1997a, Chen et al. 2009, Harding et al. 2010, Rideout et al. 2010, Weingärtner et al. 2011).

The changes in sterol metabolism may be influenced by phytosterol per se or their hypocholesterolemic effect depending on the model where phytosterols are studied. In wild-type mice, circulating cholesterol is mainly transported in HDL unlike in humans (Jawień et al. 2004), and although phytosterol feeding reduces cholesterol absorption, it does not affect serum non-HDL cholesterol levels in mice (Calpe-Berdiel et al. 2005). In C57BL/6J mice without genetic defects in sterol metabolism, phytosterol feeding has been demonstrated to upregulate levels of hepatic Hmgcr (Harding et al. 2010), *Abcg5* (Plösch et al. 2006, Harding et al. 2010), *Abca1*, and *Cyp27a1* (Harding et al. 2010) without affecting blood cholesterol levels.

Phytosterol feeding lowers plasma cholesterol levels in hamsters (Ntanios and Jones 1999), *apoE*^{-/-} mice (Calpe-Berdiel et al. 2005, Weingärtner et al. 2011), and *LDLR*^{-/-} mice (Calpe-Berdiel et al. 2005). Lowered levels of serum LDL-C are often

accompanied with reduced cholesterol concentration in the liver (Trautwein et al. 2002, Calpe-Berdiel et al. 2005, Weingärtner et al. 2011), and increased hepatic Abcg5, Abcg8, and Npc1l1 expression (Calpe-Berdiel et al. 2005, Harding et al. 2010). Increase in the activity or protein levels of the hepatic Hmgcr after phytosterol consumption have been widely reported in these animal models (Moghadasian et al. 2001, Batta et al. 2005, Xu et al. 2008, Harding et al. 2010).

In summary, phytosterols affect hepatic expression of genes related to cholesterol metabolism. The impact of phytosterol feeding on hepatic genes may be associated with changes in serum LDL-C. However, for instance, increased expression of Hmgcr after phytosterol ingestion has been observed with (apoE^{-/-} mice) or without (wild-type mice) changes in serum LDL-C.

2.1.7 Safety of phytosterols

Plant sterols have toxic effects in mice with genetic defects in Abcg5/8. Recently it was shown that a 0.2% plant sterol enriched diet increased liver mass and induced liver damage caused by accumulation of hepatic plant sterols (McDaniel et al. 2013). In addition, these mice developed severe myocardial lesions after plant sterol feeding. The loss of normal function in the ABCG5/8 sterol transporters leads to high serum plant sterol concentrations in sitosterolemia patients, and premature atherosclerosis is common (Lee et al. 2001). To avoid the consequences of enhanced sterol absorption, sitosterolemia is treated by dietary restrictions (a sterol poor diet) and by medication that inhibits sterol absorption (e.g. ezetimibe) or synthesis (statins).

Series of studies evaluating the safety of phytosterols have been conducted in animals and humans without mutations in the ABCG5 or ABCG8 transporter. The toxicity of a mixture of phytosterol esters was studied in doses up to 8.1% of diet for 90 days in male and female rats (Hepburn et al. 1999). Plant sterols had no effect on weight development, food or water consumption, or organ weights. Some minor changes were observed in haematological and clinical parameters, but these changes were not considered of toxicological importance. Later, the relative absorption and tissue

distribution of β -sitosterol, β -sitostanol, campesterol, campestanol, and stigmasterol were studied in the rat (Sanders et al. 2000). Although the absorption of phytosterols was low, some tissues showed increased accumulation of phytosterols. Increased accumulation of phytosterols was observed in adrenal glands, ovaries, lungs, liver and intestinal tissue. Most of the administered phytosterols were excreted in the feces (Sanders et al. 2000).

No adverse effects of phytosterols were seen on the reproduction physiology and sexual maturation in male and female rats in studies carried with two generations of rats. The dose of plant sterol esters varied up to 8.1% (Waalkens-Berendsen et al. 1999) and plant stanol esters up to 8.8% (Whittaker et al. 1999). Some adverse effects were reported, not conclusively related to the treatment: some females in plant sterol and stanol groups delivered only dead pups (Waalkens-Berendsen et al. 1999) (Whittaker et al. 1999), and two females fed plant stanol at low- or mid-dose exhibited histological changes in the uterus, including metaplasia and early carcinoma (Whittaker et al. 1999). Furthermore, the body weight was significantly decreased in pups in the high-dose group which was attributed to a reduced caloric intake (Whittaker et al. 1999). Plant sterols or plant stanols have not been proven estrogenic *in vitro* or *in vivo* (Baker et al. 1999, Turnbull et al. 1999). More on the estrogenic effect of plant sterols are described in the section "Phytosterols as phytoestrogens".

The effect of dietary phytosterols on fecal concentrations of bile acids and sterols was studied in men and women (Weststrate et al. 1999). Intake of 8.6 g of plant sterols from a test margarine significantly increased concentrations of neutral sterols and neutral sterol metabolites in the feces, whereas faecal secondary and total bile acid concentration was reduced. Dietary plant sterols increased the faecal concentration of 4-cholesten-3-one in both men and women, but the effect was significant only in women. 4-Cholesten-3-one is a breakdown metabolite produced from cholesterol by intestinal microflora, and it has been reported mutagenic (Suzuki et al. 1986, Kaul et al. 1987). The mutagenic potential of plant sterols and 4-cholesten-3-one was studied by Wolfreys and Hepburn as a part of the safety evaluation program of phytosterols (Wolfreys and Hepburn 2002). The treatment with plant sterols, plant sterol esters, or 4-cholesten-3-one did not increase chromosome aberrations in human peripheral

blood lymphocytes, nor did plant sterols induce cytotoxicity in mouse lymphoma cells or in the rat bone marrow. In addition, plant stanol esters were tested for genotoxicity in bacterial and mammalian cells and were not found genotoxic (Turnbull et al. 1999).

Oxidation products of cholesterol and phytosterols have been documented to be cytotoxic *in vitro* (Adcox et al. 2001, Roussi et al. 2005, Ryan et al. 2005). The reported cytotoxic effects included increased cell death by apoptosis or necrosis (Ryan et al. 2005), decreased cell viability, and loss of membrane integrity (Adcox et al. 2001). Lea et al. reported no genotoxic effects of a mixture of oxidized plant sterols *in vitro* (Lea et al. 2004). When oxidized plant sterols were administered in the diet in concentrations up to 1.6% for 90 days, no adverse effects were detected in haematological and clinical parameters, or in weights of selected organs in male and female rats. Only liver weights were significantly increased in females that were fed oxidized plant sterols at 1.6% of the diet when compared with control and plant sterol diet (Lea et al. 2004).

The reduction in serum β -carotene level is a widely reported side-effect of phytosterol consumption (Mensink et al. 2002, Gylling et al. 2010b, Heggen et al. 2010, Hernández-Mijares et al. 2011). Serum levels of vitamin A, the end of product β -carotene, have been reported to remain unaffected (Gylling et al. 2010b). This reduction in circulating β -carotene may result from decreased absorption of carotenoids from the intestine or lower level of LDL particles in the circulation after phytosterol consumption.

2.1.8 Phytosterols as phytoestrogens

The estrogenic potential of phytosterols has been studied *in vitro* and *in vivo*. The results are inconsistent and so far phytosterols are not regarded as strong phytoestrogens as e.g. isoflavones and lignans. Some studies suggest that phytosterols alter sex steroid hormone levels (Nieminen et al. 2003, Ju et al. 2004), but also no effects on hormone levels have been reported (Ayesh et al. 1999). Phytosterols have been shown to bind estrogen receptors (ER) (Gutendorf and

Westendorf 2001, Newill et al. 2007) with low affinity with better binding affinity to ER β than to ER α (Gutendorf and Westendorf 2001). Not all studies confirm binding of phytosterols to ER (Baker et al. 1999). Baker et al. used a mixture of plant sterols (47.9% β -sitosterol, 28.8% campesterol, 23.3% stigmasterol) and found no binding of plant sterols to estrogen receptors nor did plant sterols compete with estradiol for binding to the ER (Baker et al. 1999). In addition, plant sterols did not produce transcriptional activity of the human ER in yeast, or affected the growth of estrogen-responsive tissue *in vivo* (Baker et al. 1999). Similarly, high plant stanol intake (mixture of plant stanols) has not proven to be estrogenic in rats (Turnbull et al. 1999). Findings by Ju and coworkers (Ju et al. 2004) indicate that β -sitosterol may, however, exert estrogenic activity, and stimulate the growth of MCF-7 human estrogen-responsive breast cancer cells. Studies on stigmasterol and its oxidation products suggest that oxidized stigmasterols bind to ER with weak affinity, and by displacing estradiol from ER these compounds interfere with hormone signaling (Newill et al. 2007). So far, phytosterols per se have not been shown to activate the transcriptional targets of estrogens. Overall, the estrogenic effects appear to be different between individual phytosterols and the effects seem to be model-dependent.

2.2 Colon carcinogenesis

2.2.1 Colorectal cancer

Colorectal cancer is the third most common cancer in men (10.0% of the total cancer incidence) and the second in women (9.4% of the total cancer incidence) in the world (IARC 2008). In 2008, over 1.2 million people worldwide were given the diagnosis of colorectal cancer. About 608 000 deaths from colorectal cancer are estimated worldwide, accounting for 8% of all cancer deaths, making it the fourth most common cause of death from cancer. Almost 60% of the cases occur in developed regions. Ethnic, migrant and twin studies suggest that environmental and lifestyle factors, including diet, play a pivotal role in the etiology of CRC. It is estimated that dietary factors could contribute to CRC incidence by 30%-50% (Vargas and Thompson 2012). The evidence on lifestyle factors and CRC risk is covered thoroughly in the report of the World Cancer Research Fund (WCRF/IARC 2011).

Majority (75%) of colon cancers develops sporadically and the remaining colon cancer cases are caused by an inherited predisposition (Boyle and Levin 2008). The most common inherited colon cancer syndromes are familial adenomatous polyposis (FAP), hereditary non-polyposis colorectal cancer (HNPCC), also known as the Lynch syndrome, and MUTYH-associated polyposis (MAP). Patients with FAP carry an inherited germline mutation in the *APC* tumor-suppressor gene that causes development of hundreds to thousands of adenomas in the colon and rectum in early age (Kinzler and Vogelstein 1998). Lynch syndrome is caused by mutations in mismatch repair genes (Peltomäki 2001). In addition to colorectal cancer people with Lynch syndrome develop cancer in other organs.

Mutations in the *APC* gene are common in non-inherited colon cancers, too, and the incidence of *APC* mutations in the colon increases at middle-age (Luebeck and Moolgavkar 2002). Approximately, 70-80% of sporadic colorectal adenomas and carcinomas have somatic *APC* mutations (Fearon 2011). Adenomas, or adenomatous polyps, are tumors that develop from glandular epithelium, and they seem to be an important precursor of colorectal cancer (Fearon 2011); however, small amount of adenomas develop into malignant carcinomas. The process of a dysplastic cell to turn

into a carcinoma is a multistep sequence that involves mutations in other tumor-suppressor genes and proto-oncogenes, as well as alterations in the genomic stability. These transformations in the genome lead to disturbed regulation of cell growth and differentiation, and eventually to the development of an early adenoma to a metastatic tumor (Figure 3.).

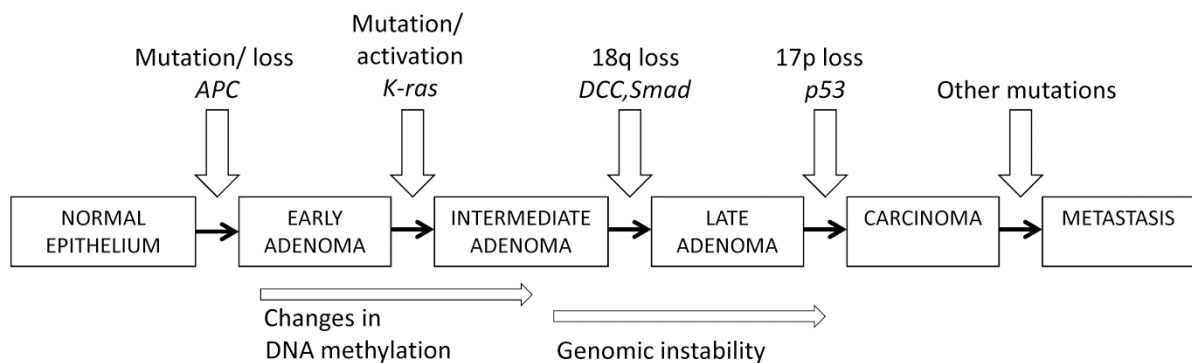


Figure 3. The multistep development of colon cancer. Mutations in tumor-suppressors and proto-oncogenes are involved in the adenoma-carcinoma sequence. Changes in DNA methylation and genomic instability contribute to the malignant transformation. (Adapted from Fearon and Vogelstein 1990, and Fearon 2011).

2.2.2 Construction and maintenance of intestinal epithelium

The epithelial cells in the intestine are under a rapid but steady process of renewal. While new cells are generated in the intestinal crypts, old cells are removed by shedding from the tips of villi. The intestinal epithelium is constructed of invaginations called crypts, and intestinal stem cells responsible for tissue regeneration are located in the lower part of the crypts (Clevers 2013). Epithelial cells produced by the stem cell daughters migrate toward the upper part of crypt and villus where they lose their capacity to divide and start to differentiate. The differentiated cells found in the colon and in the small intestine are the predominant enterocytes, mucus-producing Goblet cells, and the peptide hormone secreting enteroendocrine cells. In the small intestine, cells migrating down the crypt differentiate into Paneth cells that modulate innate immune system (Santaolalla and Abreu 2012).

The homeostasis in the intestinal epithelium is tightly regulated by maintaining a balance between proliferative and anti-proliferative signals (reviewed by Crosnier et al. 2006 and Clevers 2013). The Wnt and Notch signaling play a pivotal role in the proliferation and maintenance of intestinal stem cells (Clevers 2013). On the other hand, anti-proliferative signals such as the Hedgehog and bone morphogenetic proteins (BMP) repress Wnt signaling and stem cell proliferation (Crosnier et al. 2006). Cell proliferation is regulated by signals received from the cell microenvironment. Neighboring cells may activate Notch signaling in intestinal stem cells and through Wnt signaling maintain the proliferative state of stem cells (Medema and Vermeulen 2011). The BMP's are secreted by the mesenchymal microenvironment and their signaling is active in differentiated cells along the epithelial lining (Medema and Vermeulen 2011).

The lifespan of an intestinal cell is only few days long (Näthke 2004), and the active clearance of cells protects the intestinal epithelium from oncogenic mutations. However, the rapidly renewing tissue appears to be a potential target for mutational changes. Several theories on the primary cells for oncogenic transformation in the intestinal epithelium have been proposed. Whether oncogenic mutations accumulate in the founder stem cells or in the stem cell daughters in intestinal crypts have been suggested (the bottom-up model), but initiating transformation of migrated and differentiated cells have been proposed (the top-bottom model), too (reviewed by Medema and Vermeulen 2011). Despite the controversies on the site of the transformation, growing evidence supports the model that human cancers arise from cancer stem cells that are capable of initiating and sustaining tumor growth (Clevers 2011, Verga Falzacappa et al. 2012). Like stem cells, cancer stem cells have the potential of self-renewal, as well as the ability to expand and differentiate (Clevers 2011). The generation of colon cancer stem cells is dependent on genetic factors as well as micro-environmental signaling (Medema and Vermeulen 2011, Verga Falzacappa et al. 2012). Cancer stem cells produce a progeny of cells that forms the bulk of the tumor, and a single tumor may have subclones of multiple cancer stem cells (Clevers 2011).

2.2.3 Apoptosis

Programmed cell death by apoptosis is important in maintaining homeostasis in tissues with rapid cell turnover such as the intestinal epithelia. Apoptosis regulates the removal of normal and transformed cells and acts as a natural barrier for cancer development. Resistance of cell death is a trait that cells have to acquire during the process of cancer, and this is established by mutations in the regulatory machinery (Hanahan and Weinberg 2011).

