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Phytosterol-mediated inhibition of intestinal cholesterol absorption is independent of ATP-binding cassette transporter A1

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An increased activity of ATP-binding cassette transporter (ABC) A1 has been proposed as a mechanism underlying the hypocholesterolaemic effect of phytosterols. In the present study, ABCA1-deficient mice (ABCA1 −/− mice) were used to examine the involvement of the ABCA1 in the reduction of intestinal cholesterol absorption in response to a phytosterol-enriched diet. A decrease in intestinal cholesterol absorption of 39 and 35 % was observed after phytosterol treatment in ABCA1 +/+ mice and in ABCA1 −/− mice, respectively. No statistically significant changes in plasma lipoprotein profile or in intestinal ABCG5, ABCG8 and Niemann-Pick C1-Like 1 gene expression levels were found when phytosterol-treated ABCA1 −/− mice and untreated ABCA1 −/− mice were compared. We conclude that phytosterol inhibition of cholesterol absorption in mice is independent of ABCA1.

Phytosterols: Intestinal cholesterol absorption: ATP-binding cassette transporter A1: Mice

Dietary consumption of phytosterols, or their saturated forms known as stanols, is a recommended therapeutic option to decrease LDL-cholesterol in the most recent guidelines of the National Cholesterol Education Program (Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, 2001). Although it has been clearly demonstrated that phytosterols decrease intestinal cholesterol absorption, the mechanisms involved in this action remain unclear (de Jong *et al.* 2003). One mechanism could be physical competition between phytosterols and cholesterol for incorporation into micelles (de Jong *et al.* 2003). However, phytosterols do not need to be present in the intestinal lumen simultaneously with cholesterol to inhibit its absorption (Plat *et al.* 2000). In recent years, important advances in the understanding of intestinal sterol absorption (Sudhop *et al.* 2005) have provided potential molecular targets of phytosterols. One of these is ATP-binding cassette transporter (ABC) A1 (Brousseau, 2003), especially considering that ABCA1 gene expression increased when Caco-2 cells were incubated with sitostanol added in a micellar solution (Plat & Mensink, 2002). This transcriptional increase, if maintained at protein level, could decrease ABCA1-mediated intestinal cholesterol absorption (Plat & Mensink, 2002; Brousseau, 2003). We have used ABCA1-deficient mice to study whether this protein is needed by phytosterols to decrease intestinal cholesterol absorption.

Materials and methods

Mice and diets

ABCA1 heterozygous mice (ABCA1 +/−) in the DBA/1 background were obtained from Jackson Laboratories (no. 003897; Bar Harbor, ME, USA) and were crossed to produce wild-type ABCA1 +/+ and ABCA1-deficient (ABCA1 −/−) mice (McNeish *et al.* 2000). Genotype of the offspring was confirmed by PCR using the wild-type and the targeted allele-specific primers recommended by Jackson Laboratories (http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh). Sex- and age-matched ABCA1 +/+ and −/− were used in our experiments. Eight-week-old ABCA1 +/+ and −/− mice were randomised into two groups and fed either a control Western-type diet (fat, 200 g/kg; PUFA:saturated fatty acids, 0·07; cholesterol, 0·8 g/kg; protein, 170 g/kg; fibre, 105 g/kg; Mucedola srl, Settimo Milanese, Milan, Italy) or a 2 % phytosterol-enriched Western-type diet (w/w) for 2 weeks. The phytosterol product was composed of 20 % campesterol, 22 % stigmasterol and 41 % β-sitosterol (Lipofoods S.L., Barcelona, Spain) (Calpe-Berdiel *et al.* 2005). All the procedures described were approved by the ethical committee of the Ministry of Agriculture, Livestock and Fishing of the Generalitat de Catalunya.

