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ORIGINAL CONTRIBUTION

Etienne B. Pouteau
Irina E. Monnard
Christelle Piguet-Welsch
Michel J. A. Groux
Laurent Sagalowicz
Alvin Berger

Non-esterified plant sterols solubilized in low fat milks inhibit cholesterol absorption

A stable isotope double-blind crossover study

Summary *Background* The cholesterol absorption inhibiting properties of plant sterols in milks are unknown. The milk fat globule membrane components may enhance the absorption of cholesterol and could make plant sterols less efficient in this complex matrix. *Aim of the study* To evaluate in hypercholesterolemic men the cholesterol absorption inhibiting properties of verified properly solubilized, non-esterified plant sterols in partly vegetable oil containing milks. *Methods* The plant sterols in milk were determined to be properly solubilized, and to have effective *in vitro* functionality. Sixteen hypercholesterolemic adult men (initial total cholesterol 5.8–8.6 mM) then consumed milk containing sterols (1.8 g of non-esterified pure plant sterols/d) and control milk, alternatively, during two 6-day periods in a double blind cross over design. During the trial, cholesterol absorption was evaluated from the ratio of plasma isotopic enrichment of [26, 26, 26, 27, 27, 27–$^{2}H_{6}$]cholesterol from oral intake (35.6 ± 0.2 μmol, ± SEM) over enrichment of [23, 24, 25, 26, 27–$^{13}C_{5}$]cholesterol from intravenous injection (77.9 ± 0.5 μmol). *Results* Plant sterols in low fat milks contained very few crystals > 11 μm in the presence and absence of bile salts and lysophospholipids, and inhibited cholesterol uptake in Caco-2 cell. This assured that the sterols were properly solubilized prior to the clinical trial. In the clinical study, compliance of volunteers was excellent. After tracer injections (72 h), the plasma [^{2}H] and [^{13}C] isotopic enrichments changed from 0.024 ± 0.001 and 0.072 ± 0.003 MPE (control) to 0.015 ± 0.001 and 0.074 ± 0.002 MPE during sterol treatment, respectively. Cholesterol absorption was reduced from 70.1 ± 4.2 % with control to 41.1 ± 4.0 % with milks containing plant sterol ($P < 0.001$). *Conclusions* These results demonstrate that properly solubilized non-esterified plant sterols in milks significantly inhibit cholesterol absorption in mildly hypercholesterolemic men.

Key words cholesterol absorption – hypercholesterolemic men – milk – plant sterols – stable isotope-labeled cholesterol

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Etienne B. Pouteau (✉) · I. E. Monnard · C. Piguet-Welsch · L. Sagalowicz · A. Berger
Nestlé Research Centre
Nestec Ltd
Vers-chez-les-Blanc
P. O. Box 44
1000 Lausanne 26, Switzerland
Tel.: +41-21/785-8830
Fax: +41-21/785-8925
E-Mail: etienne.pouteau@rdls.nestle.com

M. J. A. Groux
Nestlé Product Technology Center
Nestec Ltd
Konolfingen, Switzerland

A. Berger
Current address: Manager Lipidomics™
Markham, Ontario, Canada

Abbreviations

BMI	body mass index
BSA	bovine serum albumin
DMEM	Dubecco's modified eagle media
FCS	fetal calf serum
GC/C/IRMS	gas chromatography/combustion interface/isotopic ratio mass spectrometry
GC/P/IRMS	gas chromatography/pyrolysis interface/isotopic ratio mass spectrometry
HBSS	Hank's balanced salt solution
LDL-C	low density lipoprotein cholesterol
MPE	mole percent excess
OM	Omega® milk
OMS	Omega® milk containing sterols
PDB	Pee Dee Belemnite ^{13}C international standard

EJN 406

SMOW	standard mean ocean water ^{2}H international standard
TAG	triacylglycerol
TC	total cholesterol
TLC	thin layer chromatography

Introduction

Dietary plant sterols (plant sterols and phytostanols) and their esters have been shown to efficiently lower cholesterol absorption. Typically, 0.5–3.0 g/d of plant sterols produce a 30–80 % lowering of cholesterol absorption and a 10–15 % lowering of LDL cholesterol (LDL-C) [1–3]. The efficacy of the dose given has been shown to be dependent on the physical form and structure in which the plant sterols are dissolved or suspended. For example, sitostanol administered as a crystalline powder is less efficient at inhibiting cholesterol absorption than sitostanol solubilized in a phospholipid matrix [4].

The cholesterol absorption inhibiting properties of plant sterols have largely been studied with plant sterols dissolved in oily food matrices such as spreads and oil water dispersions [4–6], but never in milks to our knowledge. The complex milk matrix containing milk fat globule membrane components could hypothetically enhance the absorption of cholesterol relative to other food matrices, making it more difficult for plant sterols to inhibit cholesterol absorption.

In the present work, we first characterized the physical form of the plant sterols in a milk matrix, in which the plant sterols were solubilized using a proprietary crystal retardation and emulsification system. The principal objective was to accurately evaluate the effect of non-hydrogenated, non-esterified soybean-derived plant sterols in partly vegetable oil containing low fat milks on cholesterol absorption, at a daily dose of 1.8 g/d, in mildly hypercholesterolemic men.