The apoptosis pathway involves the up-stream regulators, which can be extracellular (extrinsic pathway) or intracellular (intrinsic pathway), and the down-stream effectors. The extrinsic pathway is activated by death receptor ligation, such as binding of Fas or TNF ligand to its receptor that subsequently activates caspase-8 (Wen et al. 2012). The intrinsic pathway, also named as the mitochondrial pathway, is initiated by intracellular stress that activates caspase-9 (Wen et al. 2012). Both the extrinsic and intrinsic pathways activate the down-stream effector caspase-3 that is responsible for the final execution of apoptosis. The signaling between the regulators and effectors is mediated by the Bcl-2 subfamily of pro-apoptotic (Bax, Bak, Bad, Bcl-X, Bid, Bik) and anti-apoptotic (Bcl-2, Bcl-X_L, and Mcl-1) proteins. The pro-apoptotic proteins induce apoptosis by facilitating the release of cytochrome *c* from mitochondria which activates caspase-9, whereas anti-apoptotic proteins inhibit apoptosis by binding of pro-apoptotic proteins (Adams and Cory 2007). A key sensor that responds to cellular abnormalities, such as DNA damage, hypoxia, and reduced nutrient supply, is the p53 tumor suppressor (Fearon 2011) (Sperka et al. 2012). Mutations in the *p53* gene are common in human CRC tumors and are associated with increased invasiveness (Fearon 2011).

2.2.4 Cell cycle regulation

In order to divide cells have to increase their mass and replicate their DNA. The consecutive processes and phases that lead to cell division are called the cell cycle, which is regulated by a machinery of proteins. The entry from one phase to another is controlled by several check-points along the cell cycle. The check-points respond to

mitogenic signals such as growth factors that promote cell division. In addition, check-points sense genetic errors that can be then repaired or alternatively the damaged cell is eliminated by apoptosis. Proteins that regulate the progression of cell cycle at the checkpoints are the cyclins, the cyclin dependent kinases, the cyclin dependent kinase inhibitors, the Retinoblastoma (Rb) complex, and the E2F family of transcription factors (reviewed by Satyanarayana and Kaldis 2009). Deregulation of the cell cycle leads to uncontrolled cell growth (increase in cell mass) and cell proliferation (increase in cell number).

Rapidly dividing cells in organs with high cell turnover, such as the intestine, are more susceptible to DNA damages than cells in quiescent, non-dividing state. Mechanisms that eliminate DNA damages (i.e. gene mutations) have evolved to maintain cell homeostasis. As a consequence of DNA damage signaling pathways that regulate the activation of checkpoints, cell cycle progression, DNA repair and apoptosis are activated (Patil et al. 2013). The DNA damage kinases ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3 related) activate DNA damage checkpoints and phosphorylate their downstream targets such as checkpoint kinase 1 and 2 (Chk1, Chk2). Activation of Chk1 leads to cell cycle arrest by inhibiting PLK1 (polokinese 1), DNA repair, and cell death by apoptosis independent of p53 (Patil et al. 2013). Chk2 phosphorylates p53 leading to cell cycle arrest, DNA repair or elimination of the damaged cell by apoptosis (as described in the previous section "*Apoptosis*"). p53-mediated cell cycle arrest is primarily elicited by p21 that inhibits the G1/S transition and therefore prevents replication of damaged DNA (Helton and Chen 2007). Furthermore, p53 regulates the transcription of genes involved in DNA repair such as the mismatch repair genes (Helton and Chen 2007). As already mentioned, mutations in the *p53* are common in human CRC (Fearon 2011).

2.2.5 Colon cancer and the APC protein

The *APC* gene is a tumor-suppressor essential to normal cell growth. The gene encodes APC protein that acts as a "gate-keeper" of the genome. In sporadic colon cancer, *APC* mutations are associated with the early stages of tumorigenesis (tumor development) (Fearon 2011), but in order to promote tumor development, both *APC*

alleles have to be inactivated according to the Knudsen two-hit-model (Kinzler and Vogelstein 1996). Once both alleles have been inactivated by somatic mutations or epigenetic alterations, the tumor-suppressive function of normal APC is lost. Disturbed function of APC protein results in the activation of the Wnt pathway that mediates proliferative signaling. On the other hand, Wnt signaling plays a crucial role in the regulation of cell proliferation as a morphogen during embryogenesis and organ development (Clevers and Nusse 2012). Balanced Wnt signaling is also a key regulator in the maintenance of intestinal stem cells in the intestinal crypts (Clevers 2013). The abnormal activation of Wnt signaling is considered an initiating step in the colon carcinogenesis (Bienz and Clevers 2000, Näthke 2004).

APC protein has multiple functions in the cell regulating cell migration, cell adhesion and mitosis (Näthke 2004). One of its tasks is to regulate the β -catenin-dependent Wnt signaling pathway which is thoroughly reviewed by Clevers and Nusse (2012). The APC protein forms a multiprotein complex with glycogen synthase kinase 3 β (GSK3 β) and axin that by binding together promote the phosphorylation and degradation of β -catenin by ubiquitin/ proteasome pathway (Fig. 4). A mutation in the *APC* gene leads to a truncated form of the APC protein that no longer can function normally. The absence of normal APC leads to activation of β -catenin-dependent Wnt signaling with concomitant down-regulation of β -catenin degradation and its accumulation in the nucleus. In the nucleus, β -catenin interacts with the Tcf/Lef transcription factor, and regulates transcription of genes e.g. *c-myc* and *cyclin D1* (He et al. 1998, Shtutman et al. 1999) that enhance cell proliferation and growth.

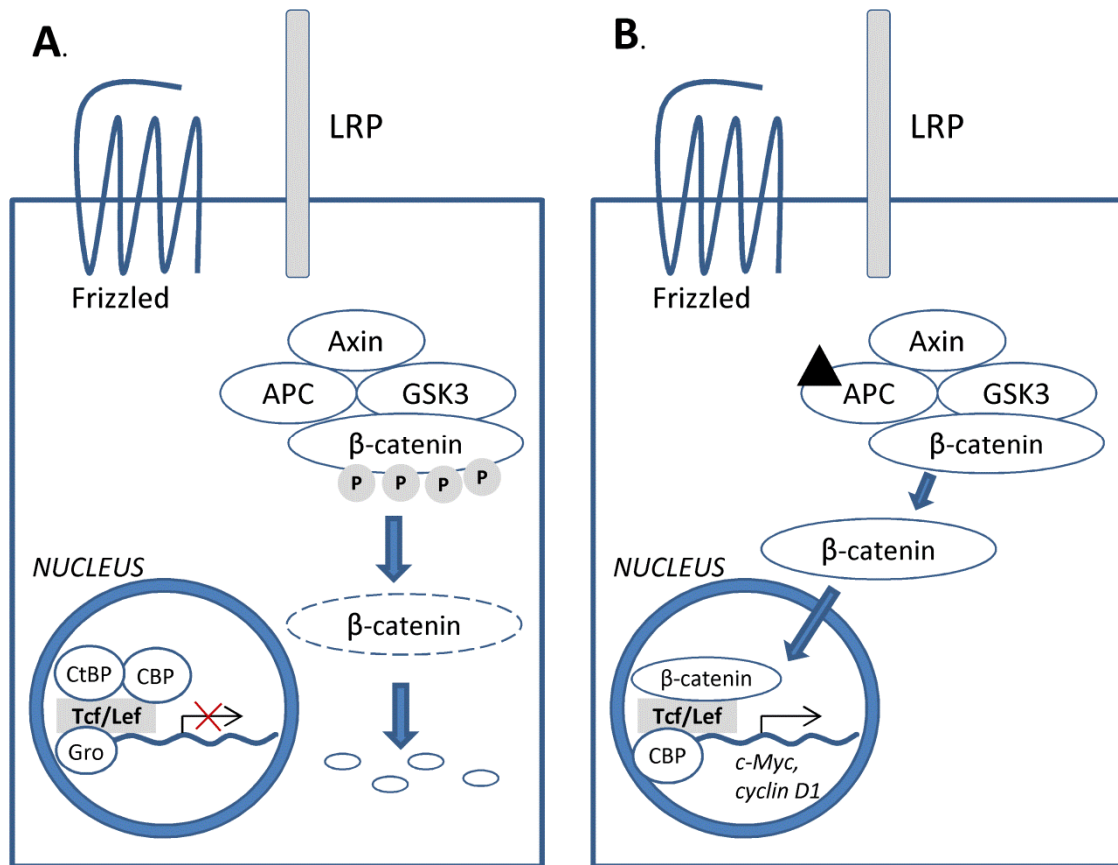


Figure 4. The Wnt – β-catenin pathway in normal colonic epithelial cells (A) and in APC mutated colon cancer cells (B). Adapted from Narayan and Roy (Narayan and Roy 2003).

2.2.6 Colon cancer and EGFR signaling

The activation of the ERK MAPK pathway plays an important role in cell proliferation in colorectal cancer. ERK is activated by growth factor signaling and proto-oncogenes contributing to increased cell proliferation (Fang and Richardson 2005). Mitogen-activated protein kinase (MAPK) signaling occurs in response to almost any change in the extracellular or intracellular milieu. MAPK regulates cell growth, differentiation, cell survival, neuronal function and the immune response by responding to growth factors, hormones, cytokines and stress (Yang et al. 2013).

One mechanism that activates ERK in colon carcinogenesis is the activation of epidermal growth factor receptor (EGFR) signaling. EGF receptors are tyrosine

kinase receptors that are located on the plasma membrane in lipid rafts rich in cholesterol and sphingolipids (Pike 2005, Patra 2008, Balbis and Posner 2010). Upon ligand binding EGFR is activated by phosphorylation of the tyrosine residues. Activated EGFR recruits several downstream targets and activates signaling through phosphorylation. These downstream signaling pathways include Ras/Raf/MEK/ERK1/2, but also the PI3K/Akt pathway. Through these two pathways EGFR regulates the homeostasis between cell proliferation and maturation in the gut (Prenzel et al. 2001, Krasinskas 2011). The Ras/Raf/MEK/ERK pathway is dysregulated in approximately 30% of all cancers (Fang and Richardson 2005).

The activation of EGFR signaling pathway results in uncontrolled proliferation of colon cancer stem cells (Feng et al. 2012). The role of EGFR signaling has been related with early stages of colon carcinogenesis such as microadenoma formation (Fichera et al. 2007), and inhibition of EGFR signaling is shown to inhibit polyp formation (Roberts et al. 2002, Buchanan et al. 2007). Previously, increased levels of both total and phosphorylated EGFR were seen in *Apc*-null tumors of *Apc*^{Min} mice as well as in the intestinal mucosa, where *Apc* function was reduced (Moran et al. 2004).

2.2.7 Colon cancer and epigenomics

Gene expression and activity can be regulated epigenetically without changing the DNA sequence of the gene. The major types of epigenetic regulation are DNA methylation, histone modification and RNA interference.

DNA methylation is a normal mechanism by which cells regulate gene activity. In DNA methylation, methyl groups are added enzymatically to the 5-position of cytosine. Cytosine-guanine dinucleotide sequences, called CpGs, are preferably methylated by DNA methyltransferase. In the mammalian genome, most of CpGs located outside of promoter regions are methylated. Unmethylated regions of CpGs are located in so called CpG islands, where CpGs exist in sequences longer than 200-500 bases. CpG islands are often located within the promoter region of genes and are normally protected from methylation. In colorectal cancer, CpG islands within the

promoter region are aberrantly hypermethylated (Goel and Boland 2012). Methylation of CpG islands suppresses gene expression by altering chromatin structure and hindering transcription factors from accessing the promoter. Hypermethylation is detected in tumor suppressor genes such as, *APC*, *CDKN2A*, *MLH1* and *CDH1* (Lao and Grady 2011, Goel and Boland 2012). Opposite to the local hypermethylation in promoter regions, global hypomethylation of DNA is an early event in the development of colorectal cancer and may contribute to genomic instability (Goel and Boland 2012).

Histone modifications and RNA interference also regulate gene expression in human cancers; however alterations in these mechanisms are less well understood than in DNA methylation. Dietary factors including folate, polyphenols and isoflavones could mediate their anti-carcinogenic effect through epigenetic modifications (Supic et al. 2013).

2.2.8 Sterol metabolism in cancer cells

Cancer cells are in high-demand of energy to support rapid cell division. Tumor cells re-programme their metabolic pathways in order to produce increasing amounts of energy-rich ATP and macromolecules (carbohydrates, proteins, lipids and nucleic acids) that are needed for cell growth and proliferation (Cairns et al. 2011). The changes in tumor cell metabolism are caused by genetic mutations or continuous exposure to growth factors (Wellen and Thompson 2010). The metabolic alteration in cancer cells, the Warburg effect, was first described by Otto Warburg in 1950's (Warburg 1956), but the role of metabolic alterations in cellular transformation has regained more attention in recent years. It now seems evident that there is cross-talk between cell cycle and metabolic regulation (Aguilar and Fajas 2010), and that altered energy metabolism should be regarded as and was recently added by Hanahan and Weinberg (Hanahan and Weinberg 2011) as one of the hallmarks of cancer.

Pathways that produce lipids are deregulated in cancer cells. These changes affect the synthesis of membrane lipids (sterols, phosphoglycerides, sphingolipids), lipids in

energy homeostasis, and lipids involved in cell signaling (Santos and Schulze 2012). Up-regulation of the mevalonate pathway, the first steps of cholesterol synthesis, has been associated with cellular transformation (Singh et al. 2003, Dimitroulakos et al. 2006). Since cholesterol is a structural component of the plasma membrane, the demand for cholesterol is increased in dividing cells. However, cholesterol biosynthesis pathway produces also mevalonate and isoprenoids that are both needed for cell growth. Isoprenoids are intermediates of the cholesterol synthesis pathway that are needed for isoprenylation of small GTPases, such as farnesylation of Ras and geranyl-geranylation of Rho, that activate their signaling inducing cell proliferation (Singh et al. 2003).

2.3 Phytosterols and cancer

2.3.1 Epidemiological studies

In addition to the serum LDL-C decreasing effect, epidemiological studies have demonstrated that increased intake of plant sterols is associated with reduced risk for several types of cancer e.g. cancer of lung (Mendilaharsu et al. 1998, Schabath et al. 2005), breast (Ronco et al. 1999), and stomach (De Stefani et al. 2000). The association between plant sterol intake and colorectal cancer is less consistent. The Netherlands Cohort Study on Diet and Cancer did not find association between high intake of dietary plant sterols and reduced risk of colorectal cancer after 6 years of monitoring. The intake of β -sitosterol was, however, positively associated with cancer of distal colon, and the intake of stigmasterol was positively associated with rectal cancer in men (Normén et al. 2001).

2.3.2 Experimental research: *in vitro* studies

The effects and mechanisms of plant sterols on cancer processes have been widely studied in several *in vitro* studies with several different cell lines. β -sitosterol, the main dietary plant sterol, is the most studied plant sterol. β -sitosterol has been reported to inhibit the growth of human colon cancer cells (Awad et al. 1996, Baskar et al. 2010), prostate cancer cells (von Holtz et al. 1998a, Awad et al. 2000, Ifere et al. 2010), breast cancer cells (Awad et al. 2003b), and leukemia cells (Moon et al. 2008). No effect on cell growth was seen with β -sitosterol or campesterol treatment in differentiated CaCo2 cells (Awad et al. 2005).

In studies where β -sitosterol was observed to reduce cell growth, β -sitosterol targets a number of cellular processes. Evidence indicates that β -sitosterol induces apoptosis in neoplastic cells (von Holtz et al. 1998, Awad et al. 2003a, Awad et al. 2007, Moon et al. 2008, Ifere et al. 2010) including human colon cancer cells (Choi et al. 2003, Baskar et al. 2010), but also in non-neoplastic cells (Rubis et al. 2008). The mechanism by which β -sitosterol drives cells into apoptosis seems to be through down-regulating the expression of anti-apoptotic Bcl-2 protein (Choi et al. 2003,

Park et al. 2007, Ifere et al. 2010) and up-regulating pro-apoptotic Bax protein (Choi et al. 2003). Furthermore, β -sitosterol supplementation has been described to increase caspase activities (Awad et al. 2003a, Park et al. 2007) and release of cytochrome c from the mitochondria (Choi et al. 2003). The activation of sphingomyelin cycle and ceramide production may mediate apoptosis after β -sitosterol treatment (von Holtz et al. 1998). Additionally, β -sitosterol has been reported to regulate cell cycle progression in cancer cells by inducing cell cycle arrest at the G2/M phase (Awad et al. 2001, Moon et al. 2008), targeting microtubule organization (Moon et al. 2008), up-regulating growth-suppressors (Ifere et al. 2010), and reducing DNA synthesis (Park et al. 2003). Studies on human breast cancer cells (MDA-MB-231) have suggested that β -sitosterol and campesterol suppress metastatic processes (Awad et al. 2001). However, β -sitosterol has been reported as pro-proliferative in MCF-7 breast cancer cells (Mellanen et al. 1996, Ju et al. 2004). At the plasma membrane, cholesterol forms lipid-rafts that are essential for cell signaling. Several studies have demonstrated that plant sterols and stanols are incorporated to cellular membranes by replacing cholesterol (Awad et al. 1996), which may result in altered membrane properties and receptor function (Mora et al. 1999, Ratnayake et al. 2000, Awad et al. 2007).

