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ORIGINAL CONTRIBUTION

Effects of non-esterified stanols in a liquid emulsion on cholesterol absorption and synthesis in hypercholesterolemic men

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Summary *Background* Numerous studies have shown that dietary plant sterols (phytosterols and phytostanols) and their esters can decrease cholesterol absorption. However, few researchers have examined the effects of plant sterols on cholesterol absorption and synthesis using stable isotope tracers, instead of relying on endogenous pathway precursors. Further, we have worked with non-esterified lecithin-solubilized stanols as opposed to the more frequently studied esterified sterols and stanols. The vehicle was an oil-in-water liquid emulsion rather than the more common spread vehicle typically employed. *Aim of the study* To determine the effects of relatively low doses of lecithin-solubilized non-esterified stanols in liquid emulsions on cholesterol absorption and synthesis in mildly hypercholesterolemic subjects. *Methods* In a randomized, double blind crossover design, 12 mildly hypercholesterolemic men received either a free phytostanol supplement (3 g/d in 3 servings) or a control treatment for 3 days. Cholesterol endogenous synthesis rate was determined using the rate of incorporation of deuterium from body water into newly formed cholesterol molecules. Cholesterol absorption at the intestinal level was determined using the dual isotope method using ^{13}C cholesterol injected intravenously and ^{18}O cholesterol given orally. *Results* Cholesterol absorption was 55.7 ± 6.5 % for the control and 33.5 ± 5.3 % for the phytostanol treatment. This massive reduction of the cholesterol absorption did not induce, on average, a difference in cholesterol endogenous synthesis which was measured at 0.074 ± 0.0015 pool/d for plant sterols and 0.0736 ± 0.0015 pool/d for controls ($p > 0.05$). *Conclusions* The results demonstrated that lecithin-solubilized stanols administrated during a short period of time (3 days) in an oil-in-water emulsion can dramatically decrease cholesterol absorption, without a consistent, concomitant increase in synthesis, which is highly suggestive of effective LDL cholesterol lowering. The effects of synthesis should be verified in a longer study with more subjects.

Key words Plant sterols – stanols – phytostanols – cholesterol – absorption – synthesis – stable isotopes – IRMS – plant sterol – hypercholesterolemia – sitostanol

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Abbreviations

APE, atom percent excess
ASR, absolute synthesis rate
BMI, body mass index
FSR, fractional synthesis rate
GC-C-IRMS, gas chromatography-combustion-isotopic ratio mass spectrometry
GC-pyrolysis-IRMS, gas chromatography-pyrolysis-isotopic ratio mass spectrometry
HDL-C, high density lipoprotein cholesterol
LDL-C, low density lipoprotein cholesterol
MPE, mol percent excess
TC, total cholesterol
TG, triacylglycerol

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Introduction

Dietary plant sterols (phytosterols and phytostanols) and their esters have been widely shown in literature to efficiently lower cholesterol percent absorption. Typically, 0.5–3.0 g/d of plant sterols produce a 30–80 % lowering of the cholesterol absorption and a corresponding 10–15 % lowering of the LDL cholesterol [1–3]. The efficacy of the dose given has been shown to be highly dependent on the physical form and structure in which the plant sterols are dissolved or suspended. As was shown by Ostlund et al. [4], sitostanol administered as a crystalline powder is less efficient at inhibiting cholesterol absorption than sitostanol solubilized in a phospholipid matrix. Finally, it is known that individual absorption responses to the exact same treatment will vary because of large subject to subject variation in absorption [5]. In some studies, following plant sterol feeding, there is a compensatory increase in cholesterol synthesis, whereas in other studies this does not occur. In a study [6] where hypercholesterolemic subjects and normcholesterolemic subjects were fed sitostanol-containing tall oil plant sterols, large variations in fractional synthesis rate (FSR) were observed. For some subjects the FSR increased sharply, in others it remained unchanged, while for still others, it decreased. Among the factors that may explain the different responses to the plant sterol treatment are glucose and insulin parameters [7] or habitual dietary intakes of cholesterol (as shown herein).

