

Hypocholesterolaemic effects of plant sterol analogues are independent of ABCG5 and ABCG8 transporter expressions in hamsters

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The hypolipidaemic effects of plant sterols are well established. However, mechanisms by which plant sterols lower plasma cholesterol levels, particularly at the molecular level, have not been clearly elucidated. The objective of the present study was to determine whether different plant sterol analogues reduce plasma cholesterol levels by up regulating the sterol transporters ABCG5 and ABCG8 in the liver and/or small intestine. Male Golden Syrian hamsters were divided into eight groups. Groups 1 and 2 were fed a maize starch–casein–sucrose-based diet that did not contain cholesterol (control; Con) or the Con diet with the addition of 0.25 % cholesterol (Ch-Con). Groups 3–8 were fed the Ch-Con diet supplemented with 1 % plant sterols, 1 % plant stanols, 1 % of a plant sterol and stanol mixture (50:50), 1.76 % plant sterol–fish oil esters, or 0.71 or 1.43 % stanol–ascorbic acid esters, respectively. After 5 weeks, the Ch-Con diet up regulated the ABCG5 mRNA expression and tended ($P=0.083$) to increase ABCG8 mRNA expression in the liver, but did not affect both genes' expression in the small intestine compared with the Con diet. Hamsters fed 0.7 % stanol esters showed lower plasma cholesterol levels ($P<0.001$) and also lower liver ABCG5 mRNA expression ($P<0.05$) compared with the Ch-Con diet. Plant stanols, stanol esters, and sterol esters did not affect the ABCG5 or ABCG8 mRNA expressions in the liver and intestine although they reduced plasma cholesterol levels. These results suggest that plant sterols and their derivatives reduce plasma cholesterol levels independently from the mRNA expression of ABCG5 and ABCG8 transporters.

Cholesterol: Plant sterols: ABCG5: ABCG8: Hamsters

It has been well documented that dietary supplementation with plant sterols and stanols reduces plasma cholesterol levels in human subjects and animals^{1–9}. Plant sterols and stanols are not water soluble and possess low solubility in fats. It is believed that solubility characteristics affect the bioavailability and cholesterol-lowering efficacy of sterols and stanols. Thus, a large body of research has been conducted attempting to modify the structure of plant sterols and stanols, and the esterification to fatty acids^{10,11} or ascorbic acid has been extensively studied in the past years^{12–14}. Accordingly, several different analogues of plant sterols and stanols are currently available as functional food ingredients or nutraceuticals.

The interference with cholesterol incorporation into micelles has long been thought to be the possible mechanism by which plant sterols and stanols inhibit cholesterol absorption^{15,16}. However, this mechanism cannot explain the observation that plasma cholesterol levels were significantly

decreased in hamsters after plant sterols were injected intraperitoneally¹⁷. Recent advances in molecular research have indicated that the sterol transporter-mediated cholesterol efflux in the enterocytes and cholesterol secretion in the liver may play important roles in cholesterol absorption and metabolism^{18–22}. It has been found that in sitosterolaemic subjects, cholesterol transporters ABCG5 and ABCG8 are mutated, resulting in sterol secretion disorders in the liver and enterocytes^{18–20}. The role of ABCG5 and ABCG8 transporters in cholesterol efflux has further been demonstrated in transgenic mice by expressing both human and mouse ABCG5 and ABCG8 genes. Results showed approximately 50 % reductions in cholesterol absorption and marked increases in biliary cholesterol secretion and faecal neutral sterol excretion compared with their wild-type littermates²². In contrast, the ABCG5^{-/-} and ABCG8^{-/-} mice presented increased absorption of dietary sterols and impaired biliary

Abbreviations: Ch-Con, control diet with 0.25 % cholesterol; Con, control diet; Sta, Ch-Con diet with 1 % plant stanols; Ste, Ch-Con diet with 1 % plant sterols; SteF, sterols esterified to fish oil; StaA, stanols esterified to ascorbic acid.

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sterol excretion and had plasma sitosterol levels 30-fold higher than their wild-type littermates²¹.