Since cancer cell lines are originally derived from cancer tissues, the cells already possess characteristics of transformed cells. As a result from genetic and metabolic defects, these cells may exhibit altered response to apoptosis, growth signaling, etc. The effect of β -sitosterol on cellular functions is usually compared with the effects of cholesterol supplementation. Whereas cholesterol treatment supports cancer cell growth, β -sitosterol has mainly shown opposite effects in these studies. The cholesterol-treated cells are also reported to grow faster than the vehicle-treated cells (Ifere et al. 2010). In summary, different cell lines have different cellular responses when treated with phytosterols. Most of the research has been conducted with β -sitosterol, therefore the effects of other phytosterols in cancer processes still remain unknown.

2.3.3 Experimental research: *in vivo* studies

The effect of plant sterols on colon carcinogenesis has been studied mainly in carcinogen-induced animal models such as the MNU (methylnitrosourea) and DMH (1,2-dimethylhydrazine) rat. Plant sterol supplementation has been shown to inhibit colon tumorigenesis in some studies with carcinogen-treated rats (Raicht et al. 1980) (Janezic and Rao 1992), but also no effects on colon cell proliferation (Jia et al. 2006) or colon tumor formation (Quilliot et al. 2001) have been reported.

Raicht and co-workers showed that dietary β -sitosterol may protect from chemically-induced colon tumors (Raicht et al. 1980). They fed MNU-treated male Fischer rats 0.2% β -sitosterol in the diet (95% β -sitosterol, 4% campesterol, 1% stigmasterol) for 28 weeks. In the control group 54% of rats developed tumors whereas 33% of rats in the plant sterol group had colon tumors, and the reduction in tumor-bearing animals was significant. Similarly, there was significantly less tumors per animal in the plant sterol group than in the control group. Furthermore, the size of the proliferative compartment in colonic crypts was reduced in MNU-rats fed 0.2% sitosterol compared with control MNU-rats (Deschner et al. 1982).

Quilliot and his group (Quilliot et al. 2001) reported that dietary plant sterols had no effect on colon tumor formation in MNU-treated female Wistar rats. They investigated the effect of plant sterols by feeding 24 mg/d of plant sterols (55% β -sitosterol, 41% campesterol, 4% stigmasterol of total plant sterols) per rat with or without saturated fat supplement for 30 weeks. There was no difference in the number of colon tumors between plant sterol supplemented rats or control rats. The cholesterol and plant sterol content in the feces was significantly higher in plant sterol supplemented rats. The authors concluded that plant sterols modified gut microflora, which was seen as increased level of fecal coprostanol, a bacterial metabolite of cholesterol associated with colon carcinogenesis (Peuchant et al. 1987, Panda et al. 1999).

More recently, Baskar et al. reported that plant sterol feeding reduced the number of aberrant crypt foci in male DMH-treated rats (Baskar et al. 2010). β -sitosterol isolated from *A. curassavica*, was given to rats at doses of 5, 10, and 20 mg/kg b.w.

for 16 weeks, and the reduction in the number of aberrant crypt foci was dose-dependent (Baskar et al. 2010).

All the studies described above have multiple distinctions in their designs which may explain the inconsistency in results. First, the amount and timing of carcinogen to rats was different. Likewise the dose and the manner that plant sterols were supplemented to rats differed. MNU-instillation and plant sterol enriched diet were introduced to rats concurrently by Raicht et al. (Raicht et al. 1980) and Baskar et al. (Baskar et al. 2010) , whereas Quilliot and co-workers (Quilliot et al. 2001) gave the plant sterol enriched diet to rats after the MNU administration. Second, the composition of diets was different in their fat and plant sterol composition. Third, gender of animals varied between studies.

Compared to chemically induced colon cancer models, the *ApcMin* mouse represents a model that resembles more human colon cancer and shares similarities in the mechanisms leading to tumor development. A study conducted by Sang et al. (Sang et al. 2006) showed that a wheat bran oil fraction containing plant sterols inhibited intestinal tumor formation in male *ApcMin* mice. The decrease in tumor number was significant only in the proximal small intestine. It must be emphasized that conclusions on the effect of plant sterols in *ApcMin* mice cannot be drawn from their study, since the investigated fraction contained other bioactive compounds, too. The effect of plant sterols or plant stanols on colon tumorigenesis has not been studied in other genetically modified animal models.

The effect of plant sterols on intestinal cell proliferation has been reported also in healthy, wild-type animals (Janezic and Rao 1992, Awad et al. 1997b, Jia et al. 2006). Janezic and Rao (Janezic and Rao 1992) fed female C57BL/6J mice a diet supplemented with either cholic acid or cholic acid and plant sterols (60% β -sitosterol, 30% campesterol, 5% stigmasterol) at 0.3%, 1.0% or 2.0% of diet for two weeks. Cell proliferation was assessed by histological staining. Whereas cholic acid increased the markers of cell proliferation in the colon epithelium, dietary plant sterols significantly decreased the level of proliferation markers to the level of the diet without cholic acid supplementation; however, there was no dose-dependent effect. Similarly, in the experiment conducted by Awad et al. (Awad et al. 1997b), a

diet containing 2% of plant sterols and cholic acid reverted the cholic acid induced cell proliferation when given to male rats for 22 days. Jia et al. (Jia et al. 2006) investigated the effect of different plant sterol and stanol analogues on the proliferation of normal colonic mucosal cells. Male Syrian hamsters were given control diet or diet supplemented with either 1% plant sterol, 1% plant stanol, 1.76% plant sterol esters of fish oil, 0.71% plant stanol esters of ascorbic acid, or 1.4% plant stanol esters of ascorbic acid for 5 weeks. The authors found a significant decrease in colon cell proliferation marker Ki-67 in animals fed the 0.7% of plant stanol ascorbate diet compared with control diet. No further decrease in cell proliferation was observed with 1.4% of plant stanol ascorbate. Other plant sterol analogues had no effect on cell proliferation. The feeding period in these three experiments ranged from 2 to 5 weeks which is seemingly short time to generate changes in normal colonic mucosa in healthy animals.

In summary, the effect of phytosterols has been studied with a wide range of animal models. Although, plant sterols reduce tumor formation in carcinogen-induced models and in cholic-acid induced models, some inconsistency exists (Quilliot et al. 2001). In wild-type animals, the results show also inconsistency, and the experiments have been rather short to detect colonic changes that usually develop over a long period of time. So far, no proper studies on the effect of phytosterols in genetically modified animals have been reported.

2.4 The *ApcMin* mouse

Colon carcinogenesis can be studied in animals by either inducing tumor development by chemical carcinogens or using animals with genetic susceptibility/predisposition to tumor formation. The Min mouse was first described by Moser et al. (Moser et al. 1990). C57BL/6J mice were exposed to ethylnitrosourea, a chemical carcinogen, and at the age of few weeks, a progeny of these mice developed multiple/dozens of adenomas in the small intestine: the mouse was therefore named the Min mouse (Min, multiple intestinal adenomas).

The Min phenotype was found to be inheritable by a dominant mutant allele. Later, the germline mutation was mapped to mouse chromosome 18. A point mutation in the *Apc* allele converts thymine to adenine resulting in a premature stop-codon (Su et al. 1992) and leading to a truncated form of the Apc protein. The Min mouse is heterozygous for the *Apc* allele meaning that they have one mutated allele and one non-mutated, a normal allele. The suppressive function of the normal allele has to be lost, too, before an adenoma starts to develop (LOH, loss of heterozygosity). Mice homozygous for the Min mutation die at gestation (Moser et al. 1995). The *ApcMin* mouse develops approximately 30-50 spontaneous adenomas in the small intestine, while adenomas in the colon are rare (Bilger et al. 1996). Most of the adenomas are benign and develop in the distal small intestine. *ApcMin* mice live to approximately 120 days, and the main cause of death is severe anaemia and/ or intestinal obstruction (Moser et al. 1990).

As already described, in humans the *APC* mutation is involved in the hereditary colon cancer syndrome, FAP, but also in non-inherited colon cancers. The murine *Apc* gene shares homology with the human *APC* gene and 90% of amino acids in the *Apc/APC* protein are identical (Su et al. 1992). In contrast to humans, *ApcMin* mice develop tumors mainly in the small intestine and tumors in the colon are rare. Despite of the differences in the phenotype, the *ApcMin* mouse has been considered a suitable model to study intestinal carcinogenesis. This animal model has been widely used to study the effect of pharmaceutical agents as well as dietary compounds on intestinal tumorigenesis. The data from dietary experiments is found in colon cancer Chemoprevention Database (<http://corpet.free.fr/>). The pre-

cancerous adenomatous polyps found in *ApcMin* mice are considered as a strong end-point marker for dietary chemoprevention in animals and humans (Rafter et al. 2004).

3 AIMS OF THE STUDY

In this thesis we used the tumor-prone *ApcMin* mouse to study the effects of plant sterols and stanols on intestinal tumor formation. The intestine of the *ApcMin* mouse is susceptible to tumor development. Furthermore, unlike in chemically induced colon cancer models (the MNU rat) where a wide range of unidentified mutations occur, tumorigenesis in *ApcMin* mice is primarily driven by the mutation in the *Apc*. Since *APC* mutations are common in human spontaneous colon cancers, the *ApcMin* mouse is a more suitable model to study human colon carcinogenesis. Previous to this work no studies on phytosterols in *ApcMin* mice have been reported. The dietary intake of plant sterols or stanols from enriched products was assessed among Finnish men and women, which has not been done earlier in Finland.

The objectives of the present work were:

- 1) To study the effect of plant sterols and stanols from enriched foods on intestinal tumor development in the *Apc* mutated intestine.
- 2) To study the effect of plant stanol and sterol feeding on the composition of sterols in the faeces and intestinal mucosa.
- 3) To identify if cell signaling pathways related to cancer were affected after plant sterol and stanol feeding.
- 4) To estimate the intake of plant sterols and stanols from enriched foods in the FINDIET 2007 Survey.

4 MATERIALS AND METHODS

This section gives a brief overview of study designs and methods that are presented in more detail in the original publications (I-III).

4.1 Studies I and II: Feeding phytosterols to *ApcMin* mice

4.1.1 Mice

The study protocol was approved by the Laboratory Animal Ethics Committee, University of Helsinki. Male and female C57BL/6J *ApcMin* (*Apc*^{Min/+}) mice were bred at the Laboratory Animal Centre in Viikki, Helsinki, from inbred mice originally obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Mouse DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) and the *Min* genotype was determined by a PCR assay described by Dietrich et al. (Dietrich et al. 1993). The 5-week-old *ApcMin* mice were assigned into the experimental groups by their weight and litter background (I, II). The mice were housed in plastic cages in humidity- and temperature-controlled facilities with 12-h light-dark cycle, and food and water was given *ad libitum*. During the experiment, the mice were weighed and monitored weekly. Mice with considerable weight-loss within one week were sacrificed before the end of experiment. These mice were excluded from the analysis. In addition, one plant sterol female was excluded from the data (II) for having over 170 intestinal adenomas. The total number of mice included in the data is 28 in Study I (8 control male and 6 control female mice; 7 plant stanol male and 7 plant stanol female mice), and 24 in Study II (6 control male and 7 control female mice; 6 plant sterol male and 5 plant sterol female mice).

4.1.2 Diets and study designs

All experimental diets were semi-synthetic AIN-93G-based (Reeves et al. 1993) high-fat diets. The fat content and fat composition of the diets were designed to mimic a typical Western-type diet. The proportion of saturated, monounsaturated and

polyunsaturated fatty acids was approximately 3:2:1. All diets were similar with respect to their energy values from carbohydrate, protein and fat. The protein, carbohydrate, fat, fatty acid, cholesterol, vitamin and mineral content of both diets were otherwise similar (Analysis conducted by Agrifood Research Finland and Division of Food Chemistry, University of Helsinki; Table 1 in Paper I). The composition of experimental diets in Studies I and II are presented in Tables 1 and 3. The sterol composition of diets (Tables 2 and 4) was analyzed by the method described in the section "Analysis of fecal and mucosal sterols".

Study I: The mice were fed either a control diet or a 0.8% (w/w) plant stanol diet. Plant stanol was added to the experimental diet in the form of freeze-dried plant stanol ester enriched food products (Benecol®, Raisio Group, Finland and Benecol®, Valio, Finland; Table 1). The food products added to the control diet did not contain plant stanol esters, but they were purchased from the same manufacturer and therefore the content of the product was more likely to be similar in other ingredients.

Study II: The mice were fed either a control diet or a 0.8% (w/w) plant sterol diet. Plant sterol was added to the experimental diet in the form of plant sterol ester enriched margarine (Becel pro.activ® 35% fat, Unilever). Similarly, margarine from the same manufacturer (Becel® 35% fat, Unilever) was added to the control diet to maintain the fat composition of diets as similar as possible. Freeze-dried food products used in the control diet in Study I were added to plant sterol diet and control diet; this was done to make these two studies as similar as possible in the diet composition.

Studies I and II: Plant stanol and plant sterol diets contained 0.8% (w/w) free phytosterols. The intake of phytosterols from the 0.8% (w/w) enriched diet was estimated to be 20 mg per day per mouse. This amount is equivalent to a calculated daily intake of 5 g in a human when the intake is adjusted to energy consumption.

Table 1. Composition of diets in Study I.

Ingredient	Control diet <i>g/kg</i>	Plant stanol diet <i>g/kg</i>
Freeze-dried products	131.5 ¹	131.5 ²
Casein	196.7	197.9
Dextrose	413.2	412.5
Butter	131.4	131.0
Sunflower oil	11.7	11.7
Rapeseed oil	54.9	54.7
Mineral mix AIN-93-MX ³	41.6	41.6
Vitamin mix AIN-93-VX ³	11.8	11.8
L-cystine	3.6	3.6
Choline chloride	3.6	3.6
BHQ	0.014	0.014

¹ Freeze-dried products were cream cheese like product (Keiju, Raisio Group), cheese-like product (Julia 17, Kyrönmaan Juustomestarit Ltd., Isökyrö, Finland), pasta (Torino, Raisio Group), and butter milk (Valio).

² Freeze-dried products were cream cheese like product (Benecol®, Raisio Group), cheese-like product (Benecol®, Valio), pasta (Benecol®, Raisio Group), and butter milk (Benecol®, Valio).

³ Composed according to Reeves et al. 1993.

Table 2. Sterol composition of diets in Study I.

Sterol	Control diet <i>mg/ 100 g</i>	Plant stanol diet <i>mg/ 100 g</i>
Cholesterol	39.2	37.4
Phytosterols ¹	56.9	832.6
Plant sterols	56.1	68.4
Brassicasterol	4.5	4.9
Campesterol	21.1	29.2
Sitosterol	30.1	33.5
Stigmasterol	0.5	0.9
Plant stanols	0.8	764.2
Campestanol	0.5	206.7
Sitostanol	0.2	557.5

¹Total of plant sterols and stanols

Table 3. Composition of diets in Study II.

Ingredient	Control diet <i>g/ kg</i>	Plant sterol diet <i>g/ kg</i>
Freeze-dried products ¹	131.5	131.5
Casein	175.8	178.6
Dextrose	376.5	372.8
Butter	64.9	65.3
Sunflower oil	4.2	4.2
Rapeseed oil	66.6	67.0
Becel® (35% fat), Unilever	120.0	-
Becel pro.activ® (35% fat), Unilever	-	120.0
Mineral mix AIN-93-MX ²	41.6	41.6
Vitamin mix AIN-93-VX ²	11.8	11.8
L-cystine	3.6	3.6
Choline chloride	3.6	3.6
BHQ	0.014	0.014

¹ Freeze-dried products were cream cheese like product (Keiju, Raisio Group), cheese-like product (Julia 17, Kyrönmaan Juustomestarit Ltd., Isökyrö, Finland), pasta (Torino, Raisio Group), and butter milk (Valio).