Methods of characterization of plant sterols in milk

Solubilization of plant sterols

Plant sterols were non-hydrogenated, non-esterified and predominately soy bean oil-derived. The plant sterols were added to partly vegetable oil containing low fat milks (Omega® milk, Nestlé, Vevey, Switzerland) using a proprietary crystal retardation and emulsification system, under pilot plant factory standardized conditions (Nestec Ltd, Konolfingen, Switzerland). The milks contained 0.40 % crude sterols (0.36 % of pure sterols since sterols were 90 % pure), thus containing 1.8 g of pure sterols in the 500 mL of milk/d, in two 250 mL servings. The quantity of plant sterols added to the milks, and the composition of plant sterol species, was verified by extracting the milks with the modified Mojonnier method [7], without saponification, in the presence of the internal standard epicoprostanol (Sigma-Aldrich Co., Buchs, Switzerland) isolating the free sterols with thin layer chromatography (TLC), and quantifying silyated sterols with gas chromatography according to validated in-house procedures. Control milks and plant sterol milks were analytically determined to have slightly higher amounts of plant sterols than that added from the soybean source because of the contribution from vegetable oil and butter fats added to the milks (0.018 % sterols). For practical purposes, we will refer to the doses as being 0 g and 1.8 g of pure sterols. The composition of the sterols used in the clinical trial contained 57.67 ± 0.39 % β-sitosterol, 24.93 ± 0.08 % stigmasterol, 14.92 ± 0.09 % campesterol, 1.83 ± 0.23 stigmastanol, 0.52 ± 0.05 % brassicasterol, and 0.15 % trace amounts of other sterols (mean of 3 replicates ± SD).

Microscopic assessment of emulsion. The presence of crystals in at least three milk samples was checked weekly for stability. A small drop of emulsion was spread between two glass plates and examined under a Zeiss Axioplan microscope (Zeiss, Jena, Germany) by polarized light microscopy and by differential interference microscopy. The samples were also heated on the slide from 30–95 °C, at 2 °C/min, under a Zeiss Axiovert 25 microscope equipped with a Planer CM3 cryomicroscope temperature control stage. Samples consisted of milks used in the clinical trials and reference milks containing plant sterols in a predominately crystalline, needle-morphology.

Particle sizing. To evaluate stability of the emulsions, and aggregation/coalescence phenomena, particle size distributions in these samples were determined with a static laser light scattering apparatus (Mastersizer 2000 equipped with a Hydro 2000G large volume sampler; Malvern Instruments, Malvern, UK). Samples were diluted 1:1000 in water, and the following optical parameters were utilized: particle refractive index of 1.460; absorption coefficient for fat globules of 0.010; and dispersing refractive index for water of 1.330.

Determination of plant sterol crystals

Determination of crystals in milk using a filtration assay. Duplicate 10 mL milk samples of each type were passed under gravity flow through a 47 mm 11 μm nylon filter (Millipore SA, Lausanne, Switzerland) in a 250 mL Millipore glass filtration system. The filter was pre-washed with water, then dried under vacuum before passing the milk. The precipitate trapped on the filter was washed

with 5 mL water to remove contaminating milk protein. The filter was then vortexed in a separate tube with isopropanol, seated on a glass funnel, rinsed on both sides of the filter with 5 mL isopropanol, and the sterols collected in the glass tube. The isopropanol solution was centrifuged to precipitate out any remaining milk proteins, transferred to a new tube, and concentrated under N_2 to 0.5 mL volume.

To quantify sterol crystals, the 0.5 mL isopropanol solution was sonicated, gently inverted, and 10 µL of isopropanol added to 1 mL Sigma Infinity Cholesterol Reagent (Sigma-Aldrich) in a separate tube. Following a 15 min incubation at room temperature, 200 µL of the above mixture was plated in quadruplicate into a flat-bottomed 96-well ELISA Plate, and the blank-corrected absorbance determined on an ELISA reader (Microplate Reader MRX, Dynatech Laboratories Inc., Embrach-Embraport, Switzerland) at 490 nm, within 30 min following the incubation. The blank consisted of Omega® milk without plant sterols. The mean molar concentration of sterols from 8 measurements was determined from a standard curve using known concentrations of identical plant sterols dissolved in isopropanol. To quantify plant sterols in the filtrate, milk filtrates were spiked with epicoprostanol, and the filtrate extracted and quantified as described for the quantification of sterols in intact milk.

Determination of crystals in milk in presence of bile acids. A bile acid solution was prepared after Ostlund et al. [8], containing 150 mM NaCl, 15 mM $NaPO_4$ and 8 mM sodium taurocholate (Sigma-Aldrich), pH 7.4. Soy lecithin (Sigma-Aldrich) in $CHCl_3$:MeOH (2:1, v/v) was dried under N_2, and the above solution was added to achieve 5 mM lecithin. The solution was gently stirred overnight at room temperature with a magnetic stirring bar and filtered through 5 µm filter paper. Milks (10 mL) were added to 5 mL of the above bile acid solution in a 250 mL flask (3-fold dilution of the bile acid solution) and gently agitated at 30 rpm for 1 h in a 37 °C water bath. The solution was filtered through an 11 µm filter and quantified for sterol crystals as described above.