Taken together, these data suggest that ABCG5 and ABCG8 transporters regulate cholesterol efflux out of the enterocytes and cholesterol secretion in the liver. The few studies conducted to date have failed to demonstrate any action of plant sterols or stanols on expression of ABCG5 and ABCG8 in the intestine^{23–25}. The effect of plant sterols or stanols on the expression of these two genes in the liver has rarely been studied. It still remains unclear about the molecular mechanisms of action of different plant sterol analogues on cholesterol metabolism. Therefore, the present study was undertaken to determine whether the cholesterol-lowering effects of different plant sterol analogues are due to ABCG5- and ABCG8-mediated alterations in cholesterol secretion from the liver and cholesterol efflux from the enterocytes in hamsters. Because hamster lipid composition and biochemistry more closely resemble those of man than those of other rodents, the hamster has been considered as a good model for studying human cholesterol metabolism^{26,27}.

Materials and methods

Animals and diets

Eighty male Golden Syrian hamsters (Charles River Laboratories, Montreal, QC, Canada), weight 100–120 g, were housed individually in stainless-steel mesh cages with a 12 h light–dark cycle. Animals were fed regular rodent chow with free access to water. After 2 weeks of adaptation, hamsters were randomly divided into eight groups and fed experimental diets for 5 weeks. Group 1 was given a semi-purified maize starch–casein–sucrose diet with no cholesterol added (control; Con). Group 2 was given the Con diet with the addition of 0.25% cholesterol (Ch-Con). Groups 3 to 8 were given the Ch-Con diet with either 1% plant sterols (Ste),

1% plant stanols (Sta), 1% Ste and Sta mixture (50:50), 1.76% sterols esterified to fish oil (SteF), or 0.71 or 1.43% stanols esterified to ascorbic acid (0.7% StaA or 1.4% StaA), respectively. All diets contained 5% fat provided in the form of a mixture of beef tallow and safflower-seed oil to yield a PUFA:SFA ratio of 0.4. Composition of the diets is shown in Table 1. Diet SteF had an equivalent amount of plant sterols as the Ste diet. Diets 0.7% StaA and 1.4% StaA provided an equivalence of 0.5% and 1% free plant stanols, respectively. Plant sterol analogues were mixed into the oil at 55°C before blending into diets. Diets were prepared every 2 weeks and stored at –20°C.

After 5 weeks on the experimental diets, hamsters were anaesthetized with halothane and killed by decapitation. Blood was collected into tubes containing EDTA and placed on ice. Plasma was separated by centrifugation and stored at –80°C until analysis. Liver and proximal small intestine were quickly removed, frozen in liquid N₂, and stored at –80°C for mRNA measurement. The experiment was reviewed and approved by the Animal Care and Research Ethics Committee of McGill University and was conducted in accordance with the guidelines of the Canadian Council on Animal Care.

Plasma lipid analysis

Plasma total cholesterol, HDL-cholesterol concentrations were measured in duplicate by enzymic methods (Roche Diagnostics, Laval, QC, Canada). HDL-cholesterol was measured after precipitation of apoB containing lipoproteins with dextran sulfate and magnesium chloride²⁸. Because the Friedewald equation²⁹ may not be applicable in hamsters, non-HDL-cholesterol (VLDL- + intermediate-density lipoprotein- + LDL-cholesterol) instead of LDL-cholesterol was used and calculated by subtracting HDL-cholesterol from total cholesterol.