² Composed according to Reeves et al. 1993.

Table 4. Sterol composition of diets in Study II.

Sterol	Control diet <i>mg/ 100 g</i>	Plant sterol diet <i>mg/ 100 g</i>
Cholesterol	21.6	22.4
Phytosterols ¹	75.0	826.5
Plant sterols	73.0	718.2
Avenasterol	3.0	11.7
Brassicasterol	5.4	25.7
Campesterol	25.1	134.5
Sitosterol	38.6	538.0
Stigmasterol	1.0	8.3
Plant stanols	2.0	108.3
Campestanol	0.5	13.7
Sitostanol	1.5	94.6

¹Total of plant sterols and stanols

4.1.3 Tumor scoring and sample collection

After the 9-week feeding experiments, the mice were killed by CO₂ asphyxiation. The small intestine, caecum and colon were removed and opened along the longitudinal axis, rinsed with ice-cold saline, and put flat on an objective glass. Tumors were scored under microscope connected to a camera and a TV screen. The intestinal tumors were cut out from the tissue, and the histologically normal-appearing mucosa was scraped off from lamina propria. The tissue samples were snap frozen in liquid nitrogen and stored at -70°C. Samples for RNA analysis were stored in stabilization solution (RNAlater, Qiagen) at -20°C. Blood was collected from the abdominal aorta, centrifuged and the plasma was stored at -20°C. Fecal samples were collected from the caecum of each mouse and were stored at -20°C.

4.1.4 Plasma lipids

Plasma triacylglycerol and cholesterol concentrations were analyzed using enzymatic colorimetric assays by KoneLab 20 analyzer (Thermo Electron Corporation, Finland), and calibrators and control samples from the manufacturer were used.

4.1.5 Analysis of fecal and mucosal sterols

Study I: The sterol composition was analyzed from faeces and the mucosa of proximal small intestine for each mouse by applying a method described by Soupas et al. (Soupas et al. 2005). Dihydrocholesterol (95%, Sigma, St. Louis, MO, USA) was added to each sample as an internal standard. Briefly, ethanol and saturated potassium hydroxide were added to each sample and the esterified sterols were hydrolyzed in a shaking incubator for 30 minutes in 85°C. After hydrolyzation, water and cyclo-hexane were added to extract sterols into solvent phase. To obtain a detectable concentration of sterols, solvent was evaporated from the samples of the control mice.

Study II: Free and esterified sterols were analyzed from faeces and the mucosa of the proximal small intestine. Dihydrocholesterol (95%, Sigma, St. Louis, MO, USA) was added to each sample as an internal standard and 5 α -cholestane (Sigma, St. Louis, MO, USA) was added to control the separation of esterified sterols from free sterols by solid phase extraction (SPE). The sample was first homogenized and incubated in a mixture of 0.5 M sodium acetate, methanol, and chloroform (1.6:4:2, vol:vol:vol). The incubation was continued by adding first chloroform and finally Milli-Q-water. The chloroform phase was transferred to a round bottom flask, and chloroform was evaporated in 50°C. The lipids were collected from the flask with heptane-diethylether (9:1, vol:vol). This extract was used to fractionate free and esterified sterols with SiOH-column (Strata SI-1 Silica 500 mg; Phenomenex, Torrance, CA, USA) in a SPE vacuum manifold (Supelco Visiprep DL, Sigma-Aldrich Co.).

Prior to the gas chromatographic analysis, derivatives of sterols were obtained using pyridine and BSTFA:TMCS (99:1, vol:vol) as silylation agents (1:1, vol:vol). The sample was injected into a RTX-5 w/Integra Guard (crossbond 5% diphenyl-95% dimethyl polysiloxane) capillary column (60 m x 0.32 mm i.d., 0.10 μ m film; Restek, Bellefonte, PA, USA) by automated injector of a gas chromatograph equipped with a flame ionization detector (Agilent 6890N Network GC System, Agilent Technologies, USA). Sterols were quantified using an internal standard method. Identification was based on retention times and GC-mass spectrometric analysis. A sample of rapeseed oil was used to control interassay variation.

4.1.6 Western blot analysis

Sample preparation and Western blot analysis have been described in more detail in original publications (I, II). Shortly, proteins were isolated from normal-appearing mucosa of the distal small intestine which represents approximately 40% of total small intestine. Tissue samples were homogenized in ice-cold buffer with enzyme inhibitors. A homogenate containing all the cellular proteins was prepared, and nuclear, cytosolic and membrane proteins were separated by centrifugation and concentrated using the Amicon Ultra-4 Centrifugal Filter Devices (Millipore, Bedford, MA, USA). The protein content for each sample was measured by the

Bradford assay (Protein Assay, Bio Rad, Hercules, CA, USA). The samples were stored in denaturing buffer in -70°C.

Proteins were separated in sodium dodecyl sulphate (SDS) gels from which they were transferred to nitrocellulose or polyvinyl membranes using an electric current. After blocking the membrane against unspecific binding of primary antibody, the membrane was incubated with primary antibody specified in the original publications. For detection and quantification, proteins were transferred to an X-ray film (Amersham), scanned and analyzed by GSA-800 Calibrated Imaging Densitometer and Quantity One Program (BioRad Laboratories), or scanned with Odyssey infrared imager (LI-COR, Inc., Nebraska, USA). β -Actin and lamin B were used to control equal loading of protein samples.

4.1.7 RNA isolation and microarray and global gene expression statistics

Total RNA was extracted from ileal mucosa sample by using the RNeasy Mini kit (Qiagen). The tissue sample was stored in stabilization solution (RNAlater, Qiagen) until RNA extraction. Purity and integrity of RNA samples were verified by using spectrophotometry and agarose gel electrophoresis (Study I) or Bioanalyzer 2100 (v2.6, Agilent Technologies, Inc.)(Study II). Equal amounts of total RNA was taken from each mouse to make a pooled sample for each group. In Study II, samples were pooled by gender.

In microarray experiment, the expression of a large number of genes can be detected using oligonucleotide probes on a single chip. The hybridization of the fluorescently labeled target gene (sample RNA) to its probe produces a signal that is then detected and quantified. In Study I, the pooled samples were analyzed by using the Affymetrix microarray platform. The labeling, hybridization and scanning were performed at the Centre for Biotechnology, University of Turku, Finland. In Study II, Agilent Whole Mouse Genome 4x44K microarray assay. The labeling, hybridization and scanning were performed at the Biomedicum Functional Genomics Unit, University of Helsinki.

For the global gene expression data statistics were applied to detect enriched functional categories or pathways associated with the genes regulated by plant stanol or plant sterol feeding by using Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (Huang et al. 2007). This analysis was conducted to diminish the effect of false positives, as only a large body of genes with similar functions will show as significant enrichment. More detailed description of analyses are provided in the original papers (I,II). For all analyses a $P < 0.05$ was considered statistically significant.

4.1.8 Statistical analyses

In Study I, genders were combined for statistical analysis since plant stanol feeding affected both genders in similar manner. In Study II, plant sterols feeding affected adenoma formation and mucosal sterol composition in different manner and therefore genders were analyzed separately for all variables. In Study I, the non-parametric Mann-Whitney U-test was used to compare the tumor, sterol and protein data between the experimental groups. In Study II, the normality of data was tested using the Shapiro-Wilkinson test, and comparisons within genders were conducted using the independent-samples t -test. The Pearson's correlation was applied to test associations between variables. The Spearman's correlation was applied to test associations between not normally distributes variables. All comparisons in adenoma number and size, fecal and mucosal sterols, and cellular proteins between groups were performed using SPSS-PASW 18.0 for Windows software, (SPSS Inc.). For all analyses a $P < 0.05$ was considered statistically significant.

In Study II, the lasso Poisson regression analysis (Tibshirani 1996) (Friedman et al. 2010) that included all measured variables simultaneously, was conducted to explore which variables had predictive value for the total adenoma number and the analysis was performed using *glmnet* package in R. The lasso regression model was used since there were more variables than observations. The number of predictors with non-zero effects was determined using cross-validation, and the overall significance

of the predictive model was assessed with permutation sampling. Lasso regression analysis was not conducted in Study I, due to the non-normality of parameters.

4.2 Study III: Estimation of phytosterol intake in the FINDIET 2007 Survey

4.2.1 Subjects and methods

The intakes of phytosterols were calculated from the data collected by the National FINDIET 2007 Survey, which was conducted as a part of the National FINRISK 2007 Study at the National Institute for Health and Welfare, THL, in Finland (Valsta et al. 2010). The FINDIET 2007 Survey collected data on dietary habits from 33% of the FINRISK 2007 Study participants by 48 h dietary recall carried out by nutritionists trained for the method. A detailed description on dietary data collection is described by Reinivuo and co-workers (Reinivuo et al. 2010). The study included 958 men and 1080 women aged 25-74 from five regions in Finland.

Users of phytosterol enriched products were identified from the 48 h dietary recall. Intakes of phytosterols, cholesterol, energy, and selected nutrients were calculated based on the 48 h dietary recall with the national food composition database Fineli® (version 2012) at THL (Reinivuo et al. 2010). Users (n=194) and non-users (n=1844) of phytosterol enriched food products were compared with respect to sex, age, education, region, cholesterol-lowering medication, and cholesterol-lowering diet. Mean daily intakes of phytosterols, energy and selected nutrients were compared between the users and the non-users. The distribution of phytosterol intake was assessed for users of phytosterol enriched products and for users of plant sterol and plant stanol enriched products separately.

4.2.2 Statistical analyses

Differences in plant sterol and stanol intakes between men and women were tested using analysis of variance. If the variances of men and women were unequal, we conducted Welch's ANOVA. Mann-Whitney U-test for untransformed values was used if the intakes were non-normal. The relationship between the use of phytosterol enriched products and the characteristic variables were tested using Fisher's exact test. Differences in nutrient intakes between users and non-users of phytosterol enriched foods were tested using analysis of variance, adjusting for age. The analyses were conducted also using education as a covariate in the model as well as the use of cholesterol medication and/or cholesterol lowering diet. The statistical analyses were performed using the SAS statistical package (SAS Institute Inc., Cary, NC, USA, version 8.2).

5 RESULTS

This section focuses on the results of plant stanol and sterol feeding on intestinal tumor formation and cell signaling proteins related to tumorigenesis. The results on Study I are presented genders combined in the original publication since the effect of plant stanol feeding on adenoma formation was similar in both genders. The effects of plant sterols and stanols on sterol composition of intestinal mucosa are compared here more closely by gender, which has not been done in a similar detail in the original publications.

5.1 Effects of phytosterol feeding on intestinal tumor formation in *ApcMin* mice

Generally, mice grew well in Studies I and II and there was no difference in body weight gain between the groups in either study. Both plant stanols (Study I) and plant sterols (Study II) significantly increased the number of intestinal adenomas, but not their size (Tables 5 and 6). In Study I, the number of adenomas in the entire small intestine was higher in the plant stanol mice (mean \pm SD; 64.9 ± 22.1) than in the control mice (40.1 ± 13.9 , $P=0.002$; Fig. 5A and Table 5), and the effect was seen in males (plant stanol 56.4 ± 20.6 vs. control 33.5 ± 8.2 , $P=0.01$) and in females (plant stanol 73.4 ± 21.7 vs. control 48.8 ± 15.7 , $P=0.054$; Fig. 5B). In Study II, plant sterol feeding increased the total number of intestinal adenomas (plant sterol 43.7 ± 7.9 vs. control 35.6 ± 8.5 , $P=0.025$; Fig. 5C). The total number of intestinal adenomas was significantly increased in plant sterol females when compared with control females (46.8 ± 7.0 and 35.0 ± 9.1 , respectively, $P=0.036$; Fig. 5D), whereas there was no significant difference between plant sterol and control males (41.2 ± 8.2 and 36.3 ± 8.5 respectively, $P=0.34$; Fig. 5D).

Both plant stanol and plant sterol feeding induced tumor formation strongly in the distal part of the small intestine (distal three fifths; Table 5). Plant stanol enriched diet increased the number of adenomas in the proximal part (proximal two fifths), too, whereas plant sterol diet had no significant effect on adenoma number in the proximal intestine. Plant stanol and plant sterol feeding had no effect on the size of

tumors in any part of the small intestine (Table 6). There was no difference in the number or size of tumors in the colon between control and plant stanol mice or control and plant sterol mice in either gender.

Since plant stanol and plant sterol feeding increased the number of intestinal adenomas, and not the size, phytosterols appear to influence the initiation of intestinal tumorigenesis rather than tumor growth in this mouse model.

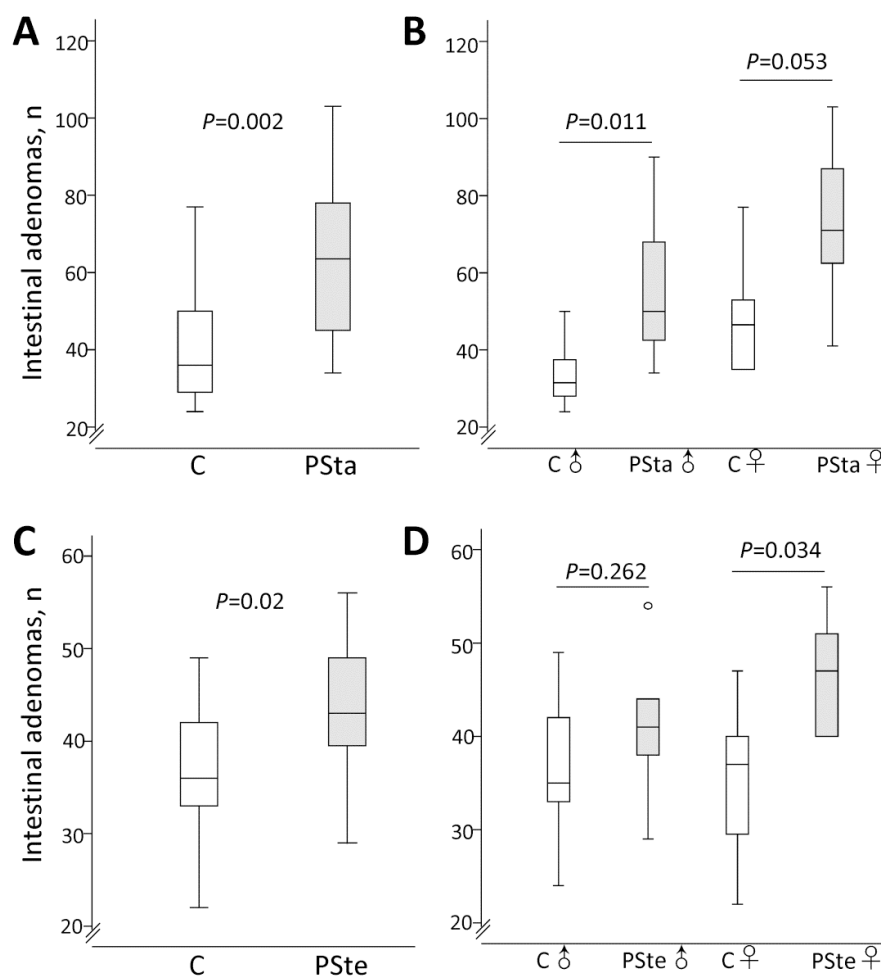


Figure 5. The number of intestinal adenomas in *ApcMin* mice in Study I by diet group (A) and by gender (B). The number of intestinal adenomas in Study II by diet group (C) and by gender (D). In Study I, mice were fed either a control diet (C= control mice, n=14) or a 0.8% (w/w) plant stanol diet (PSta= plant stanol mice, n=14). In Study II, mice were fed either control diet (C= control mice, n=13) or a 0.8% (w/w) plant sterol diet (PSte= plant sterol mice, n=11). After the 9-week feeding period the adenomas were counted and the size was measured as described in the Materials and methods. Results are presented as box-plots, where the box represents the interquartile range and contains 50% of values. The whiskers extend to the maximum and minimum values. The median is indicated by a line across the box.

Table 5. Number of adenomas in different parts of the small intestine of *ApcMin* mice¹.