In vitro efficacy of plant sterols in milk

Caco-2 intestinal cells were incubated in 24-well plates in 78.2 % Dubecco's modified eagle media (DMEM) containing 4.5 g/L glucose, 19.5 % fetal calf serum (FCS), 0.8 % penicillin-streptomycin, 0.5 % gentamycin, and 1 % 100 X DMEM for 21 d at 37 °C in a 10 % CO_2 environment. Medium was changed every 2 d. Lipids were dissolved in $CHCl_3$/MeOH (2:1 v/v), evaporated under N_2, and suspended in bile salts in DMEM plus 0.3 % bovine serum albumin (BSA), to achieve a micellar solution containing final concentrations of 0.6 mM lysophosphatidylcholine, 0.2 mM oleic acid, 50 µM cholesterol, 0.2 µCi/mL [^{14}C]cholesterol, 1 mM taurocholate, 0.5 mM taurochenodeoxycholate, 0.5 mM taurodeoxycholate, and 2 mM glycocholate (all reagents from Sigma-Aldrich). Micellar solutions (2.2 mL) were then distributed into glass tubes in quadruplicate, mixed with 8.8–88 µL of Omega® milk, plus or minus sterols and emulsifiers, to achieve a final concentration of 35–359 µM plant sterols, and a cholesterol:plant sterol ratio of about 1.4–0.14:1 (plant sterol micellar solution). Tubes were incubated for 2 h. After washing cells with Hank's Balanced Salt Solution (HBSS), and aspirating media, 0.5 mL of plant sterol micellar solution was added to cells for 2 h at 37 °C, then washed with HBSS. Cells were lysed with 1 N NaOH, agitated 30 min, and following removal of 25 µL of cells for protein determination (BioMEK 2000, Beckman, Winton, IN), remaining cell solution was counted by autoradiography.

Clinical trial

Subjects

Mildly hypercholesterolemic Caucasian adult males aged 35–57 years were enrolled in the clinical study (Table 1). Eighteen volunteers initially began the clinical trial; two subjects had an incomplete isotopic injection. Therefore, all remaining results refer to 16 subjects. Mean starting body weight was 76.0 ± 1.5 kg and body mass index (BMI) was 25.5 ± 0.6 kg/m^2. Exclusion criteria were BMI > 30, total cholesterol (TC) < 5.6 mM or > 8.6 mM, triacylglycerol (TAG) < 0.6 mM or > 3.5 mM, hemophilia, use of cholesterol-altering medication, renal or liver disease, diabetes, > 10 cigarettes/d and > 20 g of alcohol/d. Informed consent was obtained from volunteers in writing and the study protocol was approved by the Nestlé Ethical Committee (Authorization number 2000–5, Lausanne, Switzerland).

Study design

The study was a two-center (block A, Nestlé Research Center, Lausanne, Switzerland; block B, Nestlé Product Technology Center, Orbe, Switzerland), two 6 d period (period 1 and 2), double blind, randomized cross-over trial, with 4 wk washout between periods. Block A volunteers started and completed the study 2 wk later than block B volunteers. Prior to period 1, the 18 subjects were randomly distributed into two groups, 8 subjects consumed Omega® milk containing sterol (OMS) and 10 subjects consumed Omega® milk (OM). Only 16 subjects were evaluated statistically for the technical reason previously explained. The OM partly vegetable oil-containing base used in the clinical trial was similar, but not

necessarily identical to that used in commercial Omega® milk product. OMS consisted of Omega® low fat UHT milk containing soybean non-hydrogenated, non-esterified solubilized plant sterols (1.8 g/d pure material in 2 servings of 250 mL) in a proprietary crystal retardation and emulsification system. OM was identical, but lacked plant sterols and the crystal retardation and emulsification system. More specifically, both OM and OMS milks used in the clinical trials contained 0.63 % butter oil, 0.56 % rapeseed oil and 0.39 % corn oil, thus 1.6 % total fat, excluding the contribution from the plant sterols and emulsifiers. Liquid skim milk replaced the plant sterols and emulsifiers used in the OMS milk. We decided to not include the emulsification system alone in the control milk because our purpose was to evaluate the combined efficacy of the plant sterols in the proprietary crystal retardation and emulsification system, relative to the control. OM group received 2 servings of 250 mL each OM daily. Milks were consumed immediately after breakfast and lunch each day. Milks were manufactured before the study and during the wash out period and were consumed 30–79 d post-manufacture.

Subjects were fed a standardized diet for the entire duration of the period. Three different meal varieties were calculated to provide 11290 ± 326 KJ/d using food composition tables. Mean cholesterol intake was 283 ± 5 mg/d. During period 2, treatments were switched, and both groups were assigned to either OM or OMS.

On d 4 (T_0) of each study period, a fasted blood sample was collected from a radial vein. Thereafter, two tracers of cholesterol were administrated simultaneously within 30 min. A [26, 26, 26, 27, 27, 27–$^2H_6$]cholesterol tracer (98 atom%) (Medical Isotopes, Pelham, NH) was diluted in sunflower oil (15 mg/g) and placed on bread for weighted oral intake providing 14.0 ± 0.1 mg (mean ± SEM; 35.6 ± 0.2 μmol). Ready-to-inject 7.5 mL syringes containing [23, 24, 25, 26, 27–$^{13}C_5$]cholesterol tracer (99 atom%, Mass Trace, Woburn, MA) dissolved in Intralipid parenteral emulsion (Pharmacia-Upjohn, Stockholm, Sweden) was prepared by the University Hospital of Lausanne and checked for sterility, after Bosner et al. [9]. Concurrently with the oral tracer intake, [^{13}C]cholesterol tracer was injected intravenously to provide 30.5 ± 0.2 mg (77.9 ± 0.5 μmol). Blood samples were thereafter collected from 12–16 h fasted volunteers d 5, d 6 and d 7 after tracer administration during each period. Body weight and height were further monitored.

Subjects completed a daily food diary, providing information on the following: when milks were consumed; food items consumed that were not provided; quantity of non-alcoholic and alcoholic drinks consumed; number of cigarettes smoked; medicines consumed; quantity and types of sport activities; and frequency and consistency of stools.

Plasma analyses

Reagents and chemicals. Hexane, decane and diethyl ether were purchased from J. T. Baker (Mallinkrodt Baker, Holland); TLC plates, chloroform ($CHCl_3$), methanol (MeOH), and acetic anhydride were from Merck (Darmstadt, Germany); and ethanol (EtOH), pyridine and acetic acid were from Fluka Chemie (Buchs, Switzerland). Silyating reagents were from Supelco (Bellefonte, PA). Cholesterol and 5α-cholestan were from Sigma-Aldrich.