Net intestinal cholesterol absorption

Net cholesterol absorption was measured in treated and untreated ABCA1 +/+ and −/− mice by a faecal dual-isotope ratio method as previously described (Calpe-Berdiel *et al.* 2005). Briefly, five mice from each group were intragastrically administered a mixture of 2 μCi [5,6-^{3}H]sitostanol (American Radiolabeled Chemicals Inc., St Louis, MO, USA) and 1 μCi [4-^{14}C]cholesterol (NEN Life Science Products, Boston, MA, USA). They were then individually housed and stools were collected over the next 3 d. Lipids were extracted from stools with

Abbreviations: ABC, ATP-binding cassette transporter; NPC1L1, Niemann-Pick C1-Like 1.

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isopropyl alcohol–hexane (2:3, v/v) and the ^{14}C:^{3}H ratio in each sample was determined. The percentage of intestinal cholesterol absorption was calculated from these data (Calpe-Berdiel *et al.* 2005). At the end of the present study, plasma was also taken when mice were euthanised and ^{14}C counts per min determined.

Plasma and liver lipid analyses

The methods used for plasma lipid and liver analyses have been described in detail elsewhere (Escola-Gil *et al.* 2000). Size fractionation of plasma lipoproteins was performed by fast performance liquid chromatography of pooled plasma samples and total cholesterol content on each fraction was then assayed (Escola-Gil *et al.* 2001).

Quantitative real-time reverse transcriptase polymerase chain reaction analyses

The small intestine of four animals in each experimental group was removed, flushed with ice-cold saline solution, and cut into three segments with length ratios of 1:3:2 (duodenum–jejunum–ileum). From the middle of each intestinal segment, 1·5 cm of the duodenal, jejunal, and ileal tissues were cut out and pooled. Small-intestine RNA was isolated using the trizol RNA isolation method (Gibco/BRL, Grand Island, NY, USA). Total RNA samples were repurified, checked for integrity by agarose gel electrophoresis and reverse-transcribed with Oligo$(dT)_{15}$ using M-MLV RT, RNase H Minus, Point Mutant to generate cDNA (Calpe-Berdiel *et al.* 2005). Primer sequences for ABCA1, ABCG5, ABCG8, Niemann-Pick C1-Like 1 protein (NPC1L1) and β-actin have been published elsewhere (Calpe-Berdiel *et al.* 2005). PCR assays were performed on an Applied Biosystems Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) as described (Calpe-Berdiel *et al.* 2005). All analyses were performed in duplicate and relative RNA levels were determined using β-actin as the internal control.

Statistical analysis

Results are expressed as mean values and standard deviations. Two-way ANOVA with Bonferroni post hoc tests was performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA). A value of $P<0·05$ was considered statistically significant.

Results

Plasma lipid analyses

No differences were found between the lipoprotein pattern of control ABCA1 +/+ mice given phytosterols and those not given phytosterols, which showed that cholesterol was predominantly associated with HDL (Table 1 and Fig. 1 (A)). ABCA1 −/− mice exhibited a major increase in non-HDL-cholesterol compared with their ABCA1 +/+ counterparts concomitant with an HDL-cholesterol deficiency (Table 1). No lipid changes were observed between phytosterol-treated and untreated ABCA1 −/− mice (Table 1) and neither did their lipoprotein pattern differ (Fig. 1 (B)).

Net intestinal cholesterol absorption

ABCA1 +/+ mice given phytosterols presented a 39 % reduction in net intestinal cholesterol absorption compared with non-treated littermates (48·5 (SD 12·3) v. 80·0 (SD 12·1)) (Fig. 2). A similar decrease (35 %) in net intestinal cholesterol absorption was induced by phytosterol treatment in ABCA1 −/− mice (58·6 (SD 14·6) v. 90·6 (SD 7·3)) (Fig. 2).