The aim of this work was to simultaneously assess the percent cholesterol absorption and synthesis by GC-IRMS [5] following a short-term feeding of a plant stanol mixture in mildly hypercholesterolemic subjects. The work is original in that cholesterol absorption and synthesis were assessed simultaneously using newly developed methodology; the plant stanols were non-esterified and lecithin-solubilized, whereas most recent work has been conducted with esterified sterols and stanols; and finally, the vehicle was an oil-in-water liquid emulsion rather than the more common spread vehicle employed by most other authors.

Methods

Reagents and chemicals

All solvents were of analytical grade. Hexane, decane and diethyl ether "ultra resi analyzed" were obtained from J. T. Baker (Mallinkrodt Baker, Holland); TLC plates, chloroform, methanol, and acetic anhydride were from Merck (Darmstadt, Germany). Ethanol, pyridine and acetic acid were from Fluka Chemie (Buchs, Switzerland) and Sylon HTP was from Supelco, Bellefonte, PA. Standard cholesterol and 5α-cholestan were from Sigma Chemical & Co (St Louis, USA). Standard hydrogen gas (working standard) was obtained from Carbagas (Lausanne, Switzerland). The certified calibration sample for H_2 was obtained from Messer Griesheim (Duisburg, Germany). 5α-Cholestan was from Steraloids Inc (Newport, USA). [23–27]-$^{13}C_5$-cholesterol (99 at %) was purchased from Mass Trace (Woburn, USA), ^{18}O-cholesterol (87 at %) from Eurisotop (Saint Aubin, France) and ^2H_2O (99.8 at %) from Cambridge Isotope Laboratories (Andover, USA). The stanols were derived from tall oil and comprised 77.7 % β-sitostanol, 20.9 % campestanol and 1.4 % of other stanols (B. C. Chemicals LTD, Vancouver, B. C). The overall purity of the stanol powder was 98.3 %.

Subjects

The subjects were 12 mildly hypercholesterolemic men aged 42 to 62 years (average 49.8 ± 6.3 yrs). They were screened for total serum cholesterol (TC), and triglycerides (TG). TC and TG ranged from 5.8 mmol/L to 8.2 mmol/L (average 6.9 ± 0.2 mmol/L) and from 0.59 to 3.23 mmol/L (average 1.45 ± 0.22), respectively. Body weights ranged from 57.7 to 85.0 kg (average 74.8 ± 2.4 kg) and their body mass index (BMI) ranged from 22.5 to 27.4 kg/m^2 (average 25.2 ± 5.0 kg/m^2). Exclusion criteria were BMI greater than 30, total cholesterol below 5.6 mmol/L or above 8.4 mmol/L, hemophilia, use of cholesterol-altering medication, renal or liver disease, diabetes, smoking more than 1 pack of cigarettes per day and drinking more than 2 glasses of alcoholic beverage per day. The experimental protocol was approved by the Nestlé Ethical Committee (Lausanne, Switzerland).

Preparation of the stable isotopically labeled tracers

Ready-to-inject 7.5 mL syringes containing 30 mg of [23–27]-$^{13}C_5$ cholesterol dissolved in Intralipid parenteral emulsion were prepared by the Lausanne University Hospital Center. On day 1, the whole content was injected intravenously over 10–20 min. The sterility and final concentration of cholesterol in both batches of syringes was determined by the Lausanne University Hospital Center.

The [^{18}O]-cholesterol for oral administration was dissolved in sunflower oil at 30 mg/g by sonicating at 65 °C and the solution was used immediately. About 1 g of solution containing ca. 30 mg of ^{18}O-cholesterol tracer was spread on a piece of white bread on an analytical balance. It was given to subjects together with breakfast on day 1.

