# Lathosterol in plasma measures cholesterol synthesis and identifies the efficiency of dietary phytosterols in reducing the plasma cholesterol concentration

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**Research**

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## Abstract

# Background

Because the plasma campesterol/cholesterol ratio does not differ between groups that absorb different amounts of cholesterol measured by the gold standard isotopic procedure we investigated whether the intestinal absorption of phytosterols (PS) depends on the body's cholesterol synthesis rate.

# Methods

38 volunteers (58 ± 12 years; low-density lipoprotein cholesterol (LDL-C) ≥ 130 mg/dL) were randomly assigned to consume 400 mL/day of soy milk or soy milk + PS (1.6 g/day) for 4 weeks in a double-blind, placebo-controlled, cross-over study. Blood samples were collected and markers of phytosterol (PS) absorption and non-cholesterol sterol synthesis precursors measured.

# Results

PS treatment reduced plasma total cholesterol concentration (-5,5%, $p < 0.001$), LDL-C (-7.6%, $p < 0.001$), triglycerides (-13.6%, $p < 0.0085$), and apolipoprotein B (apo B) (-6.3%, $p < 0.008$), without changing high density lipoprotein cholesterol (HDL-C concentration). The lathosterol-to-cholesterol ratio in serum predicted the serum cholesterol response to PS feeding where high basal cholesterol synthesis was associated with lack of response of plasma cholesterol to PS in the diet. Cholesterol synthesis being elevated in the placebo phase in non-responders to dietary PS indicated they were resistant to further synthesis rise, whereas responders, because they have lower synthesis rate than non-responders in the placebo phase, are capable expanding synthesis under the effect of alimentary PS.

# Conclusions

responders absorbed more PS than non-responders likely resulting from responders delivering less endogenous cholesterol than non-responders into the intestinal lumen that facilitates greater absorption of PS by the intestine.

## Introduction

It is well known that phytosterols (PS) reduce plasma total cholesterol and low-density lipoproteins cholesterol (LDL-C) [1] [2] [3] due to displacement of cholesterol from the intestinal lumen micelles [4] [5] and for exerting their molecular actions inside enterocytes and hepatocytes [6]. Moreover, it was also demonstrated that PS could induce LDL receptor expression [7]. Because of the beneficial effects on lipid profile, the 2001 National Cholesterol Education Program (NCEP ATP-III) (National Cholesterol Education Program Expert Panel) included PS in the dietary treatment for moderate hypercholesterolemia [8]. However, after the publication of this guideline, some reports have shown that high PS plasma and tissue

concentrations related to increase on cardiovascular risk [9] [10]. Nevertheless, Bombo et al. [11]clearly showed that LDL receptor knockout mice fed a high saturated fat diet supplemented with 2 g of PS did not accumulate sterols on aortic valve or arterial wall. On the other hand, PS treatment prevented atherosclerotic lesion development in hypercholesterolemic mice models [11].

Plasma concentrations of PS, and of non-cholesterol sterol precursors of cholesterol synthesis, respectively, markers of the intestinal cholesterol absorption and of the body's cholesterol synthesis have been utilized as markers of atherosclerotic cardiovascular disease [12] [13] [14] [15] [16] [17]. Nonetheless, objections of diverse nature have been raised on the interpretation of the results utilizing plasma PS measurements as markers of intestinal cholesterol absorption and non-cholesterol precursors as markers of cholesterol synthesis. In this regard, high plasma PS were reported inappropriate cholesterol absorption surrogates because PS in the diet lowered the intestinal cholesterol absorption rate [18]. Furthermore, in an investigation on moderate hypercholesterolemia, the plasma campesterol/cholesterol ratio did not differ between groups that absorb different amounts of cholesterol measured by the gold standard isotopic procedure [19]. Therefore, elevation of plasma PS may represent defect in the body's efficiency to re-excrete PS and not an increase in the intestinal absorption of dietary cholesterol. In this regard it is important to take into account that the biliary excretion of plasma PS was more efficient than of plasma cholesterol [20]. Retention of PS in plasma and other tissues may not be due to their increased absorption by intestinal mucosal cells. Consequently, it is questionable whether increased intestinal uptake of PS relates to premature atherosclerosis in humans. Accordingly, cardiovascular disease (CVD) mortality related reciprocally with PS (sitosterol) as a cholesterol absorption marker: the high desmosterol/sitosterol ratio suggested high cholesterol synthesis and low absorption associated with high total and CVD mortality [21]. Nonetheless, low serum lathosterol, but not absorption markers, had also been associated with increased CVD [17]. In contrast, as expected, increased excretion of endogenous cholesterol, which represents increased synthesis, was negatively associated with carotid intima-media thickness [22]. Consequently, the validity of the role on cardiovascular risk of the serum sterol synthesis and absorption markers remains questionable.