Table 1. Composition of the experimental diets (% w/w)*

Diet...	Con	Ch-Con	Ste	Mix	Sta	SteF	0.7% StaA	1.4% StaA
Casein	20.0	20.0	19.8	19.8	19.8	19.6	19.8	19.7
Maize starch	28.0	28.0	27.7	27.7	27.7	27.5	27.7	27.6
Sucrose	36.3	36.0	35.6	35.6	35.6	35.3	35.6	35.5
Beef tallow and safflower-seed oil†	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Cellulose	5.0	5.0	4.9	4.9	4.9	4.9	4.9	4.9
D,L-Methionine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Mineral mixture	4	4	4	4	4	4	4	4
Vitamin mixture	1	1	1	1	1	1	1	1
Choline bitartrate	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Butylated hydroxytoluene	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Cholesterol	–	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Plant sterols‡	–	–	1.0	–	–	–	–	–
Mixture (Ste and Sta)‡	–	–	–	1.0	–	–	–	–
Plant stanols‡	–	–	–	–	1.0	–	–	–
SteF‡	–	–	–	–	–	1.76*	–	–
StaA‡	–	–	–	–	–	–	0.71*	1.43*

Con, control diet without cholesterol; Ch-Con, control diet with addition of 0.25% cholesterol; Ste, Ch-Con diet with 1% plant sterols; Mix, Ch-Con diet with 0.5% plant sterols and 0.5% plant stanols; Sta, Ch-Con diet with 1% plant stanols; SteF, Ch-Con diet with 1.76% plant sterols esterified to fish oil; 0.7% StaA, Ch-Con diet with 0.7% plant stanols esterified to ascorbic acid, 1.4% StaA, Ch-Con diet with 1.4% plant stanols esterified to ascorbic acid.

* All diet ingredients except safflower-seed oil were purchased from ICN Biomedicals (Aurora, OH, USA) and diets were prepared in house.

† Safflower-seed oil was purchased from a local supermarket in Montreal, QC, Canada.

‡ Plant sterols and their different analogues were provided by Forbes Medi-Tech Inc., Vancouver, BC, Canada.

Hamster ABCG5 and ABCG8 sequences

Because there was no genomic DNA or cDNA sequence available for hamster ABCG5 and ABCG8, we obtained a part of cDNA sequence of both genes using degenerated primers that were designed based on rat, mouse and human cDNA sequences. Briefly, after alignment of rat, mouse and human cDNA sequences for each gene, the most conservative sequence regions were identified by the MacVector software. Then the degenerated primers were designed within the conservative regions using the Oligo software. The target cDNA templates from one hamster were amplified with the degenerated primers by PCR (Perkin Elmer Applied Biosystems, Wellesley, USA). After separation from the templates by electrophoresis on acrylamide gel, the PCR products were purified and cloned using plasmid pCR4-TOPO (Invitrogen, Ontario, Canada). Recombinant plasmids were purified using a plasmid mini kit (Qiagen, Ontario, Canada). The target cDNA was amplified by PCR and sequenced by the Genome Quebec Innovation Centre at McGill University (Montreal, Canada). The cDNA sequences of hamster ABCG5 and ABCG8 showed over 80% homology compared with the corresponding regions of the rat and mouse sequences and were used to design primers to measure ABCG5 and ABCG8 mRNA expression by quantitative real-time PCR.

Measurement of messenger ribonucleic acid expression of ABCG5 and ABCG8

Total RNA was extracted and purified from the liver and proximal small intestine using the RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada). A sample (2 µg) of RNA was transcribed to cDNA using the Ominiscript RT kit (Qiagen). Real-time PCR was performed on a LightCycler (Roche, Switzerland) using the QuantiTect SYBR Green PCR kit (Qiagen). Primer sequences were 5'-GGA AGG GGA GGT GTT TGT-3' and 5'-GCC AGC ATC GCC GTG TAG-3' for ABCG5, 5'-CAT CAT TGG CTT CCT TTA-3' and CCG CTC CGA GTG ACA TTT-3' for ABCG8, and 5'-AGT ATT CCC CAT CGT-3' and 5'-TCA TCC CAG TTC GTC ACA-3' for β-actin. The real-time PCR reaction solution consisted of 10 µl QuantiTect SYBR Green PCR mixture containing 2.5 mM-MgCl₂, 1 µl forward primer (0.5 µM), 1 µl reverse primer (0.5 µM), and 6 µl RNase-free water. LightCycler glass capillaries were filled with 18 µl of the real-time PCR reaction solution and 2 µl cDNA templates. The capillaries were closed, centrifuged and placed into the LightCycler rotor. The real-time PCR was optimized to have initial activation at 95°C for 15 min, denaturation at 94°C for 15 s, annealing at 55°C for 20 s, and extension at 72°C for 15 s with a single fluorescence measurement and up to forty cycles. The specificity of primers was validated by acrylamide gel electrophoresis and real-time PCR to ascertain there were no significant non-specific products and primer dimers formed before the sample analysis. Data were normalized against an endogenous reference gene, β-actin. Purified plasmid, diluted to certain concentration, was used as an external calibrator across all the measurements.