The amount of labelled [^{14}C]cholesterol in plasma, taken 72 h after the isotope mixture dosage, was reduced in phytosterol-treated ABCA1 +/+ animals compared with the untreated ABCA1 +/+ group (13 160 (SD 476) *v.* 22 480 (SD 5807) counts per min; $P<0·05$) and also in phytosterol-treated ABCA1 −/− compared with untreated ABCA1 −/− mice (19 240 (SD 7266) *v.* 50 880 (SD 8394) counts per min; $P<0·05$).

Real-time reverse transcriptase polymerase chain reaction analyses

The relative intestine mRNA levels of ABCA1, ABCG5, ABCG8 and NPC1L1 were determined (Fig. 3). Two-way ANOVA of RT-PCR analysis revealed a significant effect of genotype on intestinal ABCA1 expression (Fig. 3). However, untreated control (ABCA1 +/+) and ABCA1 −/− mice showed no differences in the gene expression of transporters ABCG5, ABCG8 and NPC1L1 compared with those consuming plant sterols (Fig. 3).

Discussion

Transcriptional activation of ABCA1 has been proposed as a mechanism to explain the decrease in intestinal net cholesterol

Table 1. Effects of phytosterols on plasma lipoproteins and liver cholesterol content in ATP-binding cassette transporter (ABC) A1 +/+ and ABCA1 −/− mice after 2 weeks on each diet (eight animals per group)

(Mean values and standard deviations)

	ABCA1 +/+ mice				ABCA1 −/− mice				
	Control		2 % Phytosterols		Control		2 % Phytosterols		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Effect of genotype (P)
Plasma total cholesterol (mm)	3·5	1·5	3·3	0·9	2·0	1·6	1·9	0·6	<0·01
Plasma non-HDL-cholesterol (mm)	0·9	0·6	0·9	0·3	1·9	1·2	1·7	1·0	<0·05
Plasma HDL-cholesterol (mm)	2·5	0·8	2·5	0·8	0·1	0·1	0·1	0·1	<0·0001
Liver cholesterol (μmol/g tissue)	0·8	0·6	0·6	0·2	0·8	0·2	1·1	0·1	NS

*Two-way ANOVA.