In one study in children dietary PS altered the PS concentration in serum but not the concentrations of cholesterol synthesis precursors [23]. Contrarily, in one study on low cholesterol synthesis cases during the placebo period it was shown that high intestinal sitosterol absorption occurred on PS feeding [24] but the latter was not mentioned in the other study [25].

To investigate these previous discrepancies, we measured in plasma the concentrations of precursors of cholesterol synthesis and PS as markers of intestinal absorption of cholesterol in the placebo phase and after ingestion of PS.

# Methods

# Subject recruitment

The individuals (n = 38: female 31 and male 7) aged 38–77 years were recruited in the Dyslipidemia Outpatient Unit of the Endocrinology and Metabolism Service of the Clinical Hospital of the University of Sao Paulo, Brazil; members of the staff of the University of Sao Paulo, Brazil were also included. The participants

were invited for screening of body weight and height; blood samples were collected for lipids profile determination. The inclusion criteria were: body mass index (BMI) between 20 and 30 kg/m$^2$; total cholesterol between 200–300 mg/dL, LDL-C concentrations ≥ 130 mg/dL, and triglycerides ≤ 250 mg/dL (Table 1). Exclusion criteria were: use of lipid-lowering medication or a prescribed diet in the last month; alcohol abuse or illicit drug users; pregnancy or breast feeding; smoking; diabetes mellitus, hypothyroidism, renal or hepatic diseases or participation in another lifestyle or pharmaceutical intervention studies. All subjects provided informed written consent. The Ethics in Research Committee of the Hospital of the University of Sao Paulo Medical School approved the study protocol (CAPPesq n° 112/06).

Table 1
Subjects characteristics at baseline

| Parameter | Mean ± SD |
|---|---|
| n | 38 |
| Age (years) | 58 ± 12 |
| Weight (Kg) | 64 ± 10 |
| BMI (kg/m$^2$) | 25.3 ± 2.4 |
| Total cholesterol (mg/dL) | 245 ± 34 |
| Triglycerides (mg/dL) | 141 ± 53 |
| LDL-C (mg/dL) | 165 ± 34 |
| HDL-C (mg/dL) | 49 ± 12 |
| BMI, body mass index; LDL-C, low-density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol | |

# Study Design

The present study was a randomized, double-blind, placebo-controlled dietary intervention trial with each study period lasting 4 weeks. Initially, all the participants were submitted to a 3-week run-in period in which they received the placebo product (soy milk) to test adherence to the protocol. After baseline period the individuals were randomly assigned to placebo or to phytosterol groups for 4 weeks; after that a reverse sequence was immediately carried out. Placebo group received 400 mL of soy milk daily; phytosterol group received 400 mL of soy milk enriched with 1.6 g of PS, as follow: 78% β-sitosterol-ester, 13% sitostanol-ester, 5.3% campesterol-ester and 0.5% campestanol-ester (Table 2). Blood samples were drawn for biochemical analysis from fasting participants on the last day of each period study. All participants were advised to maintain body weight and follow a normocaloric diet based on the NCEP-ATPIII recommendation [8]: 30% of energy as fat, < 10% of energy as saturated fat, and < 300 mg cholesterol/day and was recommended not to consume products enriched with phytosterol during the study. Nutritional monitoring was carried out by a registered dietitian using a 24-hour dietary recall to ensure adherence to the prescribed diet and also to

estimate the food intake. Soy milk was weekly supplied at the same day of the body weight measurement; patients were instructed to consume the soy milk or PS-enriched soy milk twice daily, at lunch and dinner.