	Study I					
	Control	Plant stanol (PSta)	Control males	PSta males	Control females	PSta females
Mice, n	14	14	8	7	6	7
Distal small intestine ²						
Mean (SD)	32.9 (11.6)	53.8 (18.4)**	28.0 (6.0)	47.3 (16.8)**	39.5 (14.4)	60.3 (18.8)*
Median (min-max)	31 (21-63)	51 (26-86)**	27.5 (21-37)	45 (26-73)**	38 (24-63)	60 (33-86)*
Proximal small intestine ³						
Mean (SD)	7.1 (3.8)	11.1 (4.6)*	5.5 (3.3)	9.1 (4.5)*	9.3 (3.5)	13.1 (4.0)
Median (min-max)	6 (2-14)	10.5 (5-17)*	4.5 (2-13)	8 (5-17)*	10 (4-14)	15 (8-17)
Total small intestine						
Mean (SD)	40.1 (13.9)	64.9 (22.1)**	33.5 (8.2)	56.4 (20.6)**	48.8 (15.7)	73.4 (21.7)*
Median (min-max)	36 (24-77)	63.5 (34-103)**	31.5 (24-50)	50 (34-90)**	46.5 (35-77)	71 (41-103)*
	Study II					
	Control	Plant sterol (PSte)	Control males	PSte males	Control females	PSte females
Mice, n	13	11	6	6	7	5
Distal small intestine ²						
Mean (SD)	28.4 (7.5)	37.5 (6.5)**	28.3 (7.0)	36.2 (6.4)	28.4 (8.4)	39.2 (7.0)*
Median (min-max)	30 (17-39)	36 (26-46)**	28 (18-39)	36 (26-46)	31 (17-39)	44 (31-45)*
Proximal small intestine ³						
Mean (SD)	7.2 (2.8)	6.2 (2.8)	8.0 (2.8)	5.0 (2.3)	6.6 (2.8)	7.6 (3.0)
Median (min-max)	7 (4-12)	7 (3-11)	8.5 (4-11)	4.5 (3-8)	6.0 (4-12)	8 (3-11)
Total small intestine						
Mean (SD)	35.6 (8.5)	43.7 (7.9)*	36.3 (8.5)	41.2 (8.2)	35.0 (9.1)	46.8 (7.0)*
Median (min-max)	36 (22-49)	43 (29-56)*	35 (24-49)	41 (29-54)	37 (22-47)	47 (40-56)*

* Different from control, $P < 0.05$; different from control, ** $P \leq 0.01$.

¹The intestinal adenomas were counted under a light-microscope after the 9-week feeding period.

²The distal three fifths of the small intestine.

³The proximal two fifths of the small intestine.

Table 6. Size (diameter, mm) of adenomas in different parts of the small intestine of *ApcMin* mice¹.

	Study I					
	Control	Plant stanol (PSta)	Control males	PSta males	Control females	PSta females
Mice, n	14	14	8	7	6	7
Distal small intestine ² , mm						
Mean (SD)	1.0 (0.1)	1.1 (0.1)	1.1 (0.1)	1.1 (0.1)	1.0 (0.1)	1.0 (0.2)
Median (min-max)	1.1 (0.8-1.2)	1.1 (0.8-1.2)	1.1 (1.0-1.2)	1.1 (1.0-1.2)	0.9 (0.8-1.3)	1.0 (0.8-1.2)
Proximal small intestine ³ , mm						
Mean (SD)	1.9 (0.4)	1.7 (0.3)	2.1 (0.4)	1.7 (0.3)	1.7 (0.3)	1.7 (0.2)
Median (min-max)	1.8 (1.5-2.6)	1.6 (1.1-2.2)	2.1 (1.5-2.6)	1.8 (1.1-2.1)	1.6 (1.5-2.1)	1.5 (1.5-2.2)
Total small intestine, mm						
Mean (SD)	1.2 (0.1)	1.2 (0.1)	1.2 (0.1)	1.2 (0.04)	1.1 (0.1)	1.1 (0.2)
Median (min-max)	1.2 (1.0-1.3)	1.2 (0.9-1.4)	1.2 (1.1-1.3)	1.2 (1.1-1.2)*	1.1 (1.0-1.3)	1.1 (0.9-1.4)
	Study II					
	Control	Plant sterol (PSte)	Control males	PSte males	Control females	PSte females
Mice, n	13	11	6	6	7	5
Distal small intestine ² , mm						
Mean (SD)	1.2 (0.2)	1.2 (0.2)	1.3 (0.1)	1.3 (0.2)	1.1 (0.2)	1.1 (0.1)
Median (min-max)	1.2 (0.8-1.4)	1.2 (1.0-1.6)	1.2 (1.1-1.4)	1.2 (1.1-1.6)	1.1 (0.8-1.3)	1.1 (1.0-1.2)
Proximal small intestine ³ , mm						
Mean (SD)	1.6 (0.2)	1.5 (0.5)	1.6 (0.1)	1.6 (0.6)	1.7 (0.3)	1.4 (0.3)
Median (min-max)	1.7 (1.3-2.2)	1.4 (1.0-2.7)	1.6 (1.4-1.8)	1.4 (1.1-2.7)	1.7 (1.3-2.2)	1.3 (1.0-1.7)
Total small intestine, mm						
Mean (SD)	1.2 (0.2)	1.2 (0.2)	1.3 (0.1)	1.3 (0.2)	1.2 (0.2)	1.1 (0.1)
Median (min-max)	1.3 (0.9-1.4)	1.2 (1.0-1.6)	1.4 (1.1-1.4)	1.3 (1.1-1.6)	1.2 (0.9-1.4)	1.1 (1.0-1.3)

* Different from control, $P < 0.05$.

¹ Diameters of intestinal adenomas were counted under a light-microscope after the 9-week feeding period.

²The distal three fifths of the small intestine.

³The proximal two fifths of the small intestine.

5.2 Effects of phytosterol feeding on plasma lipids in *ApcMin* mice

No change was seen in plasma lipid concentrations after plant stanol feeding in Study I (Table 7). In humans circulating cholesterol is mainly transported in LDL lipoproteins but in mice in HDL (Jawień et al. 2004). Therefore phytosterol feeding does not affect the serum LDL-C levels in mice which has been also observed in other studies (Calpe-Berdiel et al. 2005). Plasma lipids were not analyzed in Study II.

Table 7. Plasma cholesterol and triacylglycerol concentrations (mean±SD; mmol/l) in the control and plant stanol group.

	Total cholesterol	HDL	LDL	TG
Control (n=14)	2.9±0.35	2.4±0.33	0.23±0.12	2.9±0.76
Plant stanol (n=11)	2.8±0.48	2.4±0.51	0.26±0.13	3.0±1.5

5.3 Effects of phytosterol feeding on fecal phytosterols in *ApcMin* mice

Both plant stanol and sterol feeding resulted in significantly higher cholesterol, plant sterol and plant stanol concentrations in the feces when compared to controls (Figure 6; values are presented in Table 2 in Paper I and in Table 1 in Paper II). Fecal total cholesterol concentration correlated positively with intestinal adenoma number in Studies I ($r=0.570$, $P<0.01$; Fig. 7A) and II ($r=0.417$, $P=0.047$; Fig. 7B).

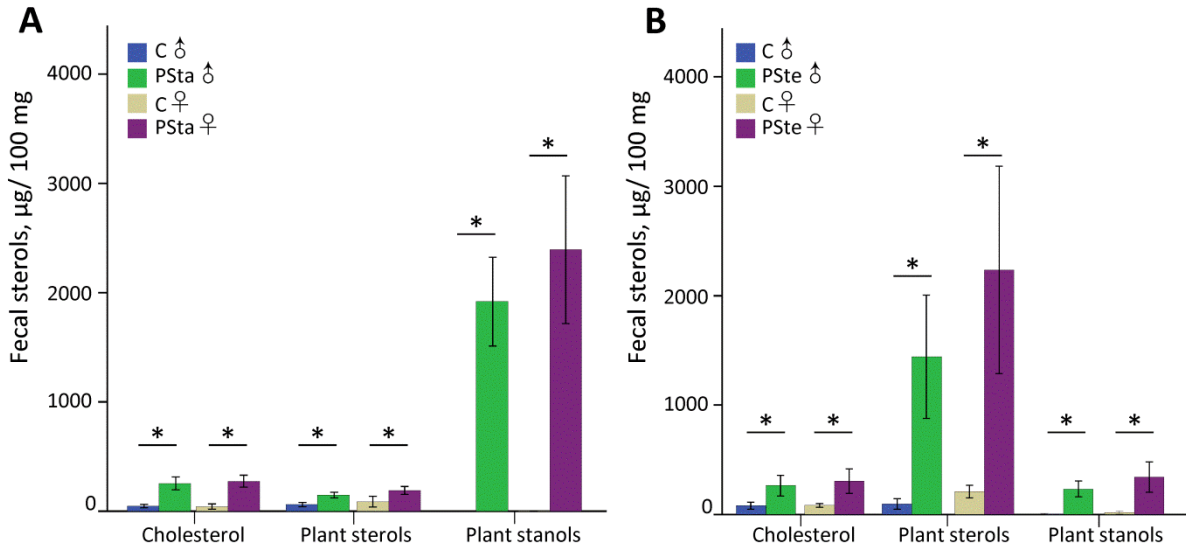


Figure 6. Fecal sterols ($\mu\text{g}/100\text{ mg}$ of wet weight) in *ApcMin* mice after plant stanol feeding (Study I) by gender (A) and after plant sterol feeding (Study II) by gender (B). Analyses were conducted from caecum content as described in Materials methods. Bars represent mean \pm SD. * Different from control $P < 0.05$. C= control, PSta= plant stanol, PSte= plant sterol.

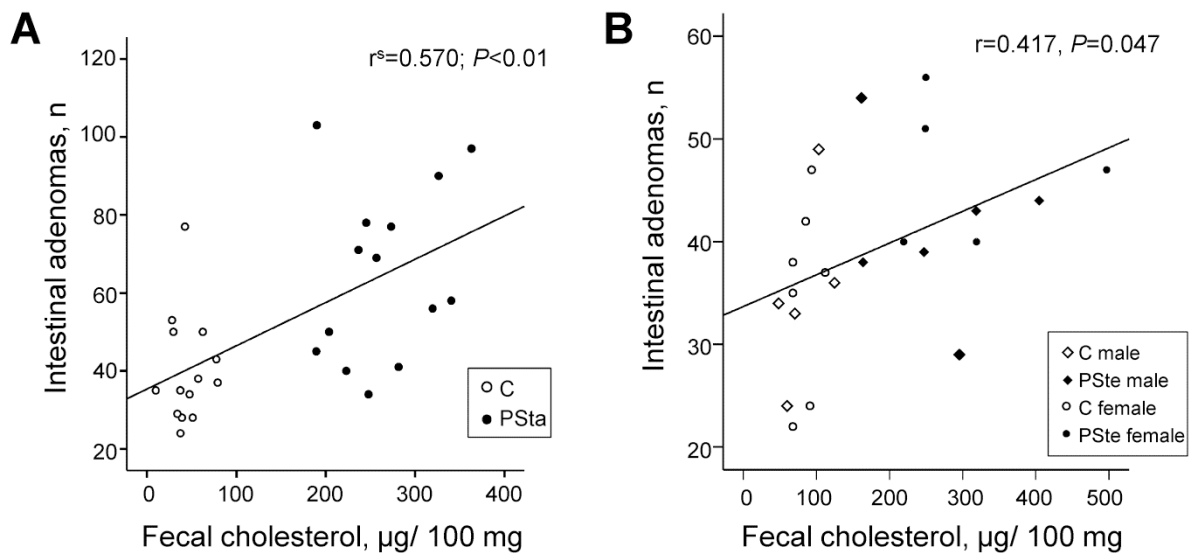


Figure 7. Correlation between total number of adenomas in the small intestine and fecal cholesterol concentration in Study I (A) and Study II (B). C= control, PSta= plant stanol, PSte= plant sterol.

5.4 Effects of phytosterol feeding on mucosal phytosterols in *ApcMin* mice

The sterol profiles of the intestinal mucosa are shown in Figure 8. In study I, sterols were analyzed as total sterols (esterified and free sterols combined) and in Study II as free and esterified sterols. Plant stanol feeding (Study I) increased the concentration of sitostanol and campestanol in the intestinal mucosa ($P < 0.001$ for both; Table 2 in Paper I). The increase in sitostanol concentration was greater than for campestanol, and was increased by almost 70 fold in both genders. In contrast, concentrations of plant sterols (campesterol, sitosterol) were decreased in plant stanol fed mice when compared with control mice ($P < 0.001$ for both). The reduction in mucosal campesterol was greater than for sitosterol in both genders. Mucosal campesterol and sitosterol concentration was reduced by more than half in both genders after plant stanol feeding (-51% in males, -61% in females), whereas the reduction in mucosal sitosterol was around 40% in both genders.

Plant sterol feeding (Study II) increased the concentration of free sitosterol and campesterol in the intestinal mucosa in both genders (males $P \leq 0.001$ for both, and females $P = 0.004$ and $P = 0.028$, respectively; Table 2 in Paper II). In plant sterol males the concentration of mucosal free sitosterol was 8-fold greater than in control males but only 2.8-fold greater in plant sterol females than in control females. Plant sterol feeding increased the concentrations of esterified sitosterol and campesterol in males ($P = 0.027$ and $P = 0.058$, respectively) but not in females. The concentration of free plant stanols was increased in plant sterol males and females (by 4.6-fold, $P = 0.009$, and by 1.7-fold $P = 0.011$, respectively) but no difference was observed in the concentration of esterified plant stanols in either gender.

In control mice (Studies I and II), mucosal phytosterols were in descending order campesterol > sitosterol > campestanol > sitostanol. In plant stanol mice (Study I), the order was sitostanol > campestanol > campesterol > sitosterol. In plant sterol male mice (Study II) the order was sitosterol > campesterol > sitostanol > campestanol and in plant sterol female mice (Study II) sitosterol \cong campesterol > sitostanol > campestanol.

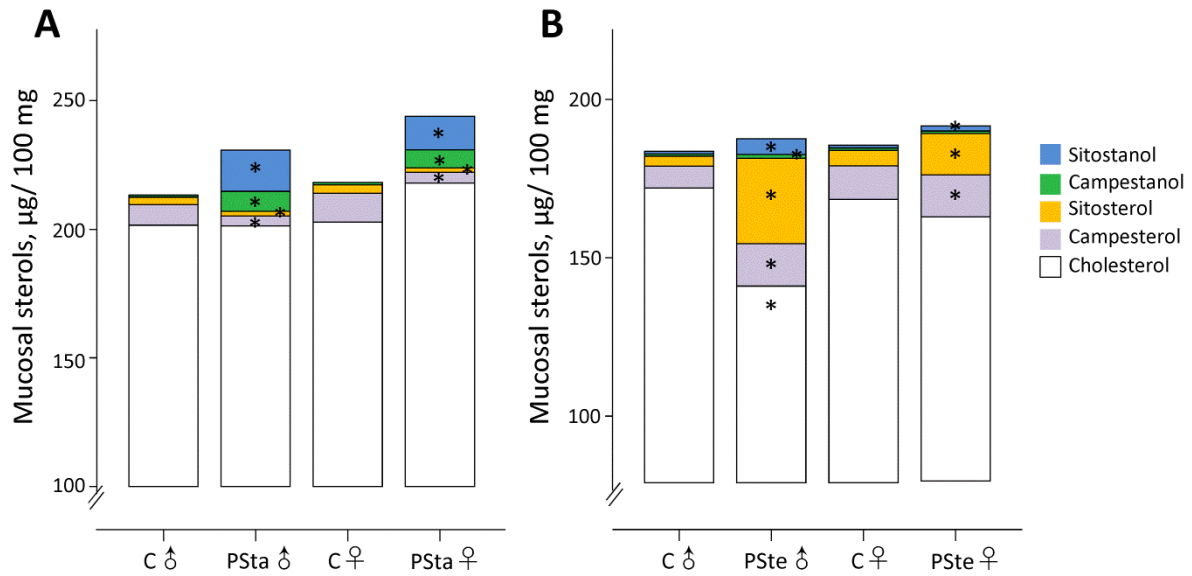


Figure 8. Mucosal sterols ($\mu\text{g}/100\text{ mg}$ of wet weight) after plant stanol (A) and plant sterol (B) feeding by gender in the small intestine of *ApcMin* mice. Analyses were conducted from the intestinal mucosa as described in Materials methods. Bars represent the mean concentration of total sterols including both free and esterified sterols. * Different from control $P < 0.05$. C= control, PSta= plant stanol, PSte= plant sterol.