Analysis: preparation of plasma samples. Plasma samples were prepared to separate free cholesterol for stable isotope measurements after Gremaud et al. [10]. Briefly, plasma (200 μL) was gently mixed with MeOH (480 μL) and $CHCl_3$ (240 μL) and left on ice 5 min. The precipitate was removed by filtration. The filter was rinsed again with $CHCl_3$ (320 μL). The filter was removed and water (360 μL) was added to the filtrate. After vortexing, the double-phase-solution was centrifuged (5 min, 2500 rpm). The organic phase was then removed and dried under N_2. The recovery of extracted cholesterol in separate experiments using radiolabeled cholesterol as a marker was 88 ± 5 % (SD). The dry residue was dissolved in EtOH/$CHCl_3$ (40 μL, 1:2, V/V), purified on TLC plates (silica gel, 20 × 20 cm; Merck), and developed in hexane/diethyl ether/acetic acid (80:20:1, v/v). Cholesterol standards were run on the right lane of the TLC plate and the migration of cholesterol was determined with ethanolic Rhodamine G (0.05 %, W/V; Sigma-Aldrich) at 254 nm. The free cholesterol band was scraped from samples, eluted from silica with 7 mL diethyl ether and evaporated to dryness. Recovery from TLC plates in separate experiments using radiolabeled cholesterol as a marker was 97 ± 4 % (SD). Free cholesterol was derivatized with acetic anhydride (150 μL) and pyridine (40 μL) by heating 10 min at 90 °C. Following evaporation, the cholesterol acetate was solubilized with hexane (50 μL) in autosampler vials.

Determination of isotopic enrichments. The same sample vial was used for ^{2}H and ^{13}C isotopic enrichment measurements. [^{13}C]cholesterol enrichment was determined using a Gas Chromatography/Combustion interface/Isotopic Ratio Mass Spectrometry (GC/C/IRMS; MAT 252, Finnigan Mat, Bremen, Germany). Duplicate acetylated cholesterol samples (0.2 μL) were injected into the gas chromatograph in the splitless mode (injector temperature at 280 °C). The capillary column was a DB5-ms column (J&W, Koeniz, Switzerland), 15 m × 0.32 mm i. d. × 250 nm film thickness. Helium carrier gas flow was 1.5 mL/min. Temperature programming was from 185 °C to 290 °C at 30 °C/min then 3.2 min at 290 °C. The retention time of cholesterol acetate and the run time were 5.2 and 6.7 min, respectively. A [^{13}C]choles-

terol enrichment calibration curve was analyzed before each sequence of plasma cholesterol samples. ^{13}C isotopic enrichment was expressed in δ ‰ against Pee Dee Belemnite ^{13}C international standard (PDB) and then converted into Mole Percent Excess (MPE).

[^2H]cholesterol enrichment was analyzed using a Gas Chromatography/Pyrolysis interface/Isotopic Ratio Mass Spectrometry (GC/P/IRMS; Delta $^+$XL, Finnigan Mat, Bremen, Germany). Conditions were different from the analysis for ^{13}C enrichment due to the larger amount of [^2H]cholesterol required for optimal detection. Triplicate samples (2 µL) were injected into the gas chromatograph in the splitless mode (injector temperature at 280 °C). The capillary column was a 20 m × 0.32 mm × 250 nm DB5-ms (J&W Scientific, Folsom, CA). Helium flow was 1.3 mL/min. Runs were performed isothermally at 290 °C, and run time was 10 min. The retention time of cholesterol acetate was 5.7 min. A [^2H]cholesterol enrichment calibration curve was analyzed before each sequence of plasma cholesterol samples. ^2H isotopic enrichment was expressed in δ ‰ against SMOW (standard mean ocean water, ^2H international standard) and further transferred into MPE.

Measurement of total cholesterol and triacylglycerol. TC and TAG in plasma were measured by enzymatic bioassays (Bio-Merieux, Lyon, France).

Calculations

Absorption of cholesterol. From [$^2H_6$]cholesterol and [$^{13}C_5$]cholesterol isotopic enrichment determinations in plasma following oral and intravenous tracer administrations, respectively, the absorption of cholesterol was calculated as follows:

$$\text{Absorption}(\%) = \frac{[^2H_6]\text{cholesterol enrichment (MPE)} \cdot [^{13}C_5]\text{cholesterol dose (mol)}}{[^{13}C_5]\text{cholesterol enrichment (MPE)} \cdot [^2H_6]\text{cholesterol dose (mol)}} \cdot 100$$

where enrichments are expressed in MPE and tracer dose in mole. Absorption was calculated for each individual 48 and 72 h after tracer administrations, the average expressing the absorption percent.

Turnover of cholesterol. Turnover of cholesterol (pools/d) was determined from [$^{13}C_5$]cholesterol enrichment decay curve throughout 3 d. First order exponential curves were fitted to 24, 48 and 72 h [$^{13}C_5$]cholesterol enrichments expressed in MPE.

Statistics

Data management was with ClinTrial software (Waltham, MA), and statistical analyses with SPSS statistical software (Chicago, IL). Sample size calculations predicted the need for 20 persons, and the trial started with 18 persons, due to inability to obtain 20 subjects. The sample size estimation for 20 subjects was approximate since we had not previously evaluated statistical variance and the decrease in absorption with plant sterols in milk. Thus, we decided to start the trial with 10 % less subjects than estimated from our calculations. Retrospectively, an absence of period effects model was used, since there were no differences in experimental or social situation between the two short periods, leading statistically to a paired *t*-test. There were likely to be no carry-over effects of treatment since the length of the washout period was long (4 wk) and plant sterols have predominately acute effects on cholesterol absorption. Unless stated otherwise, data are given as mean ± SEM.