Data analysis and statistics

Data from plasma lipids as well as the liver and intestine ABCG5 and ABCG8 mRNA were analysed by one-way

ANOVA. The gene expression data were logarithmically transformed before analysis. When a significant ANOVA result was obtained, the least-squares means test was used to test differences between treatments. Relationships between plasma total cholesterol concentrations and ABCG5 or ABCG8 mRNA levels were analysed using Pearson's correlation coefficients. All statistical analyses were conducted using the general linear model procedure of SAS software (SAS Institute Inc., Cary, NC, USA). Significance level was set at $P < 0.05$. Data are presented as mean values with their standard errors.

Results

Body weight and food consumption

The dietary supplementation of cholesterol and different phytosterol analogues did not affect body weights of hamsters as compared with the Con diet. Similarly, there were no changes in the average or total food intakes during the last 2 weeks.

Lipid profiles

The effects of experimental diets on hamster plasma lipid profiles are shown in Table 2. The inclusion of 0.25% cholesterol in the diet increased ($P < 0.0001$) plasma total cholesterol level by 58.4% as compared with the Con diet. When animals were supplemented with 0.7 or 1.4% stanol-ascorbic acid esters, their plasma total cholesterol levels were reduced ($P < 0.0001$) by 34.0 and 45.7%, respectively, compared with those fed the Ch-Con diet. The addition of stanols or sterol-fish oil esters to the Ch-Con diet lowered ($P < 0.05$) total cholesterol levels by 13.7 and 19.7%, respectively. Sterols and the mixture of sterols and stanols did not have a significant effect on plasma total cholesterol levels. Plasma non-HDL-cholesterol concentrations were 16.6, 39.2 and 54.2% lower ($P < 0.05$) in hamsters supplemented with sterol-fish oil esters, 0.7 and 1.4% stanol-ascorbic acid

Table 2. Effects of phytosterol analogues on lipid profiles in hamsters* (Mean values with their standard errors)

Diet	Total cholesterol (mmol/l)		Non-HDL-cholesterol (mmol/l)†		HDL-cholesterol (mmol/l)	
	Mean	SEM	Mean	SEM	Mean	SEM
Con	4.01 ^d	0.11	2.18 ^c	0.10	1.83 ^{c,d}	0.12
Ch-Con	6.35 ^a	0.27	3.67 ^{a,b}	0.33	2.68 ^a	0.15
Ste	6.68 ^a	0.26	4.30 ^a	0.24	2.38 ^{a,b}	0.09
Mix	5.96 ^{a,b}	0.47	3.84 ^{a,b}	0.22	2.12 ^{b,c,d}	0.15
Sta	5.48 ^{b,c}	0.26	3.32 ^{a,b}	0.25	2.16 ^{b,c}	0.15
SteF	5.10 ^c	0.17	3.06 ^c	0.12	2.04 ^{b,c,d}	0.09
0.7% StaA	4.19 ^d	0.28	2.23 ^c	0.22	1.96 ^{c,d}	0.13
1.4% StaA	3.45 ^d	0.12	1.68 ^c	0.06	1.77 ^d	0.11

Con, control diet without cholesterol; Ch-Con, control diet with addition of 0.25% cholesterol; Ste, Ch-Con diet with 1% plant sterols; Mix, Ch-Con diet with 0.5% plant sterols and 0.5% plant stanols; Sta, Ch-Con diet with 1% plant stanols; SteF, Ch-Con diet with 1.76% plant sterols esterified to fish oil; 0.7% StaA, Ch-Con diet with 0.7% plant stanols esterified to ascorbic acid, 1.4% StaA, Ch-Con diet with 1.4% plant stanols esterified to ascorbic acid.