Table 2
Soy milk nutritional composition per portion (200 mL)*.

| Nutritional composition | Soy milk | Soy milk + PS |
|---|---|---|
| Energy (kcal) | 138 | 144 |
| Protein (g) | 6.5 | 6.5 |
| Total fat (g) | 4.4 | 5.0 |
| Polyunsaturated fat | 2.3 | 2.5 |
| Monounsaturated fat | 1.0 | 1.1 |
| Saturated fat | 0.7 | 0.9 |
| Trans fatty acid | 0 | 0 |
| Cholesterol (mg) | 0 | 0 |
| Carbohydrates (g) | 18.2 | 18.2 |
| Total sugar | 14.1 | 14.1 |
| Lactose | 0 | 0 |
| Phytosterol (g) | 0 | 0.8 |
| β-sitosterol-ester | | 0.63 |
| Sitostanol-ester | | 0.10 |
| Campesterol-ester | | 0.05 |
| Campestanol-ester | | 0.005 |
| Sodium (g) | 0.1 | 0.1 |
| *Provided by Nestle Company, São Paulo, Brazil | | |

# Blood Sampling

After fasting for 12 hours, blood samples were collected into tubes containing Ethylenediamine tetraacetic acid (EDTA). Plasma was immediately separated by centrifugation (1300 *g*, 15 min, 4 °C; RT6000B; Sorvall Instruments, DuPont Co, Newton, CT), and the following preservatives were added: 0.25% chloramphenicol plus 0.5% gentamycin (20 µL/mL), 2 mmol benzamidine/L (5 µL/mL), 10 mmol phenyl-methyl-sulfonyl fluoride/L (0.5 µL/mL), and aprotinin (0.5 µL/mL). For HDL-C measurements, plasma was mixed and kept at room temperature for 10 min and then centrifuged (*700 g*, 30 min, 4ºC). Aliquots (500 µL) were stored at -70ºC.Total plasma and serum were stored at -70ºC. All measurements were performed in duplicate at the end of the study. All samples from one subject were analyzed within the same analytical run.

# Serum Sterols Analyses

Plasma precursors of cholesterol synthesis (desmosterol, lathosterol) and phytosterols (campesterol and sitosterol) were measured in samples (100 µL) added 5α-cholestane (1 µg) as the internal standard, hydrolyzed with KOH in ethanol (1 mol/l, 1 ml) at 60 °C (1 h) and extracted with hexane. Sterols were derivatized with a sylilating solution (pyridine and BSTFA (N,O-bis (trimethylsilyl) trifluoroacetamide) + 1% TMCS (trimethylchlorosilane) (1:1, v/v) (Supelco 33155-U) for 1 h at 60 °C [28]. The quantification was performed comparing the peak areas of the standard curve and corrected for internal standards. Plasma non-cholesterol sterols (µg) were expressed as ratio of plasma total cholesterol (mg).

# Statistical analysis

Comparisons between the placebo and phytosterol groups were analysed by paired Student's t test. The influence of degree of hypercholesterolemia over the PS response and PS response patterns related to LDL-C were analysed by unpaired Student's t test. Data are shown as means and standard deviation. The analyses were performed utilizing the GraphPad Prisma version 4.00 and significance level considered as $p < 0.05$.

## Results

The study was initiated with 40 subjects but two of them were excluded for presenting more than 5% of weight variation along the study. The body weight and the BMI of the participants remained unaltered throughout the study (Table 3). As demonstrated in several studies, PS reduced total cholesterol, LDL-C, apo B and triglycerides without affecting HDL-C plasma concentrations. As expected, PS supplementation increased plasma lathosterol as well as campesterol and β-sitosterol (indicating compliance to the diets). Lathosterol increased in plasma as a consequence of the blockade of the absorption of cholesterol exerted by PS ingestion. However, the lathosterol / phytosterols ratios decreased due to the higher absorption of PS.