Results

Characterization of plant sterols in milk

Microscopy confirmed the lack of significant crystals in the milk samples relative to a sample containing large amounts of needle-like crystals (Fig. 1 A–C). In the reference sample containing needle-like crystals, the needles were still observed at temperatures higher than 75 °C, which is consistent with the presence of sterol crystals. The filtration assay demonstrated that about 75 % of the sterol originally added to the milk was located within these needle crystals. Polarized light microscopy revealed particles less than 5 µm in diameter in OMS. There were no obvious changes in the particle size and number of these particles observed microscopically during 6 mo. These small particles were probably plant sterol containing crystals for the following reasons: 1) they were not observed in milk without sterol; 2) they were still observed following heating under the microscope at temperatures greater than 70 °C, a melting pattern consistent with sterol crystals, having a fusion temperature about 140 °C, but not fat crystals, which would melt (Fig. 1 D). Particle size distribution was performed weekly throughout the course of the experiment to check for any changes in distribution. The small particles observed in Fig. 1 were not detected, probably because their number was too low. Particle size distribution did not change over an 85 d period, with samples stored at room temperature, indicating that crystal growth did not occur and that the emulsion was stable. We demonstrated in our filtration assay that only 1.3 % of the added sterols could be trapped in an 11 µm filter and that, in the presence of bile, only 0.5 % of the sterols were trappable. In comparison, in the crystalline reference sample, about 75 % of the added sterols were trapped in an 11 µm filter and in the presence of bile, 63 % of the sterols were trappable. It was confirmed by

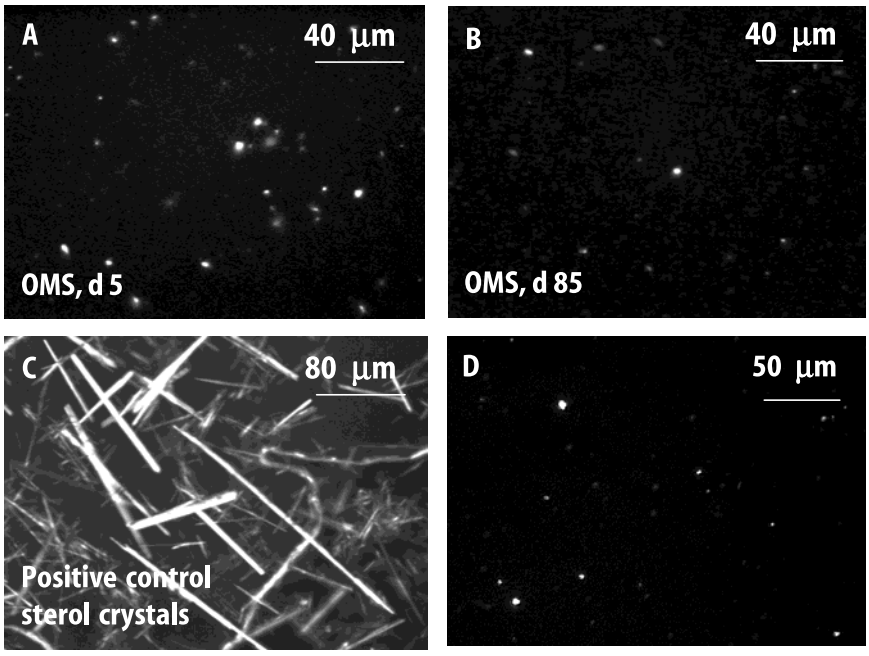

Fig. 1 Microscopy of 0.40 % sterol-enriched milk revealed no visible changes in crystal formation during 85 d at room temperature (**A**, **B**). A reference sample with 0.40 % sterol had large crystals after 267 d (**C**). A control milk without sterols showed no visible crystals (not shown). Polarized light microscopy revealed that when milks in **A** and **B** were heated to 75 °C, the small crystals remained (**D**)

microscopy that all the large needles observed in the reference sample in Fig. 1 were trapped in the filter.

Inhibition of cholesterol uptake in Caco-2 cells

Milks containing plant sterols were found to reduce the uptake of cholesterol in intestinal Caco-2 cells from 1.1–3.6-fold, whereas crystalline sterols were less effective in this regard (Table 1, Experiments 1–2). Milks containing plant sterols and emulsifiers reduced the uptake of cholesterol in a statistically significant manner, although there were quantitative differences in the two experiments shown. In experiment 2, there was evidence that cholesterol uptake was more strongly inhibited as the ratio of cholesterol: plant sterol decreased.

Clinical trial randomization results. Compliance and protocol violations

Compliance of study volunteers was excellent, and protocol violations were minor. In 3 of 216 man-days, 3 volunteers exceeded the alcohol limit, and were accepted for further analyses. One volunteer had the last blood measurement taken 21 h in advance, and missed the last 250 mL milk sample. Excluding two subjects (omitted from Table 2), the OM and OMS groups had initial TC values of 6.85 ± 0.94 ($n = 9$) and 6.96 ± 1.11 ($n = 7$), respectively.