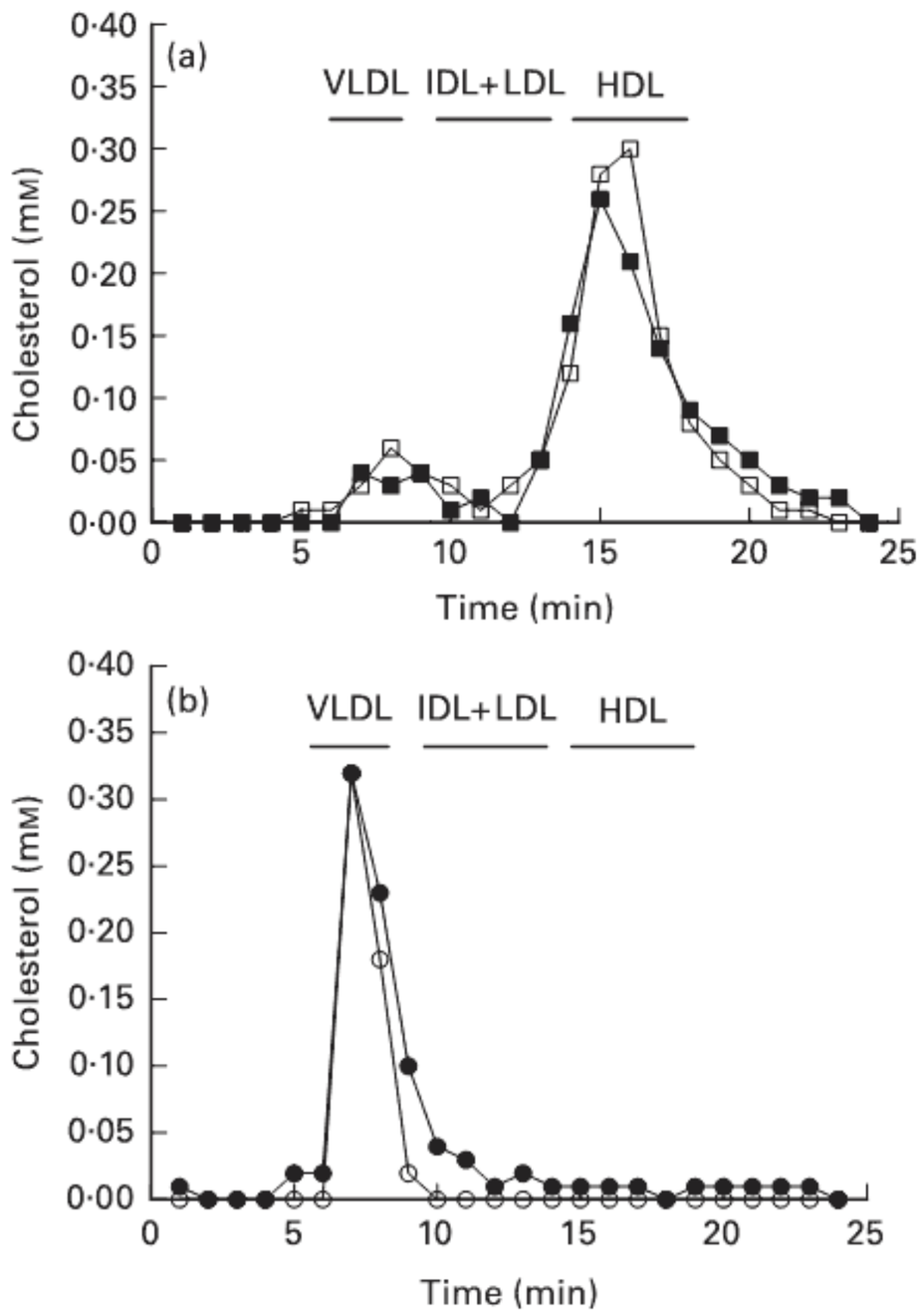

Fig. 1. Plasma lipoprotein cholesterol distribution determined by fast performance liquid chromatography in (a) ATP-binding cassette transporter (ABC) A1 + /+ and (b) ABCA1 −/− mice, both fed a Western-type diet with or without phytosterols. Pooled mouse plasma (200 μl) from the different genotypes was analysed. The positions of elution of the VLDL, intermediate-density lipoproteins (IDL) + LDL and HDL are represented by horizontal lines. (−□−), ABCA1 + /+ mice fed control diet; (−■−), ABCA1 + /+ mice fed diet with 2% phytosterols; (−○−), ABCA1 −/− mice fed control diet; (−●−), ABCA1 −/− mice fed diet with 2% phytosterols.