^{a,b,c,d} Mean values within a column with unlike superscript letters are significantly different ($P < 0.05$; pairwise comparisons among the eight groups for each lipid parameter).

* For composition of the experimental diets, see Table 1.

† VLDL- + intermediate-density lipoprotein- + LDL-cholesterol.

esters while not affected by sterols, stanols, and the sterol–stanol mixture, respectively, relative to hamsters fed the Ch-Con diet. Plasma HDL-cholesterol levels were lower ($P < 0.05$) in hamsters supplemented with sterol–stanol mixture, stanols, sterol–fish oil esters, and the two doses of stanol–ascorbic acid esters relative to animals given the Ch-Con diet.

Gene expression of ABCG5 and ABCG8

The inclusion of cholesterol in the diet increased ($P < 0.05$) plasma cholesterol levels. The Ch-Con diet also increased the ABCG5 mRNA and tended ($P = 0.083$) to increase ABCG8 mRNA expression in the liver compared with the Con diet (Fig. 1). Plasma total cholesterol levels were positively correlated with liver ABCG5 ($r = 0.36$; $P = 0.0018$) and ABCG8 ($r = 0.29$; $P = 0.011$) mRNA levels. The addition of 0.7% stanol–ascorbic acid esters to the Ch-Con diet reduced ($P < 0.05$) plasma cholesterol levels and also the expression of ABCG5 levels as compared with the Ch-Con diet. The other sterol analogues appeared to lower the expression of ABCG5 and ABCG8 mRNA in the liver, but these reductions were not significant as compared with the Ch-Con diet (Fig. 1). No differences were observed in both ABCG5 and ABCG8 mRNA levels in the proximal small intestine among all the treatments (Fig. 2).

Discussion

The present study demonstrated that different phytosterol analogues possess various cholesterol-lowering efficacies, with the stanol–ascorbic acid esters showing the strongest effect, followed by sterol–fish oil esters and stanols. The supplementation of plant sterols and the mixture of sterols and stanols did not result in significant changes in plasma cholesterol levels. Plant sterols and their analogues did not up regulate the expression of ABCG5 and ABCG8. In contrast, they reduced or tended to reduce the expression of both genes in the liver. The expression of ABCG5 and ABCG8 in the intestine was

not affected by plant sterols and their different analogues. The addition of cholesterol in the diet significantly up regulated ABCG5 mRNA expression and showed a tendency of increasing the expression of ABCG8 in the liver, while showing no effect on either gene in the small intestine.

Accumulating evidence has demonstrated that plant sterols inhibit cholesterol absorption, and thus lower plasma cholesterol levels^{5,30}. The interference with cholesterol incorporation into micelles in the intestinal lumen is the most widely accepted mechanism by which plant sterols reduce plasma cholesterol levels^{15,16}. Recent advances in molecular research have revealed that the sterol transporters ABCG5 and ABCG8 are critical to cholesterol efflux in the enterocytes and consequently cholesterol absorption. It has been speculated that plant sterols may inhibit cholesterol absorption, at least in part, through up regulating the expression of ABCG5 and ABCG8 in enterocytes. However, data from the present study do not support this hypothesis. Plant sterols and stanols and their esterified forms had no effect on either ABCG5 or ABCG8 mRNA levels in the small intestine, which is in agreement with previous studies in hamsters supplemented with stanol esters²³ or in mice given plant sterols or stanols^{24,25}. The present results demonstrated that different phytosterol analogues possess various cholesterol-lowering efficacies which are independent from the expression of sterol transporters ABCG5 and ABCG8 in the intestine.