Protocol and diet

The experimental design was of a double blind randomized crossover (Fig. 1) nature. Each phase consisted of a 4-day feeding followed by a 6-week washout. In a first period, the twelve subjects were randomly distributed into two groups (n = 6 each) and assigned to either a plant stanol containing beverage (3 g/d) or to a control. In a second period, treatments were switched. The plant stanols were dissolved in phospholipids and vegetable oil using in-house technology, then emulsified with water. The serving size of ~30 g given at each major meal provided the subjects with approximately 1.0 g plant stanols (983 mg if correcting for purity of the starting material). The control emulsion, provided with each major meal, contained vegetable oil emulsified with 260 mg whey proteins and 87 mg vanilla aroma. All meals were prepared for the subjects. Breakfast and lunch were given under supervision. Evening meals were administered refrigerated in an insulated bag for consumption at home. Meals were based on precooked ready-to-eat frozen meals using a 4-day meal cycle. The average energy provided by the meals was calculated at 2685 ± 178 Kcal/d using food composition tables. Average cholesterol intakes during the study were measured at 255 ± 8 mg/d by gas chromatographic analysis. With the exception of day 0, where no samples were collected, blood samples were collected every morning before breakfast at approximately 08:00. On day 1 at 08:00, a fasted blood sample was taken from the subjects. Immediately afterwards, they received the ^{13}C-cholesterol infusion followed by oral administration of the two other tracers. The ^{18}O-cholesterol was dissolved in sunflower oil and $^{2}H_2O$ was dissolved in orange juice. ^{2}H-, ^{18}O- and ^{13}C-cholesterol enrichments were measured in plasma. At the beginning of each phase of the study we also measured body weight, height, blood serum lipid levels (TC, low density lipoprotein cholesterol [LDL-C], high density lipoprotein cholesterol [HDL-C], triacylglycerols [TG]).

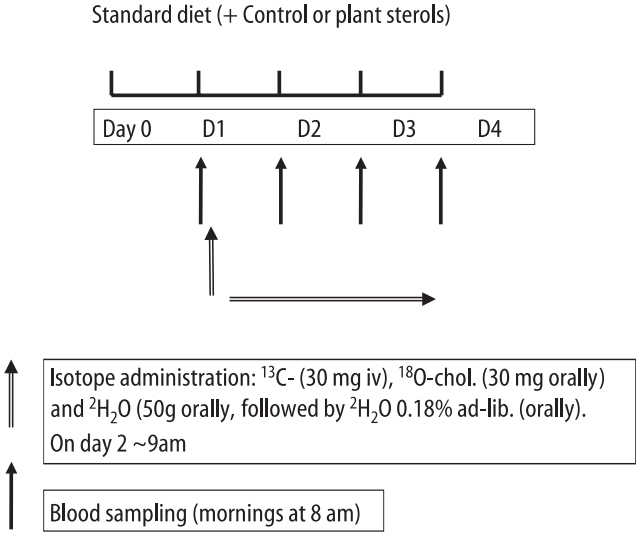

Fig. 1 Experimental design of study. There were 12 mildly hypercholesterolemic men. Half received controlled diet plus placebo; half received controlled diet spiked with plant sterols. After 6 weeks, the treatments were crossed over.

Assessment of the subjects' dietary cholesterol intakes

Each subject's habitual intake of cholesterol was determined using a food frequency questionnaire [5]. The compliance of the subjects to the diets and treatments was checked by direct observation and encouraged by use of a diary that the subject had to complete to monitor compliance. Overall compliance was very good and no subjects dropped out.

GC-FID measurements of the cholesterol contents of the study diet

The homogenized freeze-dried food items were solubilized at 70 °C in 1 N NaOH in ethanol/water 9:1 (v/v) and 5α-cholestan (200 nmol) was added as an internal standard. After water addition, neutral sterols were extracted with cyclohexane and the organic layer was recovered and dried under nitrogen. Then the samples were silylated with Sylon HTP and allowed to react 30 min at room temperature. The reagent was evaporated and the derivatized sample was dissolved in n-decane. One microliter was injected in split mode (1/20) into a J&W DB5 30 m × 0.25 mm GC column (MSP Friedli, Koeniz, Switzerland). Oven program was 250 °C (1 min), 2 °/min, 280 °C (3 min), 20 °/min, 300 °C (6 min). Helium was used as carrier gas at a pressure of 10 psi.

Measurement of total cholesterol, LDL-C, HDL-C and TG

Total cholesterol was measured using a Cobas Bio (Hoffman La Roche, Basel, Switzerland) after hydrolysis using cholesterol esterase. For HDL- and LDL-cholesterol, the lipoproteins were first disrupted using surfactants and the cholesterol was precipitated, then redissolved and analyzed with the Cobas.