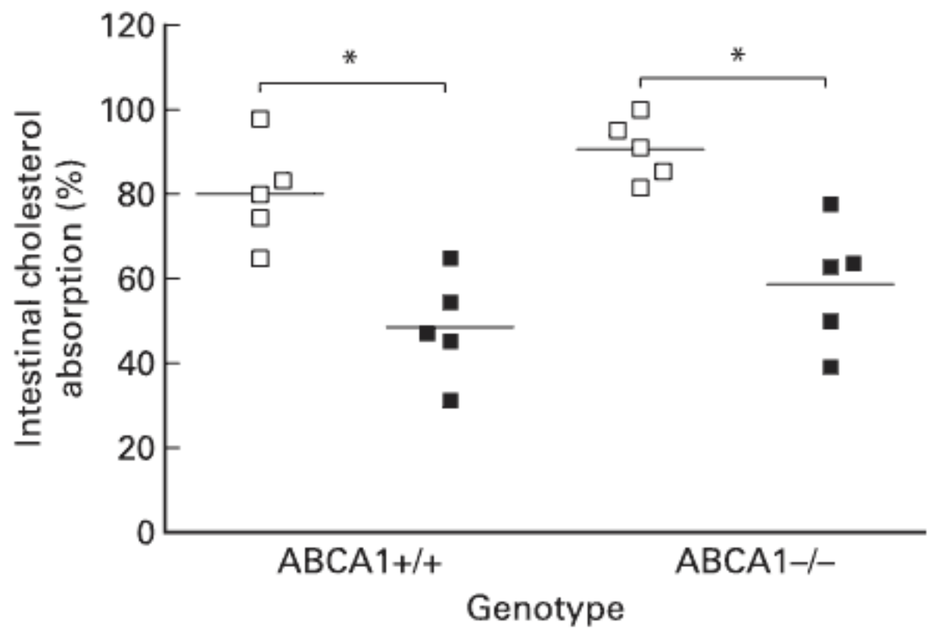

Fig. 2. Effects of phytosterols on intestinal net cholesterol absorption in ATP-binding cassette transporter (ABC) A1 + /+ and ABCA1 − /−; mice. The group means from five animals in each group are indicated by horizontal bars. (□), Control diet; (■), diet with 2% phytosterols. * Two-way ANOVA and Bonferroni *post hoc* tests revealed significant effects of phytosterols ($P < 0{\cdot}01$).

absorption induced by phytosterols (Plat & Mensink, 2002). Furthermore, phytosterols or their derivatives could act as liver X receptor ligands and increase ABCA1 expression at a transcriptional level (Kaneko *et al.* 2003; Plat *et al.* 2005). However, the importance of ABCA1 in net intestinal cholesterol absorption is unclear (Brousseau, 2003). Several reports in animal models, though not all (Groen *et al.* 2001), have provided substantial *in vivo* evidence that ABCA1 influences intestinal net cholesterol absorption (McNeish *et al.* 2000; Drobnik *et al.* 2001; Mulligan *et al.* 2003; Temel *et al.* 2005). However, ABCA1 is dominantly expressed on the basolateral surface of intestinal cells (Ohama *et al.* 2002) and liver X receptor activation increases intestinal cholesterol excretion independently of ABCA1, probably by increasing the intestinal transcription of ABCG5 and ABCG8 (Plosch *et al.* 2002). ABCG5/G8 heterodimers may exchange phytosterols and cholesterol in the intestinal lumen (Plosch *et al.* 2002; Sehayek, 2003). NPC1L1 is a critical mediator of cholesterol absorption as revealed in mice lacking a functional NPC1L1 (Altmann *et al.* 2004). Thus, activation of these ABC and the reduction in NPC1L1 could also explain the phytosterol-mediated inhibition of intestinal net cholesterol absorption (Duan *et al.* 2004; Davies *et al.* 2005).

We have reported that dietary plant stanols and sterols decrease intestinal net cholesterol absorption regardless of increases or decreases in intestinal gene expression of ABCA1, ABCG5/G8 and in NPC1L1 in apoE −/− mice, LDL receptor-deficient mice and control mice (Calpe-Berdiel *et al.* 2005). Other authors have reported similar results in hamsters (Field *et al.* 2004). An oxidised plant sterol metabolite has also been found to enhance intestinal ABC expression in C57BL/6 mice (Kaneko *et al.* 2003). Liver X receptor activation with increased ABCA1 gene expression has also been described in Caco-2 cells after the addition of plant sterols and stanols from the 4-desmethylsterol family (Plat *et al.* 2005). However, in our opinion, there is no conclusive explanation for the differences observed between the different models and thus it cannot be ruled out that phytosterols changed the activity of these transporters through post-transcriptional mechanisms. Furthermore, these results do not permit differentiation between primary and compensatory changes (Kaneko *et al.* 2003; Field *et al.* 2004; Calpe-Berdiel *et al.* 2005; Plat *et al.* 2005).