Table 3
Body weight, BMI, biochemical analysis, plasma sterol concentrations of moderately hypercholesterolemic patients.

| | n | Placebo | Phytosterol | p |
|---|---|---|---|---|
| Body weight (kg) | 38 | 64.9 ± 10.2 | 65.1 ± 10.3 | 0.08 |
| BMI ($kg/m^2$) | 38 | 25.4 ± 0.4 | 25.4 ± 0.4 | ns |
| Total cholesterol (mg/dL) | 38 | 261 ± 7.1 | 244 ± 5.8* | < 0.001 |
| HDL-C (mg/dL) | 38 | 46 ± 1.7 | 48 ± 1.9 | ns |
| LDL-C (mg/dL) | 38 | 183 ± 5.9 | 169 ± 5.2* | 0.001 |
| ApoB (mg/dL) | 38 | 126 ± 3.7 | 118 ± 3.2* | 0.006 |
| Triglycerides (mg/dL) | 38 | 154 ± 10 | 133 ± 7* | 0.008 |
| **Plasma sterols expressed as µg/mg cholesterol** | | | | |
| Lathosterol | 38 | 1.53 ± 0.09 | 1.69 ± 0.06* | 0.012 |
| Campesterol | 38 | 1.96 ± 0.12 | 2.34 ± 0.11* | 0.02 |
| β-sitosterol | 38 | 1.64 ± 0.09 | 2.02 ± 0.09* | < 0.001 |
| Lathosterol/campesterol ratio | 38 | 0.85 ± 0.05 | 0.76 ± 0.03* | < 0.001 |
| Lathosterol/β-sitosterol ratio | 38 | 1.03 ± 0.05 | 0.88 ± 0.04* | < 0.001 |

BMI: body mass index; LDL-C: low-density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; apoB: apolipoprotein B. Data shown as means and standard deviation. Student's t test.

In order to investigate whether the degree of hypercholesterolemia influenced the PS response, patients were divided according to tertiles of LDL-C at baseline (< 168 mg/dL and > 187 mg/dL) (Table 4). Placebo minus phytosterol data variations are expressed as delta values. PS intake effectively reduced LDL-C and apo B concentrations in both groups but failed to modify triglycerides concentrations

Furthermore, serum concentration of lathosterol, campesterol and sitosterol were higher in the LDL-C < 148 than in LDL-C > 187 tertiles, but failed to change on PS feeding.

Table 4
Patients according to the averages of LDL-C tertiles at baseline (< 168 mg/dL and > 187 mg/dL)

| | | LDL-C < 168 | LDL-C > 187 | p |
|---|---|---|---|---|
| LDL (mg/dL) | Placebo | 150 ± 14<br>n = 13 | 215 ± 20<br>n = 12 | < 0.0001 |
| | Phytosterol | 148 ± 15<br>n = 13 | 192 ± 24<br>n = 12 | < 0.0001 |
| Delta LDL-C (%) | | -0.5 ± 13 | -10.3 ± 12 | ns |
| Delta LDL-C (mg) | | -1.9 ± 18.1 | -23.0 ± 26.1 | 0.0269 |
| ApoB (mg/dL) | Placebo | 110 ± 10<br>n = 13 | 151 ± 22<br>n = 13 | < 0.0001 |
| | Phytosterol | 112 ± 14<br>n = 13 | 131 ± 22<br>n = 13 | 0.0120 |
| Delta ApoB (%) | | 1.8 ± 10.1 | -12.5 ± 10.2 | 0.0019 |
| Delta ApoB (mg) | | 1.8 ± 11.5 | -19.3 ± 17.5 | 0.0013 |
| Triglycerides (mg/dL) | Placebo | 96 ± 7<br>n = 13 | 130 ± 14<br>n = 13 | < 0.0001 |
| | Phytosterol | 115 ± 38<br>n = 13 | 148 ± 44<br>n = 13 | 0.0472 |
| Delta triglycerides (%) | | 22 ± 36 | 15 ± 38 | ns |
| Delta triglycerides (mg) | | 19 ± 42 | 18 ± 47 | ns |
| Lathosterol (µg/mg cholesterol) | Placebo | 1.689 ± 0.314<br>n = 11 | 1.372 ± 0.303<br>n = 12 | 0.0221 |
| | Phytosterol | 1,887 ± 0,250<br>n = 11 | 1,522 ± 0,233<br>n = 12 | 0.0016 |
| Delta lathosterol (%) | | 13,73 ± 15,42 | 14,15 ± 22,77 | ns |
| Delta lathosterol (µg/mg) | | 0,197 ± 0,214 | 0,150 ± 0,286 | ns |
| Campesterol (µg/mg cholesterol) | Placebo | 2.370 ± 0.633<br>n = 11 | 1.391 ± 0.345<br>n = 12 | 0.0002 |