Quantification of total plant sterols in plasma

Total plant sterols (squalene, cholestanol, brassicasterol, lathosterol, campesterol, stigmasterol, lanosterol, β-sitosterol and sitostanol) were determined in plasma

following completion of each phase of the study after extraction according to [8] and quantification by GC/MS, using epicoprostanol as the internal standard.

Measurement of the endogenous cholesterol synthesis and absorption

Cholesterol endogenous synthesis rate was determined using the rate of incorporation of deuterium from body water into newly formed cholesterol molecules [5]. Deuterated cholesterol was measured by GC-pyrolysis-IRMS after extraction from plasma and acetylation. Cholesterol absorption at the intestinal level was determined using the dual isotope method using ^{13}C cholesterol injected intravenously and ^{18}O cholesterol given orally. ^{13}C-cholesterol was determined by GC-combustion-IRMS, whereas ^{18}O-cholesterol measurements were carried out by GC-pyrolysis-IRMS.

Quantitative estimation of crystals in the emulsions

Ostlund et al. [4] observed that crystalline sitostanol had one fourth the efficiency of solubilized sitostanol in inhibiting cholesterol absorption. Therefore, the presence of crystals was checked daily in the emulsions by polarized light microscopy. A small drop of emulsion was spread between two glass plates and examined under a Zeiss Axioplan microscope in H mode (polarized light). In almost all cases, some crystals were visible (Fig. 2). It was not possible to estimate the ratio of solubilized to crystalline forms on the basis of the micrographs. However, there was no major differences between the two experimental periods with respect to the amount of crystals visible by polarized microscopy.

Statistical analysis

Analysis of variance was used to test the effect of treatment, period, and treatment sequence on absorption and synthesis [9]. This analysis assumes that no biological carry-over effect of the treatment is present, which can safely be assumed given the length of the washout period (6 wks) and the fact that plant sterols have predominately acute affects on cholesterol absorption. In trials measuring LDL cholesterol, levels typically return to baseline within 2 wks following cessation of plant sterols; thus any affects on synthesis should not be present following a 6-wk washout period. The relationships between absorption and synthesis or cholesterol intakes were analyzed using linear regression analysis. Unless stated otherwise, all values are given as average ± standard deviation (SD).

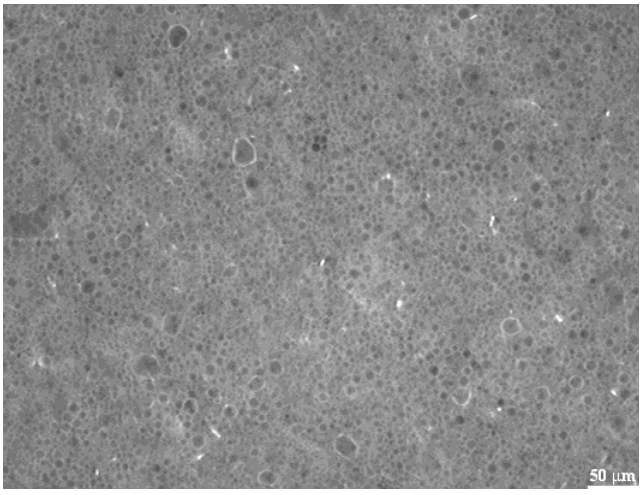

Fig. 2 Polarized micrographs showed a few crystals in the plant stanol preparation (Zeiss Axioplan microscopy). The emulsions were freshly prepared each morning for the breakfast and before noon for the lunch and the dinner.