In addition to cholesterol absorption in the intestine, the liver plays a central role in cholesterol homeostasis. Cholesterol secretion is one of the mechanisms by which the liver excretes cholesterol. Because the sterol transporters ABCG5 and ABCG8 mediate cholesterol secretion into bile^{21,22,31}, plant sterols have been thought to up regulate the expression of these two genes in the liver and thus cholesterol excretion, thereby lowering plasma cholesterol concentrations. Paradoxically, the present study showed that stanol esters (0.7% in the diet) decreased liver ABCG5 mRNA levels and appeared to lower ABCG8 mRNA expression. The insignificant effect of the other phytosterol analogues on ABCG5 and ABCG8

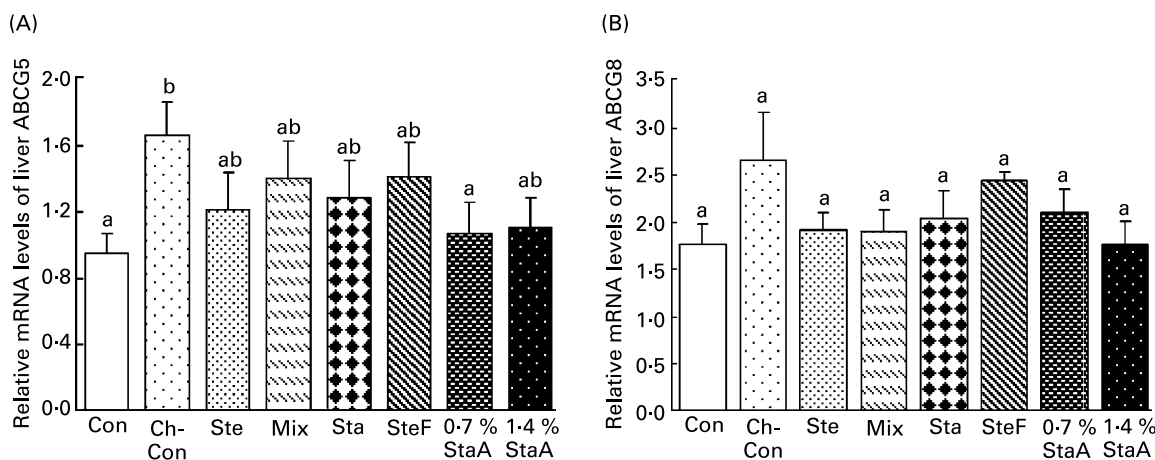


Fig. 1. Effects of cholesterol and different phytosterol analogues on sterol transporters ABCG5 (A) and ABCG8 (B) mRNA expressions in the liver. Con, control diet without cholesterol; Ch-Con, control diet with addition of 0.25% cholesterol; Ste, Ch-Con diet with 1% plant sterols; Mix, Ch-Con diet with 0.5% plant sterols and 0.5% plant stanols; Sta, Ch-Con diet with 1% plant stanols; SteF, Ch-Con diet with 1.76% plant sterols esterified to fish oil, 0.7% StaA, Ch-Con diet with 0.7% plant stanols esterified to ascorbic acid, 1.4% StaA, Ch-Con diet with 1.4% plant stanols esterified to ascorbic acid. Values are means ($n = 10$), with their standard errors represented by vertical bars. Values were normalized against endogenous internal reference β -actin and an external calibrator for every run. Data were logarithmically transformed before the statistical analysis. ^{a,b} Mean values with unlike letters were significantly different ($P < 0.05$).