| | | LDL-C < 168 | LDL-C > 187 | p |
|---|---|---|---|---|
| | Phytosterol | 2,881 ± 0,754<br>n = 11 | 1,941 ± 0,518<br>n = 11 | 0.0028 |
| Delta campesterol (%) | | 25,43 ± 32,30 | 40,71 ± 20,73 | ns |
| Delta campesterol (µg/mg) | | 0,511 ± 0,704 | 0,550 ± 0,280 | ns |
| Sitosterol<br>(µg/mg cholesterol) | Placebo | 1.952 ± 0.445<br>n = 11 | 1.196 ± 0.283<br>n = 11 | 0.0001 |
| | Phytosterol | 2,484 ± 0,518<br>n = 11 | 1,693 ± 0,451<br>n = 11 | 0.0011 |
| Delta sitosterol (%) | | 30,16 ± 26,87 | 41,91 ± 17,95 | ns |
| Delta sitosterol (µg/mg) | | 0,532 ± 0,506 | 0,497 ± 0,242 | ns |

LDL: low-density lipoprotein; apoB: apolipoprotein B. Placebo minus phytosterol data variations are expressed as delta values. Data shown as means and standard deviation. Unpaired Student's t test.

We also examined whether PS response patterns related to LDL-C response patterns: some patients were non-responders (n = 10), but most cases were responders (n = 27) (Table 5). Placebo minus phytosterol data variations are expressed as delta values. We found in the placebo phase lathosterol higher in non-responders than in responders to PS. However, on PS feeding the concentrations of lathosterol did not differ between the two groups. On the other hand, lathosterol percent variation on PS in relation to the placebo period did not vary in non-responders and increased in responders. This means that non-responders, because they have high synthesis before treatment, could not further expand synthesis on PS treatment. Responders synthesize less in the placebo phase but expand the synthesis rate on PS treatment.

Table 5
Plasma sterols response patterns defined by LDL-C changes defining patients non-responders and responders to PS treatment.

| | | Non-responders (n = 10) | Responders (n = 27) | p |
|---|---|---|---|---|
| LDL-C (mg/dL) | Placebo | 171 ± 24 | 191 ± 37 | ns |
| | Phytosterol | 184 ± 29 | 165 ± 32 | ns |
| Delta LDL-C (%) | | 8 ± 5 | -13 ± 7 | < 0.0001 |
| Delta LDL-C (mg/dL) | | 14 ± 9 | -26 ± 15 | < 0.0001 |
| Lathosterol | Placebo | 1,929 ± 0,954 | 1,463 ± 0,363 | 0.0379 |
| (µg/mg cholesterol) | Phytosterol | 1,658 ± 0,411 | 1,686 ± 0,344 | ns |
| Delta lathosterol (%) | | -8 ± 16 | 18 ± 19 | 0.0009 |
| Campesterol | Placebo | 2,242 ± 0,898 | 1,831 ± 0,610 | ns |
| (µg/mg cholesterol) | Phytosterol | 2,168 ± 0,582 | 2,401 ± 0,755 | ns |
| Delta campesterol (%) | | 3 ± 25 | 34 ± 26 | 0.0053 |
| Sitosterol | Placebo | 1,958 ± 0,860 | 1,529 ± 0,469 | ns |
| (µg/mg cholesterol) | Phytosterol | 1,841 ± 0,532 | 2,075 ± 0,595 | ns |
| Delta sitosterol (%) | | 1 ± 26 | 38 ± 23 | 0.0007 |

LDL: low-density lipoprotein. Placebo minus phytosterol data variations are expressed as delta values. Data shown as means and standard deviation. Unpaired Student's t test.