5.5 Effects of phytosterol feeding on mucosal cholesterol and total sterols in *ApcMin* mice

In Study I, the concentration of total cholesterol in the intestinal mucosa was not altered after plant stanol feeding in either gender (Fig. 9A). In Study II, plant sterol fed males had a significantly lower concentration of total and free cholesterol in the intestinal mucosa when compared with control males (-17%; $P=0.008$; Fig. 9B and 9C). There was no difference in the free cholesterol between females in Study II. The concentration of esterified cholesterol in the intestinal mucosa was significantly decreased in male and female mice after plant sterol feeding when compared with control mice (-56%; $P=0.012$ and -38%; $P=0.024$, respectively; Fig. 9D).

There was no difference in the total concentration of mucosal sterols (total of cholesterol and plant sterols) between control and phytosterol fed mice in either gender (Study I: males $P=0.25$ and females $P=0.57$; Study II: males $P=0.94$ and females $P=0.55$). There was a trend towards a higher concentration of total sterols

and stanols (total of cholesterol, plant sterols and stanols) in plant stanol males and females ($P=0.083$ and $P=0.063$, respectively). In Study II, no difference was found in total mucosal sterols and stanols between control and plant sterol males ($P=0.67$) and females ($P=0.49$).

Overall, no change was seen in the mucosal total sterol and stanol concentration after plant sterol feeding. The accumulation of plant sterols, sitosterol in specific, in the intestinal mucosa of plant sterol males was accompanied by decrease in mucosal cholesterol concentration (Study II). By contrast plant stanol feeding increased the total concentration of sterols and stanols in the intestinal mucosa resulting from the accumulation of plant stanols as demonstrated in Fig. 9.

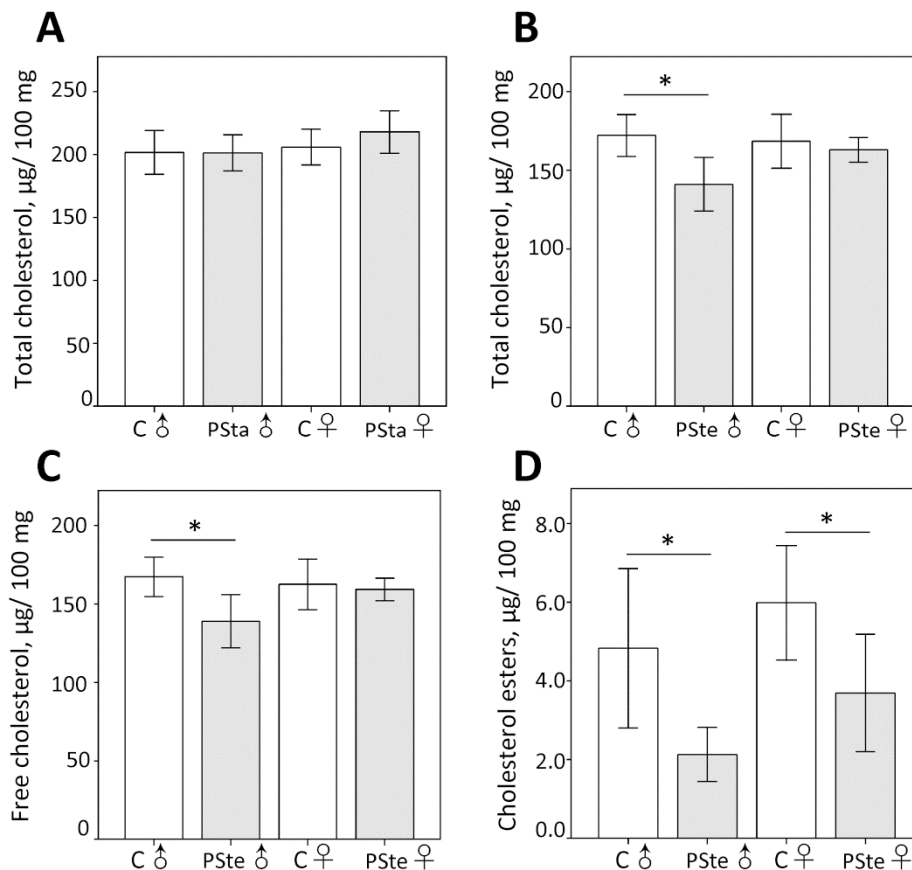


Figure 9. Total cholesterol concentrations ($\mu\text{g}/100\text{ mg}$) in the intestinal mucosa of *ApcMin* mice after plant stanol (A) and plant sterol (B) feeding. Concentrations of free (C) and esterified cholesterol (D) were analyzed separately from the intestinal mucosa in the plant sterol study as described in Materials and methods. Bars represent mean \pm SD. * Different from control $P < 0.05$. C= control, PSta= plant stanol, PSte= plant sterol.

5.6 Effects of phytosterol feeding on the regulation of cholesterol synthesis in the intestinal mucosa of *ApcMin* mice

The sterol regulatory element binding proteins (SREBP) act as transcription factors for genes that up-regulate cholesterol biosynthesis. When cellular free cholesterol decreases, the level of nuclear SREBP-2 increases (Brown and Goldstein 1997). In Study I, no difference was detected in the level of active nuclear SREBP-2 between plant stanol and control mice in either gender (Fig. 10A). In Study II, the level of nuclear SREBP-2 was significantly increased in plant sterol males when compared with control males ($P=0.045$; Fig. 10B). There was no difference in the level of nuclear SREBP-2 between plant sterol and control females. Moreover, phytosterol feeding did not affect the level of precursor SREBP-2 in Studies I and II.

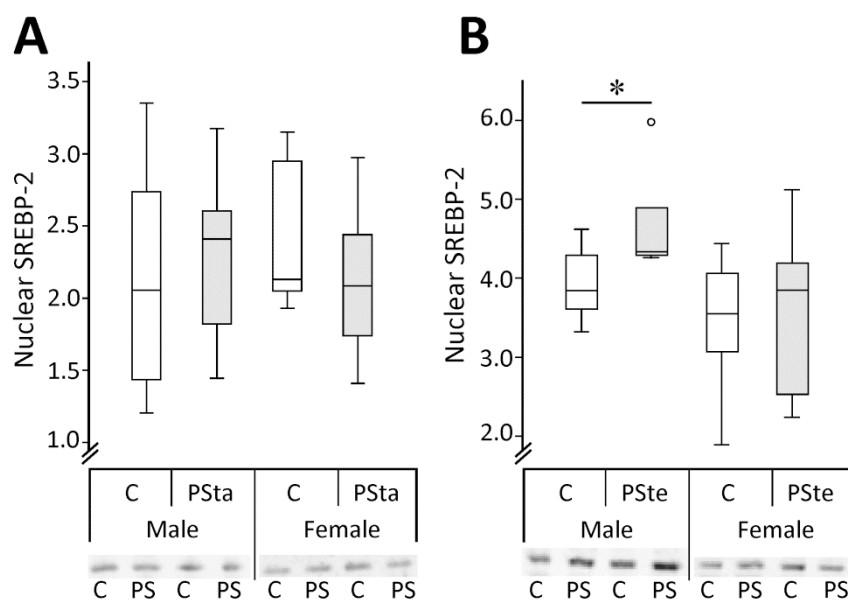


Figure 10. Levels of nuclear SREBP-2 (arbitrary units) in the intestinal mucosa of *ApcMin* mice after plant stanol (A) and plant sterol (B) feeding. Proteins were extracted from the intestinal mucosa and fractionated into different cellular compartments. After separation by SDS gel electrophoresis, the proteins were detected by immunoblotting (Western blot) and visualized using infrared dye as described in Materials and methods. Results are presented as box-plots, where the box represents the interquartile range and contains 50% of values. The whiskers extend to the maximum and minimum values. The median is indicated by a line across the box. Representative bands of SREBP-2 from Western blot are shown below the box plots. *Different from control $P<0.05$. C= control, PSta= plant stanol, PSte= plant sterol.

Global gene expression was analyzed by microarray assay using RNA isolated from the histologically normal-appearing mucosa of the distal small intestine. After enrichment analysis, the cholesterol biosynthesis pathway showed significant enrichment for upregulated genes in the intestinal mucosa of plant stanol mice ($P=1.06E-6$; Fig. 11) and in plant sterol females ($P=0.014$, Fig. 11) but not in plant sterol males.

Overall, the nuclear SREBP-2 level was increased only in plant sterol males with reduced cholesterol concentration in the intestinal mucosa. In contrast, the genes of the cholesterol biosynthesis pathway were upregulated in plant stanol mice and plant sterol female mice, but not in plant sterol male mice. This result is in concordance with the observed effects of plant sterol and stanol on the number of intestinal adenomas in *ApcMin* mice.

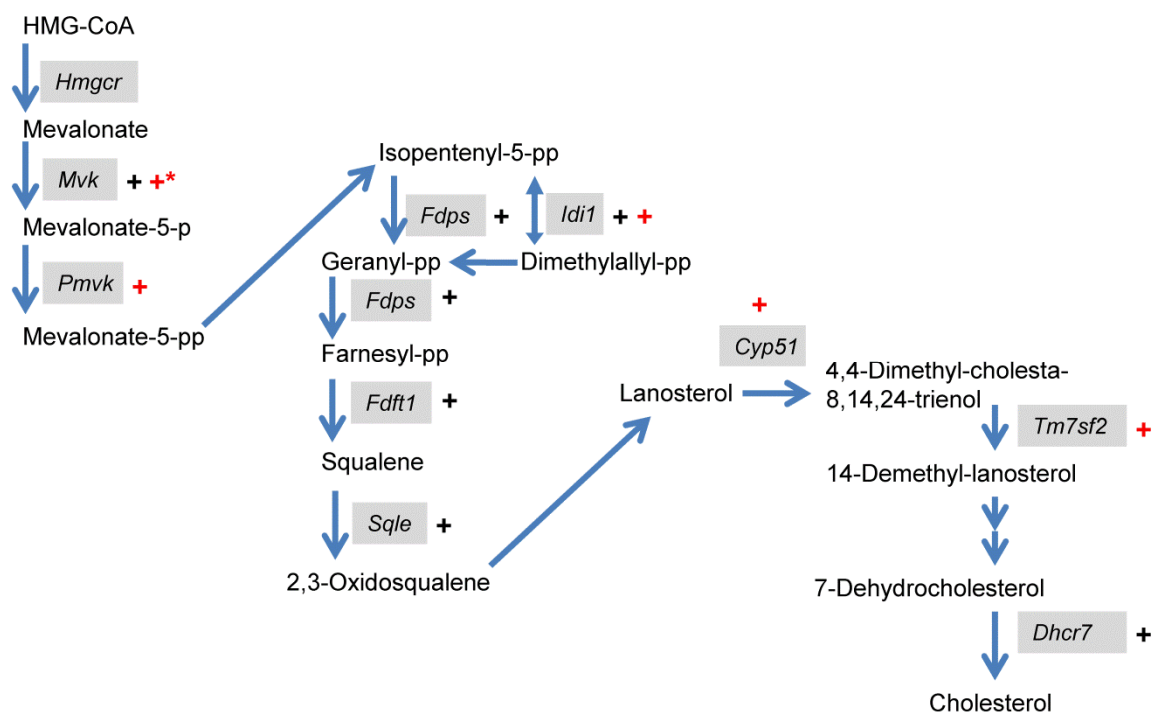


Figure 11. Cholesterol biosynthesis pathway. A black cross indicates an upregulated gene in plant stanol mice and a red cross plant sterol female mice when compared with control mice. The red asterisk indicates an upregulated gene in plant sterol male mice when compared with control mice. (Produced according to the KEGG database.)

5.7 Effects of phytosterol feeding on cell signaling proteins in ApcMin mice: Wnt and Egfr pathways

Plant stanol feeding increased levels of nuclear β -catenin and cyclin D1, a β -catenin target ($P=0.043$ and $P=0.065$, respectively; Table 8; Fig. 3A and B in Paper I). Furthermore, the level of phospho-Ser675- β -catenin, a transcriptionally active form of β -catenin (Fang et al. 2007),(Galisteo et al. 1996), was significantly higher in plant stanol mice than in the control mice (Fig. 4 in Paper I). In Study II, there was no difference in the level of nuclear β -catenin or cyclin D1 in either gender between plant sterol and control group (Table 9).

Levels of Egfr, ERK1/2 and Akt were analyzed from whole mucosa lysate. In Study I, levels of total and phosphorylated (activated) Egfr were significantly higher in the plant stanol group when compared with the control group (Table 8; Fig. 3C and D in Paper I). Plant stanol feeding resulted in significantly higher level of phosphorylated ERK1/2 (p44/42) with no difference in the total level of ERK1/2 (Table 8; Fig. 3E in Paper I). The total level of Akt was elevated in the mucosa of plant stanol fed mice ($P=0.077$), whereas no difference was seen in the level of phosphorylated Akt (Table 8). In Study II, plant sterol feeding had no effect on the levels of Egfr and ERK1/2 in either gender (Table 9).

Increased protein levels of nuclear β -catenin and cyclin D1 after plant stanol feeding demonstrate upregulation of the Wnt pathway. Similarly Egfr signaling was upregulated in plant stanol mice. Activation of Wnt and Egfr pathways are related with early stages of tumorigenesis, and increased signaling of these pathways is associated with increased cell proliferation, aberrant crypt and adenoma formation (Bienz and Clevers 2000, Roberts et al. 2002, Näthke 2004, Fichera et al. 2007). Therefore the increased formation of intestinal adenomas in plant stanol mice appears to be caused by upregulation of Wnt and Egfr signaling.

Table 8. Results from Western blot analysis after plant stanol feeding (Study I)¹.

Protein	Control mice	Plant stanol mice	Statistical difference, <i>P</i> -value ²
β -Catenin			
membrane	2.7 (2.5)	3.8 (2.7)	0.18
cytosol	1.8 (1.7)	2.5 (1.2)	0.081
nucleus	3.7 (2.4)	6.0 (2.7)	0.043
Phospho-Ser675- β -catenin	11.3 (8.3)	21.7 (12.9)	0.027
Cyclin D1, nucleus	4.1 (4.8)	6.9 (5.1)	0.065
Egfr	1.1 (1.5)	3.2 (2.6)	0.031
Phospho-Egfr	0.47 (0.30)	0.72 (0.32)	0.089
ERK1/2	5.0 (0.71)	5.3 (0.81)	0.44
Phospho-ERK1/2	2.2 (1.5)	3.8 (2.5)	0.039
Akt	0.87 (0.63)	1.4 (0.77)	0.077
Phospho-Akt	0.96 (0.72)	1.2 (1.1)	0.49

¹ Values represent mean (std. deviation) in arbitrary units

² Difference between groups was analyzed by non-parametric Mann-Whitney U-test

* Different from control *P*<0.05.

Table 9. Results from Western blot analysis after plant sterol feeding (Study II)¹

Protein	Control male mice	Plant sterol male mice	Control female mice	Plant sterol female mice
β -Catenin				
membrane	2.3 (1.8)	2.7 (1.3)	2.7 (2.0)	4.0 (1.1)
cytosol	0.18 (0.2)	0.16 (0.1)	0.52 (0.4)	0.61 (0.2)
nucleus	1.5 (0.7)	1.3 (0.4)	1.9 (1.3)	2.2 (0.8)
Cyclin D1, nucleus	0.56 (0.2)	0.92 (0.8)	1.8 (1.6)	1.4 (0.8)
Egfr	2.0 (1.1)	1.9 (1.2)	2.3 (1.8)	3.0 (0.7)
Phospho-Egfr	0.86 (0.3)	1.0 (0.5)	0.96 (0.4)	0.89 (0.1)
ERK1/2	5.5 (0.4)	5.7 (0.6)	5.5 (0.5)	5.3 (0.6)
Phospho-ERK1/2	1.1 (0.9)	1.3 (1.0)	0.93 (0.3)	0.77 (0.4)
Caveolin-1, membrane	2.7 (2.5)	6.6 (10.9)	2.1 (1.9)	3.7 (3.7)
ER α	0.82 (0.6)	0.40 (0.2)	0.94 (0.7)	0.91 (0.5)
ER β	2.4 (0.5)	2.7 (1.4)	2.3 (1.2)	3.2 (0.7)

¹ Values represent mean (std. deviation) in arbitrary units, statistical testing between groups was performed using parametric *t*-test.