Table 1 *Inhibition of cholesterol uptake by plant sterols in Caco-2 cells.* With a cholesterol:plant sterol ratio of 1.4:1, milks containing plant sterols reduced the uptake of cholesterol in intestinal cells by 2.7-fold of the (OM + emulsifier + sterol)/(OM + emulsifier) ratio (*$P < 0.05$, unpaired T-test), whereas crystalline sterols were ineffective (Experiment 1). In experiment 2, the uptake was only slightly reduced with a cholesterol:plant sterol ratio of 1.4:1, whereas at a cholesterol:plant sterol ratio of 0.14:1, the uptake of cholesterol was reduced 3.6-fold. Values represent the mean ± SEM of 3–4 determinations. Relative to control in each experiment (OM + emulsifier), all milks containing emulsifiers and sterols lowered cholesterol uptake statistically significantly (**$P < 0.05$, unpaired T-test), as indicated with two asterisks. *OM* Omega® milk

Experiment 1		[^{14}C]cholesterol in Caco-2 cells (cpm/mL/mg protein)
OM + sterol (crystalline)		39298 ± 1014
OM + emulsifier		40143 ± 1774
OM + emulsifier + sterol		14720 ± 2946*
Experiment 2	**Cholesterol/ plant sterol ratio**	**[^{14}C]cholesterol in Caco-2 cells (cpm/mL/mg protein)**
OM + emulsifier	0	5490 ± 163
OM + emulsifier + sterol	1.4/1	4882 ± 146**
OM + emulsifier + sterol	0.57/1	3967 ± 111**
OM + emulsifier + sterol	0.28/1	3714 ± 171**
OM + emulsifier + sterol	0.14/1	1544 ± 184**

Isotopic enrichments

Analysis of isotopic enrichments of [^{2}H]cholesterol and [^{13}C]cholesterol derivatives by IRMS (Delta $^{+}$XL and

Table 2 Individual data and cholesterol absorption in hypercholesterolemic men. Weight, body mass index (BMI), total cholesterol (TC), and triacylglycerol (TAG) plasma concentrations were determined prior to the clinical study. Cholesterol absorption was lowered during plant sterol-enriched milk diet compared to control milk (* $P < 0.001$). Relative change in cholesterol absorption was evaluated

Subject	Age	Weight	BMI	TC	TAG	Cholesterol Absorption		
						Control	Plant sterol	Relative change
	Years	Kg	Kg/m^2	mM	mM	%	%	%
A	49	70.5	22.8	8.3	0.79	87.2	46.8	−46.3
B	43	68.8	26.9	5.8	0.61	66.1	55.9	−15.4
C	41	75.0	23.9	6.2	0.68	66.2	84.3	27.3
D	35	69.0	21.3	6.3	0.83	86.7	54.1	−37.6
E	38	75.0	21.9	7.2	1.51	73.5	41.6	−43.4
F	38	86.1	27.5	7.8	2.25	46.1	36.5	−20.8
G	43	89.1	28.1	8.0	2.44	99.4	48.3	−51.4
H	51	80.6	26.9	6.4	1.53	59.1	34.0	−42.5
I	43	75.1	25.4	7.1	1.30	89.0	35.6	−60.0
J	51	73.6	24.6	6.0	0.95	43.9	37.7	−14.1
K	47	71.1	24.6	6.1	0.95	57.2	37.6	−34.3
L	44	77.0	28.6	8.6	1.11	69.9	37.3	−46.6
M	36	68.8	24.1	6.5	1.12	47.5	18.5	−61.1
N	44	82.0	28.0	6.1	1.14	77.2	45.6	−40.9
O	57	80.0	28.3	6.4	1.50	64.1	23.4	−63.5
P	46	75.0	25.4	7.7	0.75	87.8	19.7	−77.6
	44.1 ± 1.5	76.0 ± 1.5	25.5 ± 0.6	6.9 ± 0.2	1.2 ± 0.1	70.1 ± 4.2	41.1 ± 4.0*	−39.3 ± 5.9

MAT252, respectively) showed good signal detection (> 1 volt). The mean isotopic enrichment of [^{13}C] and [^{2}H]cholesterol during the first, second and third days of kinetics for the control and plant sterol treatments are shown in Fig. 2. The isotopic enrichment of [^{13}C]cholesterol in plasma following intravenous injection of [^{13}C]cholesterol tracer showed an identical level of enrichment the first day of kinetics during both periods for both treatments. Values were 0.129 ± 0.028 MPE and 0.139 ± 0.018 MPE for control and plant sterol treatments, respectively, without accounting for tracer doses; these values were not significantly different from one another (NS). In contrast, the first day of kinetics, following the [^{2}H]cholesterol tracer intake by oral route, a higher [^{2}H] enrichment was noticed during the control treatment (0.030 ± 0.006 MPE) compared to the plant sterol treatment (0.018 ± 0.007 MPE, $P < 0.001$). The steady-state ratio of [^{2}H] to [^{13}C]cholesterol enrichments was reached for the second and third days during both treatments (Fig. 3), showing that calculation of cholesterol absorption could be performed equally during these two days. This ratio was dramatically decreased during plant sterol treatment (by 43 % on the third day).