# Discussion

As expected, the consumption of PS-enriched soy milk significantly lowered total cholesterol (-5.5%) and LDL-C (-7.6%). The very mild cholesterol reduction could be attributed to a small PS intake in this study as compared to other investigations [29] [30]. However, the blood cholesterol variation was intense enough to identify different patterns of metabolic changes. Furthermore, a wide variability in individual LDL-C plasma reduction in response to PS intake was previously reported by us [1]. PS also reduced apoB-LP likely belonging to LDL, but increased TG plasma concentrations as compared to placebo especially in participants presenting higher LDL-C concentrations at baseline.

Present investigation confirms previous studies showing that the lathosterol-to-cholesterol ratio in serum predicted the serum cholesterol response to PS feeding where high basal cholesterol synthesis is associated with lack of response of plasma cholesterol [24, 25], and contradicts another study in children in which dietary PS alters the concentration of PS in serum but not the serum concentration of cholesterol synthesis precursors [23]. In one study during the placebo period it was shown in the low cholesterol synthesis cases

that high intestinal sitosterol absorption occurred on PS feeding [24] but was not mentioned in the other study [25]. Since the degree of cholesterol absorption indicated by plasma phytosterol concentration could influence cholesterol synthesis in the placebo phase and its response to PS intake, we measured plasma phytosterols concentrations before and after PS feeding. We noted that absorption markers did not differ between responders and non-responders in the placebo phase. Therefore, the difference in responses between the two groups is strictly dependent on the intensity of cholesterol synthesis. However, unlike the non-responders, responders are capable of increasing the absorption of PS most likely because there are small amounts of cholesterol in the intestinal lumen to compete for the intestinal absorption with the alimentary PS. We conclude that elevated synthesis during placebo in non-responders makes them resistant to further synthesis rise on PS treatment, whereas responders, because they have lower synthesis than non-responders in the placebo phase, are capable expanding synthesis under the effect of alimentary PS.

Interestingly, in the placebo phase, as well as after ingestion of PS, the plasma concentrations of campesterol and sitosterol did not differ between the non-responders and the responder cases. However, as occurred for lathosterol, the percent variation of these markers of absorption on PS feeding over the placebo period was significantly greater in the responders than in the non-responders. This is compatible with the responders absorbing more PS than non-responders. Our data contribute to explain investigation in cases of metabolic syndrome in which decreased intestinal absorption of cholesterol is associated with lower efficiency of PS esters in reducing blood cholesterol although the cholesterol synthesis markers had not been measured [31]. Such a result may be consequent to elevated cholesterol synthesis in cases of metabolic syndrome [16]. In summary, responders absorbed more PS than non-responders likely resulting from responders delivering less endogenous cholesterol into the intestinal lumen that facilitates greater absorption of PS by the intestine.

# Abbreviations

**BMI**
body mass index
**BSTFA**
N,O-bis (trimethylsilyl) trifluoroacetamide
**EDTA**
ethylenediamine tetraacetic acid
**HDL-C**
high-density lipoproteins cholesterol
**LDL-C**
low-density lipoproteins cholesterol
**PS**
phytosterols
**TMCS**
trimethylchlorosilane

# Declarations

# Ethics approval and consent to participate

The Ethics in Research Committee of the Hospital of the University of Sao Paulo Medical School approved the study protocol (CAPPesq n° 112/06). All participants were informed of the objectives of the protocol and signed an informed written consente.

# Consent for publication

All the authors have consented for the publication of this study.

# Availability of data and materials

The data used to support the findings of this study are included within the article. Additional data or information can be requested by contacting the corresponding author.

# Conflict of Interest

The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results

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This work was supported by UNILEVER (SP, Brazil)

# Author contribution

VSN: Conceptualization, Methodology, Writing - Review & Editing. AOGI: Conceptualization, Writing - original draft, Investigation, Methodology; MSA, RPAB, RMM: Investigation, Methodology; GSF: Formal analysis; ERN: Formal analysis; ECRQ: Writing - review & editing; AMPL: Writing - review & editing, Supervision, Funding acquisition.

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