Results and discussion

Blood lipid parameters

Blood lipids were measured during each phase of the study in order to be able to calculate pool size of cholesterol and check that the subjects had the same physiological condition during phase I and II of the study. TC was not significantly less in phase II (6.2 ± 0.4 mmol/L) as compared to phase I (7.0 ± 0.3 mmol/L). The HDL-C decreased from 1.29 ± 0.08 mmol/L to 1.19 ± 0.09 mmol/L (not statistically significant) and LDL-C decreased from 4.66 ± 0.28 mmol/L to 3.53 ± 0.32 mmol/L ($p = 0.01$). TG were decreased from 1.67 ± 0.20 mmol/L to 1.40 ± 0.18 mmol/L, but the difference was not statistically significant. The observed lowering of LDL-C was likely due to the subjects consuming more cholesterol-conscious diets during the 6-wk washout period, as opposed to any impact of the 4-day plant sterol feeding. Typically, a minimum of 10–14 days is required for plant sterol-induced changes in LDL-C. Dietary consumption was not directly assessed during the wash-out period.

Plant sterols in plasma

The plasma markers cholestanol and lathosterol were assessed to determine if they were statistically correlated with cholesterol absorbance and synthesis, respectively, as previously described [3]. A meaningful treatment effect was found for stigmastanol (which represents 77.7 % of the dietary plant stanol intake), after log-transformation to correct for non-normal distribution of plasma stigmastanol levels ($p < 0.001$). Aver-

age plasma values following plant stanol and control treatments were 0.41 and 0.11 mg/mL, respectively.

Dietary plant stanols did not however decrease levels of cholestanol (a marker for decreased cholesterol absorption), despite reducing cholesterol absorption using stable isotope markers. Cholestanol was also not correlated with cholesterol absorption. Cholestanol has been shown to be a useful marker in several studies [2, 10–12] and it is not clear why there was no correlation with cholesterol absorption in the present study. We will measure cholestanol levels in an upcoming clinical trial with similar vehicles, but for a longer time period and with more subjects, to assess more precisely the variation in this parameter.

There was a positive association between plasma lathosterol (log scale) and the fractional synthesis rate of cholesterol synthesis (FSR) which is consistent with published literature [3], showing that plasma lathosterol can be a positive marker for cholesterol synthesis. Relative to the control, dietary plant sterols did not increase levels of plasma lathosterol, nor the FSR, consistently indicating that plant sterols did not cause a compensatory increase in cholesterol synthesis, over the brief 3-day time periods. FSR (lathosterol) has been shown to be upregulated following plant sterol consumption in some studies [6, 11], but not others [13, 14].

Impact of treatment on cholesterol absorption

The absorption measured for each individual on day 4 (three days after tracer) is shown in Fig. 3. Percent absorption values of control and plant sterol treatments were heterogenous. Values for phytostanol treatment ranged from 7.5 % to 73.5 %. For control, values varied from 36 % to 100 %. The value above 100 % (subject P, control) was known to be due to incomplete injection of ^{13}C tracer and therefore was validly removed for the calculations. Precision achieved on the percent cholesterol absorption was 1.9 % (absolute terms). There were striking differences in cholesterol absorption between subjects, irrespective of treatments. For example, subject P showed high cholesterol absorption values for both treatments, whereas subject K showed low values for both treatments. The absorption differences induced by treatment (sensitivity to treatment) showed large subject-to-subject differences. For example, subject E was very sensitive to treatment (52.5 %, absolute terms), whereas subject I was insensitive (minus 2.8 %). The large inter-subjects variations in responsiveness to treatment is known in the literature. For example, in a 12-month study with consumption of 2–3 grams 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

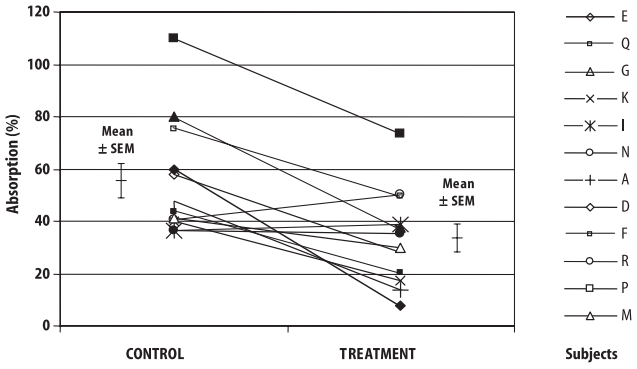

Fig. 3 Impact of phytostanol treatment on cholesterol absorption of the individuals. Subjects E–N received first the diet spiked with plant sterols, then the control diet plus placebo. Other subjects (A–M) received first control then plant sterols diet. After 6 weeks, the treatments were crossed over. Cholesterol absorption is expressed in % of administered dose.

levels [11]. The extent to which cholesterol is absorbed may be due to different apoE phenotypes and age. Apo E phenotype was originally shown to be a variable correlated with cholesterol absorption [15]. It was also shown by Vanhanen et al. [16] but not by other authors [12, 17] to be a factor associated with the LDL cholesterol 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 [18].