Absorption of cholesterol

Turnover rate of cholesterol was 0.29 ± 0.01 and 0.32 ± 0.02 pool/d for control and plant sterol treatments, respectively (values were not significantly diffe-

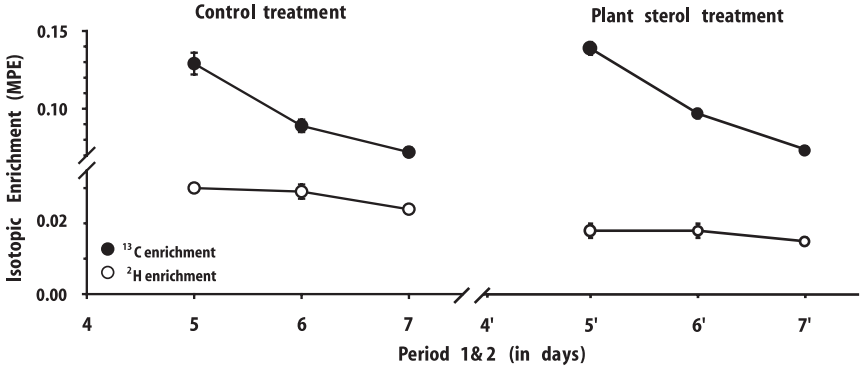

Fig. 2 Mean isotopic enrichments of [^{13}C] and [^{2}H]cholesterol during the first, second, and third day of kinetics for the control (d 5,6,7) and the plant sterol treatments (d 5′,6′,7′) (mean ± SEM)

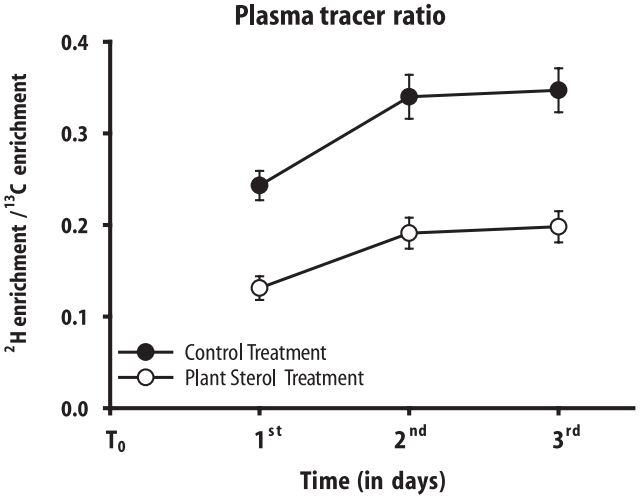

Fig. 3 Mean ratio of isotopic enrichment of [^{2}H]cholesterol over enrichment of [^{13}C]cholesterol during the first, second, and third day of kinetics of the control and the plant sterol treatments (mean ± SEM)

rent). The mean absorption of cholesterol was 70.1 ± 4.2 % and 41.1 ± 4.0 % during the control and plant sterol treatments, respectively (Table 2). The relative decrease in absorption was 39 ± 6 % ($P < 0.001$). All subjects showed a decrease in absorption between 14.1–77.6 % following the plant sterol treatment, except subject C. No significant correlation was observed for initial TC and cholesterol absorption during control milk and sterol intake periods. The Pearson correlation coefficient for initial starting TC and cholesterol absorption was 0.39, $P < 0.11$ for the OM group and –0.16, $P < 0.30$ for the OMS group. Nor was there a significant correlation for initial TC and the relative change in absorption. Initial TAG was also not correlated with these parameters.

Stools

During the OMS period of the clinical study, there was a higher percentage of 'formed' stools (73 %) compared to the OM period (63 %; $P = 0.04$). There was no difference in the hardness of stools although a slight trend to fewer soft stools with OMS could be observed (17.6 % OM vs. 11.9 % with OMS; $P = 0.11$).

Discussion

In the present study, we showed that non-hydrogenated, non-esterified plant sterol from soy incorporated into partly-filled milk decreased cholesterol absorption. Sixteen hypercholesterolemic men consumed 1.8 g/d of soy sterols, and showed a dramatic decrease in cholesterol absorption from 70 to 41 %. These data show that properly solubilized non-esterified sterols in a low fat milk matrix can decrease the absorption of cholesterol.

Unlike esterified plant sterols which are highly soluble in oily food matrices, non-esterified sterols must be properly dispersed (solubilized) with an appropriate crystal retardation and emulsification system. For this reason, we performed a series of structural and functional characterizations of the non-esterified plant sterols in our food matrix before committing to clinical trials. It can be predicted in advance that large sized plant sterol crystals (unless present in large quantities in the diet) will not be efficient at reducing the absorption of cholesterol *in vivo* [11, 12], whereas non-crystalline preparations [8] and preparations containing micro-sized crystals [13] will be efficient. Certain molecular structural characteristics, such as having 4-desmethyl moiety [14] and not having a double bond at ring position 5 [15], seem to be important for poor absorption of the plant sterols themselves, and for effective cholesterol absorption inhibition. The physical state of plant sterols in the gut matrix is particularly important in light of recent suggestions that plant sterols could interact with specific proteins, such as members of the ABC protein class, possibly implicated in the efflux of cholesterol and plant sterols [16, 17]. Our method for determining plant sterol crystals > 11 μm, in the presence or absence of bile and lysophospholipids, has not been previously published. In model bile systems, cholesterol crystal growth and nucleation have been determined by turbidometry, dialysis, light scattering, ultracentrifugation, and direct counting [18]. Due to milk's viscosity, and possible centrifugal disruption of the food matrix, these earlier approaches were deemed unsuitable. Furthermore, our method conveniently quantified plant sterols using a commercial cholesterol kit, rather than gas chromatography, as the present method is faster, easier, and more adaptable for factor use. During the filtration assay, we found that only 1.3 % of the added sterols could be trapped in an 11 μm filter, and that in the presence of bile salts and lysophospholipids (to emulate the physical state of plant sterols in the intestinal tract) roughly half of this amount became re-solubilized or reshaped in such a way as to pass through the 11 μm filter. Any tiny crystals passing through the 11 μm filter are predicted to have a better probability of becoming resolubilized in the presence of bile salts and lysophospholipids in the gut matrix, since microcrystalline plant sterol preparations are known to lower LDL-C *in vivo* [13].