5.8 Effects of phytosterol feeding on estrogen receptors in the intestinal mucosa of *ApcMin* mice

Because plant sterol feeding in Study II induced tumor formation more strongly in female *ApcMin* mice and because Wnt and Egfr pathways did not appear to be involved in the tumorigenesis, we evaluated the level of estrogen receptor (ER) α and β subtypes from the intestinal mucosa. The activity of estrogens is mediated by estrogen receptors α and β , and ER β is the dominant estrogen receptor in the intestine (Foley et al. 2000, Campbell-Thompson et al. 2001). Plant sterols bind weakly to ER β , and their estrogenic effect may be low (Gutendorf and Westendorf 2001). There was no difference in the level of ER α between the groups in either gender (Table 9). The level of total ER β was non-significantly higher in plant sterol females (Table 9 and Fig. 3 in Paper II). There was a positive correlation between the total number of tumors and ER β ($r=0.62$, $P=0.001$) and when genders were analyzed separately the correlation was significant among females ($r=0.83$, $P=0.001$; Fig. 12) but not among males ($r=0.347$, $P=0.269$).

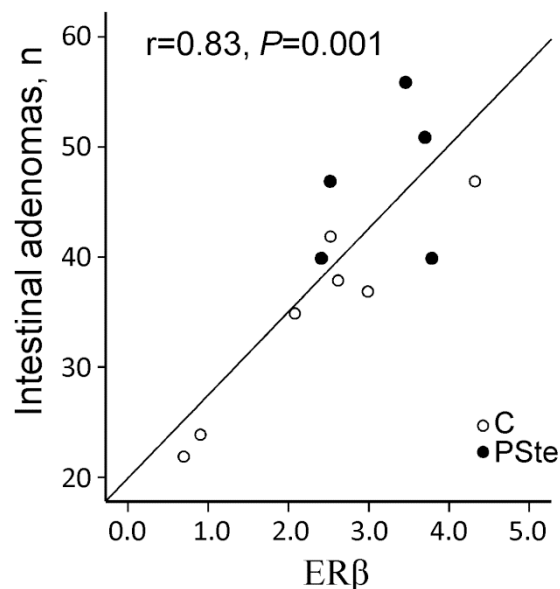


Figure 12. The correlation between the number of intestinal tumors and ER β level. Results in Fig. C= control, PS/ PSte= plant sterol.

5.9 Effects of phytosterol feeding on caveolin-1 in the intestinal mucosa of *ApcMin* mice

Plant sterols have been reported to regulate caveolin-1 levels in prostate cancer cell lines (Ifere et al. 2010). Caveolin-1 is a cholesterol binding protein that forms lipid rafts called caveolae at the plasma membrane. Caveolae function as platforms for proteins and receptors which are regulated by the cholesterol content of the plasma membrane (Pike 2005). Caveolin-1 is considered to act as a tumor suppressor by inhibiting the β -catenin mediated transcription of genes (Galbiati et al. 2000), and by down-regulating the activation of Egfr signaling (Feldman and Martinez 2009, Han et al. 2009). Caveolin-1 levels were measured in Study I and II from total mucosal homogenate and from membrane fraction. Plant stanol and plant sterol feeding had no effect on intestinal caveolin-1 level (Table 9).

5.10 Predictors of increased tumorigenesis in *ApcMin* mouse after plant sterol feeding

In Study II, a lasso regression analysis was carried out to detect a model of variables that would best predict increased tumorigenesis. The lasso regression model was used since there were more variables than observations. All measured parameters including fecal and mucosal sterols, and signaling proteins were included in the model. Those variables that received a coefficient more than 0.1 or less than -0.1 were considered strong predictors for increased adenoma number in the model. Higher levels of ER β , mucosal sitosterol esters, mucosal free cholesterol, and a lower level of caveolin-1 were the strongest predictors, and in combination predicted increased number of tumors significantly. The result is in line with the tumor suppressive role of caveolin-1. Lasso regression analysis was not conducted in Study I due to the non-normality of parameters.

5.11 Intake of phytosterols from enrichment among Finnish men and women in the FINDIET 2007 Survey

Phytosterol enriched margarines or foods were used by 9.5% (n=194) of the FINDIET 2007 Survey participants, and the use of enriched products was similar among men (9.2%) and women (9.8%). The characteristics of users of phytosterol enriched products are shown in Table 2 in Paper III. The use increased significantly with age and it was most common among subjects over 64 years of age. The level of education was significantly associated with the use of phytosterol enriched products, and the lowest frequency of users was among people with low education level. Use was more common among subjects with cholesterol-lowering medication and subjects on cholesterol-lowering diet when compared with subjects without cholesterol-lowering medication and diet, respectively (both $P < 0.0001$).

The mean intake of phytosterols among the non-users (plant sterols and stanols from natural sources) was 363 mg/d for men and 286 mg/d for women. Among the users, the mean intake of phytosterols was 2.2 g/d for men and 1.6 g/d for women, of which enrichment accounted for 1.9 g/d (86%) for men and 1.4 g/d (88%) for women (Table 3 in Paper III). More than half of the users received less than 2 g/d either plant sterol or stanol from enrichment (Table 10; Table 4 in Paper III). Among the users, 25% of men and 16% of women had an intake of phytosterols more than 3 g/d (Table 10).

Table 10. Intake of phytosterols among users of phytosterol enriched products based on 48 h dietary recall¹.

	All (n=194)	Men (n=88)	Women (n=106)
Mean (g/d)	1.9	2.2	1.6
Median (g/d)	1.6	1.9	1.3
75 th percentile (g/d)	2.6	3.1	2.2
90 th percentile (g/d)	3.7	4.1	3.3
95 th percentile (g/d)	4.1	4.3	3.6
< 2 g/d (%)	62.9	52.3	71.7
2-3 g/d (%)	17.0	22.7	12.3
> 3 g/d (%)	20.1	25.0	16.0

¹ The intake of plant sterols and stanols from all dietary sources

6 DISCUSSION

Phytosterol feeding induces intestinal tumorigenesis in *ApcMin* mice

Both plant sterols and stanols (collectively termed as phytosterols) induced intestinal tumor/ adenoma formation in *ApcMin* mice. Whereas plant stanol feeding increased the number of adenomas in both genders, plant sterol had a stronger effect in female mice. Both plant sterols and plant stanols increased the number but not the size of intestinal adenomas. The increase in adenoma number indicates that the initiation of tumor development was enhanced by plant sterol and plant stanol feeding. In other words, phytosterols affected mechanisms of the early stage in tumor development.

Phytosterols as such have not been studied earlier using the *ApcMin* mouse, and studies on plant stanols and intestinal or colon tumorigenesis have not been reported. Phytosterol feeding to carcinogen treated rodents has been shown to protect from colon tumor formation (Raicht et al. 1980), colon epithelial cell proliferation (Deschner et al. 1982), and aberrant crypt formation (Janezic and Rao 1992). In MNU-treated male rats, plant sterols were shown to reduce colon tumor formation (Raicht et al. 1980), but not in female MNU-rats (Quilliot et al. 2001). In healthy rodents, different plant sterol analogues did not influence cell proliferation (Jia et al. 2006). The experimental diets in this thesis were enriched with 0.8% (w/w) of plant sterols or plant stanols and fall within the range of earlier studies conducted in other animal models.

Plant stanol feeding (Study I) up-regulated Wnt and Egfr signaling which are both associated with early stages of colon carcinogenesis. In addition, these pathways can crosstalk and activate one another as well as act in synergy when signaling in concert (Hu and Li 2010). The activation of Egfr and Wnt signaling after plant stanol feeding may have resulted in increased cell proliferation and adenoma formation, however, the triggering mechanism behind the activation of these signaling pathways remains unresolved. On the other hand, plant sterol feeding did not affect β -catenin or Egfr levels in the histologically normal-appearing intestinal mucosa. It is possible that plant sterols and stanols affect cell signaling differentially in the small intestine.

Despite the similarity in their structure and efficacy in lowering serum LDL-C, plant stanols are absorbed less from the intestine than plant sterols are (Sanders et al. 2000). Moreover, plant stanols also decrease the absorption of plant sterols (Hallikainen et al. 2000, Miettinen et al. 2000).

Fecal sterol composition is altered after phytosterol feeding in *ApcMin* mice

In Studies I and II, the fecal cholesterol concentration was significantly increased by plant sterol and stanol feeding, and there was a significant positive association between the number of intestinal adenomas and fecal cholesterol concentration in both studies. Phytosterols interfere with cholesterol absorption, leading to increased concentrations of cholesterol and other sterol metabolites in the gut lumen (Rao and Janezic 1992, Weststrate et al. 1999). High intraluminal cholesterol concentration has been associated with enhanced cell proliferation, aberrant crypt and tumor formation in the murine colon (Kendall et al. 1992, Rao et al. 1992). The concentration of fecal neutral sterols is reported to be higher in individuals with colon cancer when compared to control subjects (Peuchant et al. 1987). Furthermore, cholesterol is metabolized by intestinal microbes to several derivatives that may be carcinogenic (Suzuki et al. 1986, Kaul et al. 1987, Panda et al. 1999).

High intake of phytosterols affects intestinal microbiota and alters the composition of intraluminal metabolites (Quilliot et al. 2001, Martínez et al. 2013). In the study conducted with female MNU rats, a mixture of plant sterols induced changes in the gut microflora that resulted in increased concentration of fecal coprostanol (Quilliot et al. 2001). Fecal coprostanol is a bacterial metabolite of cholesterol and positively associated with colon carcinogenesis (Peuchant et al. 1987, Panda et al. 1999). In a recent study, a 5% plant sterol diet induced changes in gut microbiota without affecting fecal concentrations of coprostan-3-one or coprostan-3-ol in male Syrian hamsters (Martínez et al. 2013). In addition, cholesterol, but not plant sterols, inhibited the growth of intestinal bacteria *in vitro*, and *Lactobacillus reuteri* was among the inhibited strains (Martínez et al. 2013). In humans, plant sterol intake of 8.6 g/d for 28 days reduced fecal lactic acid concentration and lactobacilli content (Ayesh et al. 1999). Changes in the intestinal microflora could potentially contribute

to intestinal tumorigenesis (Vipperla and O'Keefe 2012), however this thesis does not provide data on the effect of phytosterol feeding on intestinal microbes, since it was not in the scope of this work.

Although phytosterols increase the amount of biliary cholesterol excreted into intestinal lumen, fecal bile acid excretion has been shown to decrease (Uchida et al. 1984, Martínez et al. 2013) or remain unaffected (Raicht et al. 1980, Weststrate et al. 1999, Trautwein et al. 2002, Calpe-Berdiel et al. 2005) after phytosterol ingestion. Therefore fecal bile acids as an initiating factor in intestinal tumorigenesis after phytosterol feeding seem unlikely.

Taken together, plant sterol and plant stanol feeding resulted in increased concentrations of cholesterol and phytosterols in the feces of *ApcMin* mice, which may have caused unbeneficial alterations in the luminal environment. Increased fecal cholesterol concentration does not, however, explain the gender differences in tumor formation after plant sterol feeding.

Phytosterol feeding changes the profile of phytosterols in the intestinal mucosa of *ApcMin* mice

This thesis shows that a high intake of plant sterols (Study II) increased both plant sterol and plant stanol concentrations in the intestinal mucosa. In contrast, plant stanol feeding (Study I) resulted in increased concentrations of mucosal plant stanols, whereas concentrations of mucosal plant sterols were lower than in control mice. Even though sitosterol is the major natural plant sterol in the diet, campesterol is absorbed more efficiently than sitosterol, and campesterol is the predominant phytosterol in tissues (McIntyre et al. 1971, Sanders et al. 2000, Igel et al. 2003). This was observed in the sterol composition of intestinal mucosa in the control mice, but when plant stanol (Study I) and plant sterol (Study II) was fed to mice sitostanol and sitosterol became the predominant phytosterol in the intestinal mucosa, respectively.

In Study II, genders responded in different manner to phytosterol diet, which was observed as differences in intestinal sterol composition. Plant sterol males (Study II) had almost 2-fold higher concentration of sitosterol in the intestinal mucosa than plant sterol females. Indeed, gender has been found to affect expression and regulation of genes involved in lipid and sterol metabolism (Yang et al. 2006). The absorption of cholesterol and phytosterols is more efficient in female than in male mice (Turley et al. 1998, Sanders et al. 2000), and the effect of gender has been estimated to account for 20% of the variation in plasma plant sterol concentrations (Chan et al. 2006). For comparison, the impact of ABCG5/8 phenotype has been estimated to explain 8% of the variation (Chan et al. 2006).

To summarize, phytosterol feeding changed sterol composition of intestinal mucosa by increasing total phytosterol concentrations and altering the phytosterol profile of intestinal mucosa. Plant stanols reduced the uptake of plant sterols by enterocytes. In addition, genders responded differentially to high intake of plant sterols.

Plant sterol and stanol feeding affect mucosal cholesterol concentration in a different manner in the intestine of *ApcMin* mice

In Study I, plant stanol feeding did not affect the concentration of cholesterol in the intestinal mucosa in either gender. By contrast, plant sterol feeding (Study II) resulted in lower concentrations of free and total cholesterol in males, whereas the concentrations were unaffected in females. It would be expected that cholesterol concentration in the mucosa is tightly regulated; however, the results indicate that mucosal cholesterol homeostasis was disturbed in males after plant sterol feeding (Study II). Male and female rodents differ in their sterol metabolism as shown by Turley and co-workers (Turley et al. 1998).

Although plant sterol males had lower concentration of cholesterol, they accumulated more plant sterols in the intestinal mucosa than females. In fact, the concentration of total sterols (cholesterol, sitosterol, campesterol, sitostanol and campestanol) in the mucosa was similar between males and females in Study II. This suggests that a high concentration of sitosterol interferes with cholesterol

homeostasis in tissues as also implicated by Yang and co-workers (Yang et al. 2004). Plant stanol feeding (Study I), on the other hand, did not affect mucosal cholesterol concentrations since it *decreased* mucosal plant sterol concentrations equally in both genders.

In Study II, plant sterol feeding reduced the concentration of esterified cholesterol in both genders. The finding supports earlier evidence that plant sterols inhibit the acyl-CoA:cholesterol acyltransferase enzyme (ACAT) (Field and Mathur 1983) that esterifies cholesterol before it can be packed into chylomicrons and exported from the enterocyte. The reduction of cholesterol esterification may partially explain reduced cholesterol absorption after phytosterol feeding.

Effects of phytosterols on cholesterol synthesis in the intestinal mucosa of *ApcMin* mice

The pathway analyses on the microarray data show that plant sterol feeding (Study II) resulted in significant enrichment of upregulated genes involved in cholesterol synthesis only in females but not in males. Unfortunately, samples from both genders were pooled for analysis in the plant stanol study (Study I), and differences in gene regulation between genders were not possible to determine after plant stanol feeding. The results from the pathway analyses suggest that reduced cholesterol absorption stimulated a compensatory cholesterol synthesis in enterocytes in plant stanol mice (Study I) and plant sterol females (Study II), since no change was detected in the mucosal cholesterol concentration in these mice. When the concentration of intracellular free cholesterol is low, SREBP-controlled transcription of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), the key enzyme regulating cholesterol biosynthesis, and LDL-receptor, is increased. As a result, cholesterol synthesis and cholesterol uptake are increased. In the multistep process of cholesterol synthesis, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) is converted to mevalonate by HMGCR, and mevalonate is then used to produce isoprenoids and cholesterol.