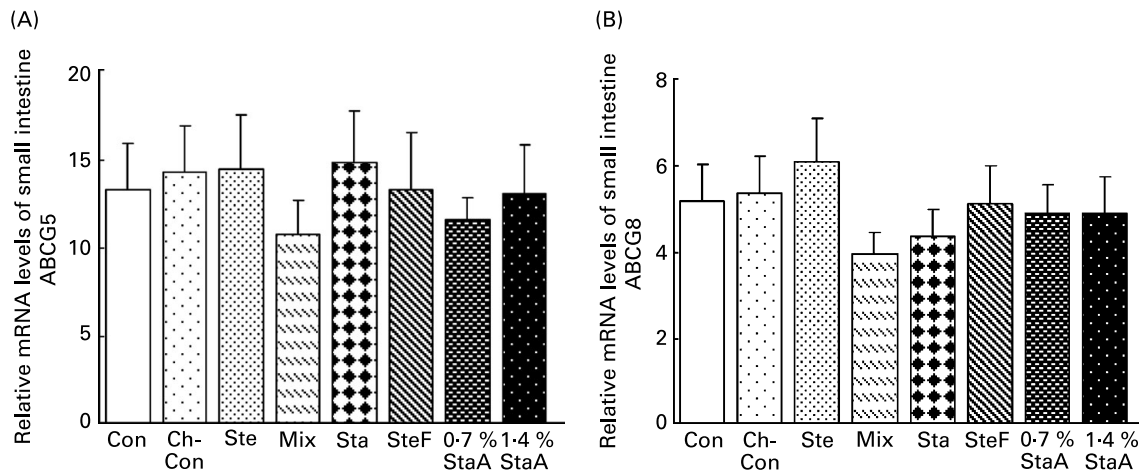


Fig. 2. Effects of cholesterol and different phytosterol analogues on ABCG5 (A) and ABCG8 (B) mRNA expressions in the proximal small intestine. Con, control diet without cholesterol; Ch-Con, control diet with addition of 0.25% cholesterol; Ste, Ch-Con diet with 1% plant sterols; Mix, Ch-Con diet with 0.5% plant sterols and 0.5% plant stanols; Sta, Ch-Con diet with 1% plant stanols; SteF, Ch-Con diet with 1.76% plant sterols esterified to fish oil, 0.7% StaA, Ch-Con diet with 0.7% plant stanols esterified to ascorbic acid, 1.4% StaA, Ch-Con diet with 1.4% plant stanols esterified to ascorbic acid. Values are means (n 10), with their standard errors represented by vertical bars. Values were normalized against endogenous internal reference β -actin and an external calibrator for every run. Data were logarithmically transformed before the statistical analysis.

mRNA expressions in the liver could be due to the large intra-group variations observed. These data suggest that the cholesterol-lowering effect of phytosterol analogues does not rely on ABCG5 and ABCG8 expressions in the liver.

The expression of ABCG5 in the liver was significantly increased by dietary cholesterol and was positively associated with plasma cholesterol levels. This observation is consistent with previous studies in mice demonstrating that ABCG5 and ABCG8 mRNA expressions in the liver were up regulated by dietary cholesterol levels in a dose-dependent manner^{18,32–34}. The effect of dietary cholesterol on the expression of ABCG5 and ABCG8 in the small intestine is controversial. The present study did not show a significant effect of cholesterol on these two genes in the small intestine in hamsters. Some studies in mice have shown that dietary supplementation of cholesterol increases the expression of ABCG5 and ABCG8 in the small intestine^{18,32,33,35} while others did not show any effect^{25,36}. Studies in hamsters²³ and rats³⁴ did not show any effect of dietary cholesterol on ABCG5 and ABCG8 expressions in the small intestine. These discrepancies may be due to differences in cholesterol absorption between different species, as shown in other studies conducted previously^{18,37}. For example, mice are resistant to 2% dietary cholesterol and show little changes in plasma cholesterol levels¹⁸, while hamsters absorb cholesterol more efficiently and quickly develop hypercholesterolaemia when 0.25% cholesterol is supplemented in the diet³⁷. The ABCG5 and ABCG8 sterol transporters in the small intestine may be less responsive to dietary cholesterol levels in hamsters than in mice.

In summary, the hypocholesterolaemic effects of different plant sterol analogues are not mediated by increases in the expression of sterol transporters ABCG5 and ABCG8 in the liver or intestine. Plasma cholesterol levels up regulate the expression of ABCG5 and ABCG8 in the liver, but not in the small intestine. To elucidate the molecular mechanism by which plant sterols and their different analogues lower

plasma cholesterol levels, the effect of plant sterols and their analogues on the expression of other genes involved in cholesterol absorption and metabolism warrants further investigation.

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