In the present study, we analysed the involvement of ABCA1 as a molecular target of phytosterols. The major finding was that ABCA1 do not play an essential role in the phytosterol-mediated reduction in net cholesterol absorption since the effect of phytosterols was similar in ABCA1 −/− and ABCA1 +/+ mice. Of note, we found very low, but not undetectable, intestinal mRNA ABCA1 in ABCA1 −/− mice. This has also been found by other groups (Plosch *et al.* 2002; Timmins *et al.* 2005) and may be due to the fact that the PCR primers were located outside the disrupted exons (McNeish *et al.* 2000).

We did find a modest increase in net cholesterol absorption in untreated ABCA1 −/− mice with respect to untreated ABCA1 +/+ animals. Although the cause of this increase is unknown, this observation is consistent with previous observations made during the characterisation of these animals (McNeish *et al.* 2000) but in contrast to that of another group that studied intestinal net cholesterol absorption in ABCA1 −/− mice fed a cholesterol-free diet (Drobnik *et al.* 2001). We found no major compensatory increase in the intestinal gene expression of other sterol transporters such as ABCG5, ABCG8 or NPC1L1.

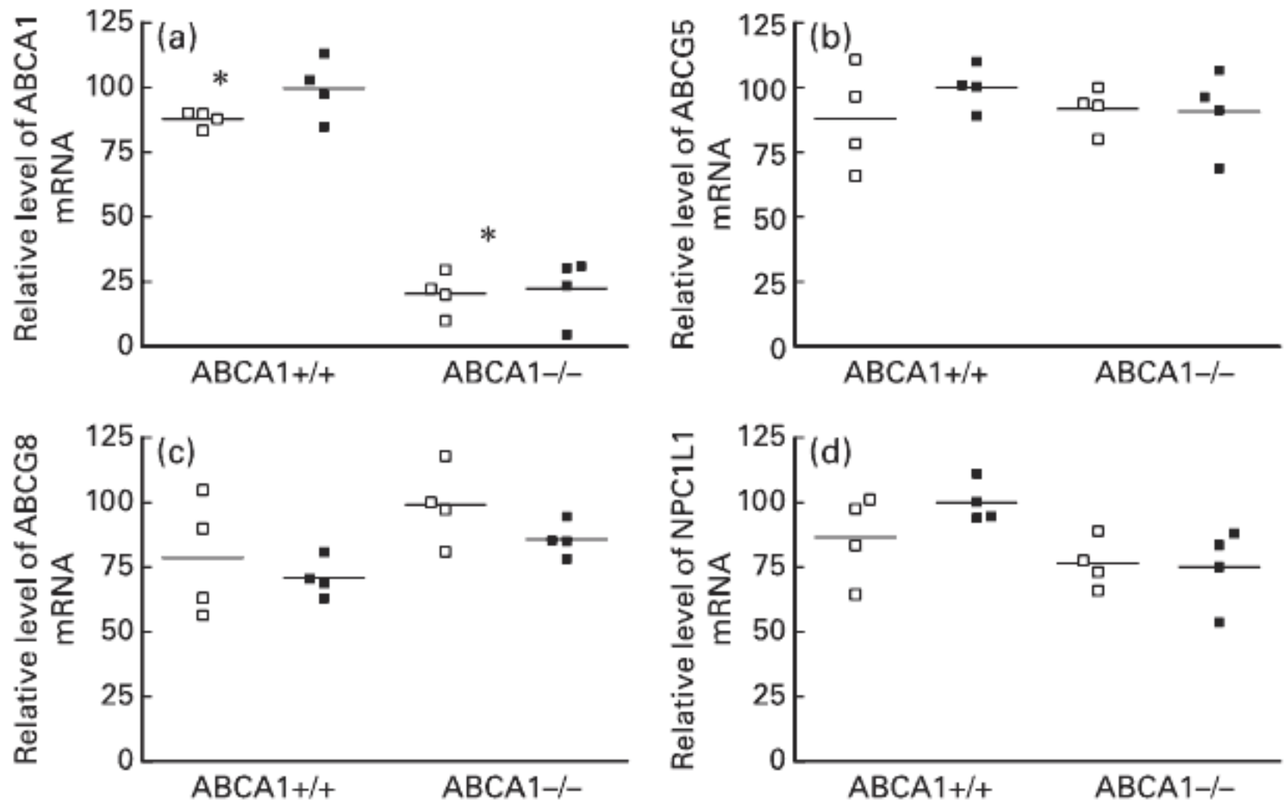

Fig. 3. Relative intestinal ATP-binding cassette transporter (ABC) A1 (a), ABCG5 (b), ABCG8 (c) and Niemann-Pick C1-Like 1 (NPC1L1) (d) mRNA levels in ABCA1 +/+ and ABCA1 −/− mice after 2 weeks fed a Western-type diet with or without phytosterols. The most abundant signal was set to a normalised value of 100 arbitrary units. The group means from four animals in each group are indicated by horizontal bars. Putatively inactive mRNA was detectable in ABCA1 −/− mice, as the PCR primers were located outside the disrupted exons. (□ or □), Control diet; (■), diet with 2 % phytosterols. * Two-way ANOVA and Bonferroni *post hoc* tests revealed a significant effect of genotype ($P<0{\cdot}01$).

The decreased plasma labelled [^{14}C]cholesterol at 72 h in phytosterol-fed ABCA1 +/+ mice compared with non-treated ABCA1 +/+ mice may be due to the decreased intestinal net cholesterol absorption in the first group. In contrast, the increased plasma radioactivity in ABCA1 −/− mice compared with ABCA1 +/+ could be due only in part to their increased intestinal net cholesterol absorption. The increase in VLDL particles observed in ABCA1 −/− animals fed a Western-type diet has also been described previously (McNeish *et al.