As expected, the decreased concentration in the mucosal free cholesterol of plant sterol males (Study II) was accompanied by increased levels of nuclear SREBP-2. Notwithstanding, the cholesterol biosynthesis pathway was not enriched for upregulated genes in plant sterol males. In this thesis, nuclear SREBP-2 was increased only when mucosal cholesterol was reduced as seen for plant sterol males, whereas no changes were seen in nuclear SREBP-2 level when genes of the cholesterol synthesis were upregulated, as observed in plant stanol mice (Study I) and plant sterol females (Study II). One explanation for this could be that the genes of cholesterol biosynthesis were upregulated due to mitogenic signaling instead of a compensatory mechanism to regulate cellular cholesterol balance. In proliferating cells, cholesterol synthesis appears to be up-regulated by nuclear SREBP-1 after activation of protein kinase B (PKB/Akt) (Porstmann et al. 2005). Like SREBP-2, SREBP-1 is a transcription factor that regulates the expression of enzymes involved in lipid and cholesterol biosynthesis (Osborne 2000). The up-regulated cholesterol biosynthesis in plant stanol mice (Study I) and plant sterol females (Study II) may therefore be a secondary response due to proliferative signaling that does not involve SREBP-2. In addition, the synthesis of SREBP-2 precursor may be under regulation, too (Kim, Takahashi, and Ezaki 1999, Field et al. 2001). Harding and co-workers (Harding et al. 2010) demonstrated that plant sterol feeding to hamsters increased concentrations of hepatic plant sterols and reduced the hepatic free cholesterol concentration. Since hepatic cholesterol synthesis and inactive SREBP-2 were increased without an increase in active nuclear SREBP-2, they speculated that plant sterols downregulated the conversion of SREBP-2 into its active form. Similarly, it is possible, that conversion of the SREBP-2 precursor into its mature form was affected after phytosterol feeding although genes of the cholesterol synthesis were upregulated.

Upregulated cholesterol biosynthesis and increased production of cholesterol intermediates, such as mevalonate and isoprenoids, has been associated with increased cell proliferation (Singh et al. 2003, Dimitroulakos et al. 2006), and inhibitors of the mevalonate pathway may act as antitumor agents (Thurnher et al. 2012). Patients with hypercholesterolemia are often treated with statins that inhibit the HMGCR activity. A meta-analysis of twenty case-control studies found a significant association between statin usage and reduced risk of colon cancer (Taylor

et al. 2008). Later, a meta-analysis combining both clinical trials and observational studies concluded that statin usage was associated with only a modest reduction in CRC (Bardou et al. 2010), however the duration of clinical trials was too short to assess CRC incidence. Moreover, atorvastatin (Swamy et al. 2006) and pitavastatin (Teraoka et al. 2011) reduced intestinal polyp formation in the *ApcMin* mouse.

Statins inhibit the conversion of HMG-CoA to mevalonate and reduce the synthesis of the downstream products, such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GPP). A higher FPP synthase activity is found in human colorectal cancer samples when compared to normal surrounding mucosa and it is shown to regulate cell proliferation (Notarnicola et al. 2004). In this thesis, the expression of enzymes producing FPP and GPP was upregulated in the intestinal mucosa of plant stanol fed mice (Study I). FPP and GPP are post-translational modifiers of several proteins including Ras, Rho, Rab and lamin B (Dimitroulakos et al. 2006, Guruswamy and Rao 2008). These intermediates of the sterol biosynthesis could modify and activate Ras, which in turn activates EGFR and its downstream effectors (Dimitroulakos et al. 2006).

Whether increased *Egfr* activity in plant stanol fed *ApcMin* mice (Study I) was a result of an increased synthesis of cholesterol and modifiers that activate Ras, is not certain. More research is needed to address the effect of plant stanols in cancer. So far, mechanistical studies on the effect of plant stanols using cancer cells are lacking.

Effects of phytosterols on cell signaling pathways in *ApcMin* mice

This thesis demonstrates that plant stanol feeding (Study I) upregulated Wnt and *Egfr* signaling, which was not seen after plant sterol feeding (Study II). It is possible that the small number of animals in Study II may not allow detecting differences in signaling proteins between the plant sterol and the control group. However, it is very probable that plant sterols and plant stanols affect cell signaling differentially within enterocytes. In fact, plant sterol and plant stanol diets changed mucosal sterol composition in completely different manner as discussed earlier. Therefore, it can be assumed that although very similar in their molecular structure and LDL-C lowering

efficacy, plant sterols and stanols may diverge in other properties. Even specific plant sterols, e.g. sitosterol and stigmasterol, differ in their physiological responses (Awad et al. 2003a, Yang et al. 2004, Sabeva et al. 2011).

After all, plant sterols seem to affect other cellular targets in the *ApcMin* mouse than plant stanols do. Plant sterols could contribute to changes in signal transduction by replacing cholesterol from the plasma membrane (Mora et al. 1999, Ratnayake et al. 2000, Awad et al. 2007). Plant sterols have been found to upregulate the gene expression of caveolin-1 (Ifere et al. 2010), which is a cholesterol binding protein that forms lipid rafts called caveolae at the plasma membrane. Caveolae function as platforms for proteins and receptors which are regulated by the cholesterol content of the plasma membrane. Caveolin-1 is considered to act as a tumor suppressor by inhibiting the β -catenin mediated transcription of genes (Galbiati et al. 2000), and by down-regulating the activation of Egfr signaling (Feldman and Martinez 2009, Han et al. 2009). In Study II, where plant sterol was fed to mice, low caveolin-1 levels when combined with increased concentrations of mucosal free cholesterol and esterified sitosterol, and ER β content, was associated with increased number of intestinal adenomas.

The lasso regression analysis (Study II) proposed a higher ER β level to be among the predictors of increased adenoma number. Plant sterols themselves bind to ER β with low affinity, although their estrogenic effect seems to be weaker than estrogens (Gutendorf and Westendorf 2001). In the colonic epithelium, ER β is the predominant estrogen receptor (Foley et al. 2000, Campbell-Thompson et al. 2001). Plant sterols may disturb estrogen metabolism by competing of binding to estrogen receptors α and β with estrogens (Newill et al. 2007).

Plant sterols have been reported to accumulate in organs that are involved in steroid biosynthesis such as the adrenal gland and ovaries (Sanders et al. 2000). Plant sterols could disturb the synthesis of female sex hormones, and plant sterol supplementation has been reported to reduce serum progesterone and estrogen levels in humans and in animals (Ayesh et al. 1999, Nieminen et al. 2003, Ju et al. 2004). Epidemiological and clinical studies suggest that estrogens and progestin, a synthetic progestogen, could protect against colon cancer development (Grodstein et

al. 1999, Rossouw et al. 2002, Lin et al. 2012). *ApcMin* females have lower progesterone levels compared to wildtype female mice, suggesting that sex hormone synthesis is disturbed in *ApcMin* females at the baseline (Cleveland et al. 2009). It could be that plant sterols further disturbed estrogen and/or progesterone signaling in the small intestine or reduced hormone synthesis in ovaries of *ApcMin* females.

ER β deficiency is often associated with increased intestinal tumorigenesis, (Cho et al. 2007, Giroux et al. 2008); however, the positive correlation between tumor number and ER β level among females (Study II) could be explained by the findings reported also for ovariectomized mice (Weyant et al. 2001). Weyant and coworkers (Weyant et al. 2001) saw that the number of intestinal tumors was increased in ovariectomized *ApcMin* mice, and this loss of endogenous estrogen production upregulated the expression of ER β (Weyant et al. 2001). If plant sterol feeding disturbed estrogen signaling, a compensatory mechanism due to reduced estrogen signaling could explain the non-significant increase in ER β level in plant sterol females.

Plant stanols, on the other hand, are absorbed from the intestine less efficiently than plant sterols, and their circulating levels are low. The accumulation of plant stanols in tissues such as ovaries is therefore low or even non-existent, and it is unlikely that plant stanols affect the hormone synthesis in the ovaries. This may in part define the different mechanisms behind plant sterol and plant stanol induced tumorigenesis in *ApcMin* mice, and why genders were affected in a different manner in the feeding studies.

Other plausible mechanisms behind intestinal tumorigenesis in *ApcMin* mice after phytosterol feeding

It has been suggested that in addition to targeting plasma membrane, phytosterols could direct at mitochondria (Rubis et al. 2008, Danesi et al. 2011, Lizard 2011). *In vitro* experiments have consistently shown that plant sterols induce apoptosis by activating pro-apoptotic proteins (von Holtz et al. 1998, Awad et al. 2003a, Choi et al. 2003). Since mitochondria are essential to energy metabolism and in the regulation of apoptosis, the indication that phytosterols could alter the function of

these cell organs maybe important, especially in carcinogenesis. As described already in the literature review, the energy metabolism is altered in cancer cells. The fact that phytosterols could affect mitochondria raises a concern if they could disturb energy metabolism in cells by affecting mitochondrial function. As a matter of fact, a mixture of phytosterols was found to reduce the metabolic activity of non-neoplastic rat cardiomyocytes when using an assay dependent on mitochondrial reductase activity (Danesi et al. 2011). Similarly, the metabolic rate and cell growth was reduced in normal human endothelial cells treated with β -sitosterol *in vitro* (Rubis et al. 2008). Sitosterol has been found to inhibit the mitochondrial CYP27 enzyme that converts cholesterol to 27-OH-cholesterol, an oxysterol ligand that activates LXR (Brauner et al. 2012).

The oxidation products of phytosterols could be potentially toxic to cells (Adcox et al. 2001, Newill et al. 2007). The epoxy- β -sitosterol was found less cytotoxic than its parent compound, β -sitosterol, when delivered to cells as a readily-formed oxidized phytosterol (Newill et al. 2007). If phytosterols were oxidized within cells, their oxides could be more harmful than food-derived oxy-phytosterols since they could generate free radical reactive oxygen species inside the cells. However, phytosterol oxides are produced mainly during storage rather than metabolically *in vivo* (Newill et al. 2007).

Intake of phytosterols from enriched food products among Finns

Phytosterol intakes from natural sources and enriched foods were evaluated in the Finnish population based on the national FINDIET 2007 Survey (Study III). The intake of phytosterols from natural sources was 363 mg/d for men and 286 mg/d for women and from enrichment 2.2 g/d for men and 1.6 g/d for women.

Currently, producers of phytosterol enriched foods are advising the customer to consume 2 g/d of plant sterols or stanols in order to reach the beneficial effect on serum LDL cholesterol. The majority of Finnish men and women who used phytosterol enriched food items did not reach the 2 g advised daily intake of phytosterols. On the contrary, 20% of people that consumed enriched foods had

mean intakes above the recommended 3 grams. Although the intake values may have some measurement error due to the 48 h dietary recall method, similar intake levels for phytosterol from enrichment have been reported for Belgian (Sioen et al. 2011) and Irish population (Hearty et al. 2009). In the Irish study, 23% of people had intake greater than 3 g/d and 58% of users had consumed phytosterol enriched foods more than one year (Hearty et al. 2009).

The use of phytosterol enriched products is common among those with increased risk for CHD. In the national FINRISK 2002 Study almost 80% of those who used phytosterol enriched foods reported having high or elevated blood cholesterol level (de Jong et al. 2004). In contrast, around 20% of Finnish users of enriched foods were not aware of their blood cholesterol level (de Jong et al. 2004). Similar results were reported for Irish population consuming phytosterol enriched products and over 35% of users had no diagnosis of a medical condition (Hearty et al. 2009). Over half of those using enriched products reported advertising to have introduced enriched products in their diet whereas a doctor or a dietitian had introduced enriched products to less than 20% of subjects (Hearty et al. 2009). If the decision to use enriched products is based on advertising the information on the use of these products is mainly based on product labels.

The European Commission requires the producers to label plant sterol and plant stanol enriched products with necessary information concerning the target group and intake levels of plant sterols and stanols from enrichment (Regulation EC No 608/2004). According to post-launch monitoring studies it seems that the label information does not reach the customer. In a study conducted in Belgium, 21% of pre-school children aged between 2.5 and 7 years consumed phytosterol enriched foods, and the mean plant sterol intake was 0.7 g/d and the highest intake 2.1 g/d (Sioen et al. 2011). Phytosterol enriched products are not recommended for children under 5 years of age (Regulation EC No 608/2004); however children living in the same household with parents who consume phytosterol enriched bread spreads are more likely to use the same enriched spreads.

The long-term effects of phytosterols from enrichment at high intake have not been studied in humans. So far, only one epidemiological study on phytosterol intake and

CRC has been published concluding that a high intake of phytosterols did not reduce CRC risk (Normén et al. 2001). In fact, there were positive associations between the intake of β -sitosterol and risk of distal colon cancer and the intake of campesterol and stigmasterol and risk of rectal cancer for men. In that study, the dietary phytosterols originated from natural sources and the intake of plant sterols even in the highest percentile was few hundred milligrams (Normén et al. 2001). When the intake of phytosterols from natural dietary sources is high, intakes of other bioactive dietary factors, such as vitamins, minerals, antioxidants and dietary fibre, is high, too. In contrast, when phytosterols originate from enriched sources, a high intake of other health promoting compounds is not guaranteed.

The accumulation of phytosterols in serum of sitosterolemic subjects with a mutation in ABCG5/8 transporters has raised the question whether phytosterols could be potentially atherogenic (Sudhop and von Bergmann 2004). In animals without a genetic defect in sterol transporters, plant sterols have been observed to accumulate in several organs and tissues (Sanders et al. 2000, Vanmierlo et al. 2012). In humans, the customary (long-term) use of plant sterol and plant stanol enriched margarine was shown to increase serum phytosterol concentrations (Fransen et al. 2007). The impact of increased serum phytosterol concentrations and accumulation of phytosterols in human tissues is yet to be defined.

SUMMARY AND CONCLUSIONS

Phytosterols are added to functional foods for their blood LDL lowering effect. The customary use of phytosterol enriched foods appears to be a cost-effective and easy way to treat people with high or elevated serum cholesterol levels. Whereas the daily intake of phytosterols from natural sources varies between 150-400 mg (Katan et al. 2003), the intake can reach several grams when enriched foods are consumed (Hearty et al. 2009, Sioen et al. 2011). The long-term effects of phytosterols at high intakes have not been thoroughly studied in humans.

The effects of plant sterols and plant stanols were examined in the *ApcMin* mouse, which is a widely used model to study the impact of diet on intestinal tumor development. This thesis shows that plant sterols and stanols regulate several signaling pathways in the small intestine of *ApcMin* mice. Plant stanol feeding increased the number of intestinal tumors, affected sterol metabolism in the mucosa by increasing plant stanol concentrations and decreasing plant sterol concentrations, upregulated genes of the mevalonate (cholesterol synthesis) pathway and increased Wnt and Egfr signaling in the *ApcMin* mouse. On the other hand, plant sterol feeding increased the number of intestinal tumors more pronouncedly in female mice, affected sterol metabolism in the mucosa by decreasing cholesterol concentration in male mice, and increasing plant sterol and stanol concentrations in the intestinal mucosa in both genders, and upregulated genes of the mevalonate pathway in female *ApcMin* mice.

Prior to this thesis no studies on plant stanols and intestinal/ colon carcinogenesis have been reported. In addition, phytosterols as such have not been studied using the *ApcMin* mouse. The mutation in the human *APC* is involved in more than half of spontaneous colon tumors, and the rate of *APC* mutations in the colon increases later in life (Luebeck and Moolgavkar 2002). At the same period of life the use of phytosterol enriched products often begins. Therefore the *ApcMin* mouse represents a feasible model to study the effects of phytosterols on intestinal tumor formation. Although phytosterols are poorly absorbed and their circulating levels are low, it is now evident that these compounds are taken up by enterocytes where they regulate

cell signaling and gene expression. Several hypotheses can be made on the increased initiation of tumorigenesis after phytosterol feeding. However, based on the data presented in this thesis, conclusions on the unequivocal mechanism behind the accelerated initiation cannot be made.

Phytosterol enriched food products are targeted to the customer for treating serum LDL-C levels, but instead of being considered as pharmaceuticals these products are termed as functional foods. Although the European Commission requires the producers to label plant sterol and plant stanol enriched products with necessary information concerning the target group and intake levels of plant sterols and stanols from enrichment (Regulation EC No 608/2004), it appears that consumers follow the advises poorly. Among the users of phytosterol enriched products, there is a subgroup of consumers whose phytosterol intake from enrichment clearly exceeds the advised intake of 2 g/d stated on the product label. On the other hand, the majority of people using phytosterol enriched foods do not achieve the advised intake. Since phytosterol enriched food products are freely available on the market, consumer guidance should be developed until more research has been conducted on the long-term effects of phytosterols at high intake levels.

The intake of plant sterols and plant stanols in the feeding studies with mice was equivalent to approximately 5 g/d in men when adjusted for energy consumption. As confirmed by this thesis, high intake levels are easily achieved by humans when enriched food items are consumed. The long-term effects of phytosterol consumption at high intake have not been evaluated in humans.

This thesis shows that phytosterols clearly influence sterol metabolism in the intestine, and accelerate the formation of intestinal tumors in a tumor-prone mouse model. In addition, plausible mechanisms behind the enhanced tumor development are demonstrated. The present results warrant for future research aimed to investigate the effects of phytosterols on intestinal health in human.

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