Absorptions in our study averaged 55.7 ± 6.5 % for the control treatment and 33.5 ± 5.3 % for the plant sterol treatment. This 39.8 % lowering relative to control was highly statistically significant ($p < 0.01$). No period effect was recorded. As shown by Ostlund et al. [4], sitostanol or similar phytostanols or phytosterols must be dissolved in an adequate matrix to be maximally efficient at inhibiting cholesterol absorption. The authors found a 34 % and 37 % lowering of the absorption using 300 mg and 700 mg of sitostanol in lecithin micelles, respectively. In contrast, when they administered 1 g/d crystalline sitostanol powder, the reduction was only 11 %. This lack of efficiency of undissolved plant sterols was previously observed by Denke et al. [19] who administered 3 g/d of sitostanol powder in gelatin capsules to mildly hypercholesterolemic men receiving 200 mg dietary cholesterol per day (instead of 250 mg/d in our case), and observed no effects on cholesterol absorption. Similarly, chronic feeding of 700 mg unesterified sitostanol/d in rapeseed oil had no significant effect on cholesterol absorption [18]. However, caution must be exerted when comparing such studies to distinguish between long-term (effects on cholesterol levels) and short-term studies (effects on cholesterol absorption), to consider the character of the plant sterol crystalline form (large or small crystal size), and whether the plant sterol was given in gelatin capsules or food matrix).

Relationship between cholesterol absorption and habitual cholesterol intakes

Using linear regression analysis of absorption as a function of intake, we found an inverse relationship (p = 0.05) between intake (from food frequency questionnaire) and cholesterol absorption on day 3 after tracer administration (Fig. 4). This relationship between habitual diet and baseline absorption of cholesterol was previously examined in a study where cholesterol percent absorption was compared between vegetarians and normal control subjects [20]. The authors did not find a significant difference between groups (control 49.6 ± 2.3 %; vegetarians 45.4 ± 3.5 %) for this parameter, which is in contrast to our finding. The relationship between habitual intakes of cholesterol and cholesterol absorption should be confirmed in future investigations.

Impact of treatment on cholesterol synthesis rate

We calculated cholesterol endogenous synthesis rates after 24 h-throughout after Jones et al. [21]. Precision achieved for our FSR measurements was ± 0.06 % pool/d and ± 14 mg for ASR. The treatment produced, on average, no significant difference in FSR nor ASR. Average values of FSR for phytostanol and control treatments were 7.49 ± 0.52 % pool/d and 7.36 ± 0.52 % pool/d, respectively. The average values of ASR for phytostanol and control treatments were very close (680.95 and 681.05 mg/d, respectively). As for absorption, there were some large inter-subject variations in cholesterol synthesis among participants (Fig. 5). FSR ranged from 4.7 % (subject A) to 10.9 % pools/d (subject E). A strong isotopic memory effect was observed in measurement of deuterated cholesterol between period 1 and 2 of the study. Indeed, deuterated cholesterol was synthesized from remaining labeled precursor available in the first period. Therefore, baseline samples of period 1 and 2 averaged –284 ± 13 δ and +32.7 ± 69.8 δ, respectively. Baseline samples of period 2 were also less homogenous than those of period 1 as their enrichment was dependent on subject's cholesterol and water turnover. Precursor $^{2}H_2O$ has a rate constant of 0.061 d^{-1} for normal men [22]. Using this exponential rate, enrichments should have declined from 0.12 to 0.006 APE. However, this is still 40 % higher than natural deuterium enrichment. Thus, for the duration of the study, enrichment level of deuterated cholesterol was higher than natural background. This however was not an analytical problem as baseline values were subtracted and Jones et al. [6] successfully used even shorter washout times.