* 2000). Thus, elevated radioactivity in ABCA1 −/− mice may also be due to enhanced VLDL particle formation (Plosch *et al.* 2002; Sahoo *et al.* 2004) and/or reduced catabolism of apoB-containing lipoproteins (Joyce *et al.* 2003). In fact, the increase (2-fold) in plasma cholesterol radioactivity and non-HDL-cholesterol found in ABCA1 −/− mice, compared with ABCA1 +/+ mice, is consistent.

Interestingly, phytosterol treatment did not decrease plasma cholesterol in either of the two mouse genotypes, although a reduction in intestinal net cholesterol absorption was observed in both models. We and other authors have reported that wild-type mice and gerbils fed a low-cholesterol diet present unchanged low plasma cholesterol concentrations regardless of manipulations that alter intestinal net cholesterol absorption, such as treatment with ezetimibe or phytosterols (Repa *et al.* 2002; Calpe-Berdiel *et al.* 2005; Hayes *et al.* 2005). Thus, it is possible that with a non-expanded whole-body pool of cholesterol, as occurs in mice with low non-HDL-cholesterol, changes in cholesterol synthesis compensate for the decrease in net intestinal absorption. However, detailed studies will be required to prove this hypothesis. In preliminary experiments we found no change in either liver cholesterol (Table 1) or HMGCoA reductase gene expression (data not shown).

In conclusion, the present results clearly demonstrate that ABCA1 is not the primary transporter involved in the reduction in intestinal net cholesterol absorption induced by phytosterols in mice. The wide availability in the future of other GM mice, such as ABCG5-, ABCG8- and NPC1L1-deficient mice, should permit an *in vivo* evaluation of other potential targets of phytosterols.

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Q1 The style of the BJN is to have only one corresponding author. Are you happy with Francisco Blanco-Vaca as corresponding author or do you prefer Joan Carles Escolà-Gil?

Q2 'compared with ABCA1 +/+ mice, is consistent' This phrase has been amended. Is it correct?

Q3 Please provide full list of authors details upto ten Altmann *et al.* 2004; McNeish *et al.* 2000; Timmins *et al.* 2005.