Before submitting the plant sterols in milks to subjects, we confirmed that these partly filled milks containing plant sterols were also able to reduce the uptake of cholesterol in intestinal Caco-2 cells, in the presence of lysophospholipid, and bile acids, as has been previously reported [19, 20]. The uptake of cholesterol is one component in the overall process comprising cholesterol absorption. The dietary ratio of cholesterol:plant

sterol is a factor affecting plant sterol efficacy in reducing cholesterol absorption and LDL-C *in vivo*. Interestingly, the same observation was apparent in Caco-2 cells.

During the clinical trial, the present method for quantifying cholesterol absorption with stable isotopes offered tracer dosage and sensitivity improvements over earlier methods. Compared to previous clinical trials, we employed a low dosage of oral [^{2}H]cholesterol tracer (15 mg) that was evaluated in the plasma using IRMS. To our knowledge, this is the first clinical trial to employ such a low oral dosage of [^{2}H]cholesterol. Previous authors used oral administration of [^{18}O]cholesterol tracer, a costly and rare tracer [10, 21]. Bosner et al. [9, 22] used 30 mg oral administration of [^{2}H]cholesterol tracer, twice the amount of tracer used presently; and did not achieve greater accuracy in determining isotopic enrichment. Jones et al. [23] used 15 mg [$^2H_7$]cholesterol for intravenous injection, and 90 mg [$^{13}C_2$]cholesterol for the oral tracer. In the present experiment, very low isotopic enrichments of [^{2}H]cholesterol were detected using high sensitivity continuous-flow GC/pyrolysis/IRMS. From our experience, oral intake doses of [$^2H_6$]cholesterol tracer as low as 10 mg could be employed in similar clinical trials.

No plant sterol-mediated effects on cholesterol turnover were apparent. The turnover in the peripheral blood circulation was about 30 % pool/d independent of diet, as reported by Jones et al. [23]. Miettinen et al. [2] reported no changes in cholesterol synthesis after 7 d of stanol ester ingestion, whereas cholesterol absorption decreased from 50 to 22 % in control and stanol treatments, respectively. Similarly, 6 d plant sterol intake during our clinical trial was sufficient to decrease cholesterol absorption, Miettinen et al. [2] showed that even after 1 d, cholesterol absorption was decreased.

As previously described, 1.8 g/d of non-hydrogenated, non-esterified, properly solubilized, soy plant sterols incorporated into milk decreased cholesterol absorption in 16 hypercholesterolemic men from 70 to 41 % in the present trial. Jones et al. [23] similarly observed a high basal absorption of 68.7 % in 15 hypercholesterolemic subjects. In the present work, one subject showed an absorption of 99.4 % during control treatment. This high value was probably overestimated since the d 1 isotopic enrichment value following intravenous [$^{13}C_5$]cholesterol tracer administration was 30 % lower than the mean enrichment value of other volunteers. This value was not excluded from our present calculations; it had no basis for exclusion according to our "Per Protocol Intention To Treat" statistical approach. Interestingly, the basal absorption rate of 70.1 % for the control in the current study was higher than the 55.7 % value we obtained using a similar methodology with other tracers [6]. This supports our speculation that milk may be an effective vehicle for enhancing cholesterol absorption. The large reduction in cholesterol absorption with milk with sterols is similar to what we previously observed with 2.9 g tall stanols plus lecithin in an oil in water emulsion [6]. The variance in response was also similar between the two studies ($P > 0.05$ in the Snedecor F test). Generally, the maximum relative reduction in cholesterol absorption with sterols, stanols and ester varieties in various vehicles is within the range of 37–56 % [1, 4, 24, 25]. Thus, we were within the maximal range of cholesterol absorption inhibition obtained in these previous studies. The observed large inter-subject variation in responsiveness to treatment was known from literature [22, 10]. For example, in a 12-month study with consumption of 2–3 g of stanol esters/d in 153 hypercholesterolemic persons, there were 12 (8 %) non-responders (no decrease in cholesterol), and these non-responders were poor absorbers of dietary cholesterol as evidenced by low cholestanol levels [26]. The extent to which cholesterol is absorbed may be due to different apolipoprotein E phenotypes and age. Apolipoprotein E phenotype was originally shown to be a variable correlated with cholesterol absorption [27]. It was also shown by Vanhanen et al. [28] but not by other authors [29, 30] to be a factor associated with the LDL-C lowering effect of plant sterols in recent trials. Genetic parameters may also explain subject-to-subject differences observed regarding sensitivity to treatment and baseline absorption rates [11]. Numerous studies, including the present one using an original low-fat partly-filled milk matrix, show that plant sterols and stanols can potently lower cholesterol absorption. This is expected to lead to decreased total and LDL cholesterol [13, 28, 31].

Conclusions

We used newly developed methodology, or methodology previously used for other food structural characterization purposes, to characterize and quantify plant sterol crystals in milks. Our clinical trial using stable isotope approach and ^{13}C and ^{2}H IRMS detection to quantify cholesterol absorption also had improvements over earlier techniques. We showed that non-esterified plant sterols solubilized in a partly-filled milk matrix using our proprietary crystal retardation and emulsification system resulted in a product with very few large crystals in the presence of lysophospholipids and bile salts. This milk product inhibited both the uptake of cholesterol in intestinal Caco-2 cells and the absorption of cholesterol in hypercholesterolemic men in the present clinical trial. We are currently evaluating the LDL-lowering properties of our particular plant sterol milk preparations in clinical trial. LDL-lowering properties of milk derived product with sterols [32, 33] and stanol esters [34, 35] have already been demonstrated. Adding properly solubilized non-esterified plant sterols to low fat

milk offers potential advantages over the traditional commercial approach of adding plant sterol or stanol esters to high fat foods from both nutritional and economic perspectives.

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