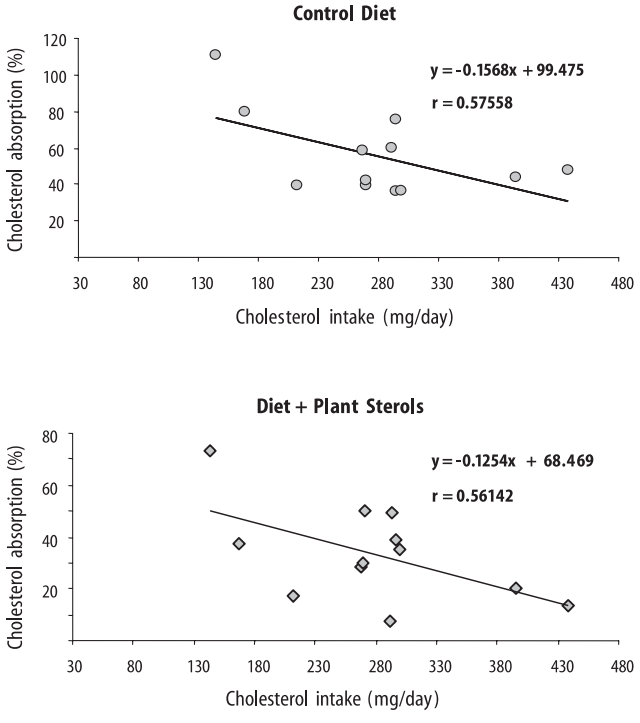

Fig. 4 Relationship between cholesterol absorption and cholesterol habitual intakes. Cholesterol absorption is expressed in % of administered dose and cholesterol intake is expressed in mg/d.

Conclusion

To assess cholesterol absorption in mildly hypercholesterolemic men we determined the ratio of $^{13}C_5$-cholesterol (intravenous) to ^{18}O cholesterol (oral) by GC-pyrolysis-IRMS and GC-combustion-IRMS. Cholesterol endogenous synthesis was measured using the incorporation rate of deuterium from body water. Herein we showed that treatment of human subjects with phospholipid-solubilized plant stanols produced a statistically and biologically significant lowering of cholesterol

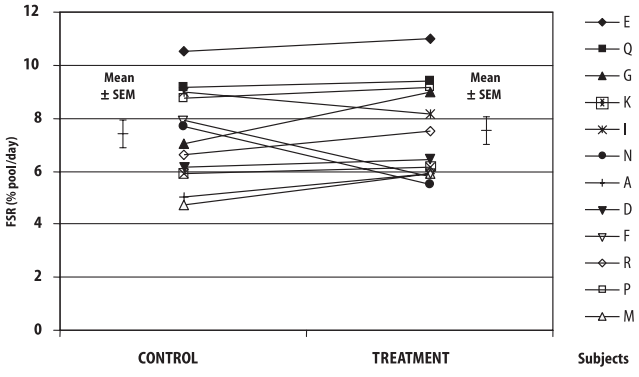

Fig. 5 Impact of phytostanol treatment on cholesterol synthesis of the individuals. Subjects E–N first received the diet spiked with plant sterols, then the control diet plus placebo. The other subjects (A–M) first received the control diet then the plant sterols-enriched diet. After 6 weeks, the treatments were crossed over. Cholesterol synthesis is expressed in % pool/d.

absorption, without a compensatory stimulation of cholesterol synthesis. However, the short-time course of the present experiments may not have been sufficient to reach steady-state conditions and reliably assess cholesterol synthesis. Further, large variations in compensatory cholesterol synthesis (present data and refs. [6, 23] may have precluded attaining statistically significant results. This work has shown that properly solubilized free sterols can effectively lower cholesterol absorption, which should lead to reduced LDL cholesterol levels [24, 25]. In a recent work, Vanstone et al. [26] showed that unesterified sitosterol reduced cholesterol absorption more effectively than sitostanol (56.1 % vs. 33.3 % reduction, respectively), but that both forms of sterols lowered LDL cholesterol equivalently, indicating that reduction of cholesterol absorption is not the only component responsible for LDL cholesterol lowering.

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