

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Phytosterol-Enriched Yogurt Increases LDL Affinity and Reduces CD36 Expression in Polygenic Hypercholesterolemia

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/65217> since

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)



UNIVERSITÀ DEGLI STUDI DI TORINO

This is an author version of the contribution published on:

Questa è la versione dell'autore dell'opera:

[Lipids. 2009 Feb;44(2):153-60. doi: 10.1007/s11745-008-3259-1. Epub 2008 Nov 8]

The definitive version is available at:

La versione definitiva è disponibile alla URL:

[<http://link.springer.com/article/10.1007%2Fs11745-008-3259-1>]

<http://creativecommons.org/licenses/by-nc-nd/4.0/>

**PHYTOSTEROL-ENRICHED YOGURT INCREASES LDL AFFINITY AND REDUCES
CD36 EXPRESSION IN POLYGENIC HYPERCHOLESTEROLEMIA.**

**Gianluca RUIU, Silvia PINACH, Fabrizio VEGLIA§, Roberto GAMBINO, Saverio
MARENA, Barbara UBERTI, Natalina ALEMANNI, Davina BURT, Gianfranco PAGANO,
Maurizio CASSADER.**

Department of Internal Medicine - University of Turin-Italy

§ Centro Cardiologico Monzino IRCCS, Milan, Italy.

RUNNING TITLE: Phytosterols and Lipids

**Key Words: phytosterols; low-density lipoprotein receptor; low-density lipoprotein affinity;
CD36.**

CORRESPONDING AUTHOR: Prof. Maurizio CASSADER,

Dipartimento di Medicina Interna, Corso A.M. Dogliotti 14.

10126 Torino (Italy)

Telephone (+39)(011)6335493

Telefax (+39)(011)6335493

e-mail: maurizio.cassader@unito.it

ABSTRACT

Dietary enrichment with phytosterols, plant sterols similar to cholesterol, is able to reduce plasma cholesterol levels due to reduced intestinal absorption. The aim of this study was to investigate the effect of phytosterol-enriched yogurt consumption on the major serum lipid parameters, low density lipoprotein (LDL) receptor activity, LDL receptor affinity, and CD36 expression in hypercholesterolemic subjects.

Fifteen patients affected by polygenic hypercholesterolemia were evaluated in a single-blind randomized crossover study after a 4 weeks treatment with a phytosterol-enriched yogurt containing 1.6g esterefied phytosterols (equivalent to 1.0 g free phytosterol). Lipid parameters were compared with a phytosterol-free placebo controlled diet.

The effect of the two treatments on each variable, measured as percent change, was compared by paired-samples *t*-test and covariance analysis.

The treatment induced a modest but significant decrease in LDL-cholesterol levels (4.3%, $p=0.03$) and a significant increase in high density lipoprotein (HDL) 3-cholesterol (17.1%, $p=0.01$). Phytosterol consumption had no effect on LDL-receptor activity whereas patient LDL receptor affinity significantly increased (9.7%, $p=0.01$) and CD36 expression showed a marked significant decrease (18.2%, $p=0.01$) in the phytosterol-enriched yoghurt patients.

Our data show that the oral administration of a phytosterol-enriched yogurt has modest but significant effects on commonly measured lipid parameters. The improvement of LDL receptor affinity and the reduction in CD36 expression may reflect an important antiatherogenic effect.

INTRODUCTION

Changes in dietary habits represent an important action in the strategy to reduce cardiovascular risk not only in at-risk patients but also in the whole population (1).

Phytosterols, a commonly used term for plant sterols and stanols, are a complex class of compounds chemically resembling cholesterol present in vegetable and other food sources (1). Previous studies demonstrated that phytosterols dissolved in food fats, such as margarine or in a liquid formula, show long term recognized cholesterol-lowering properties (2) due to their ability to reduce intestinal cholesterol absorption (3).

The lower cholesterol (Chol) flux to the liver results in a decreased Apolipoprotein (Apo) B-lipoproteins production and in a compensatory increase in LDL-receptor activity resulting in a reduction of total and LDL-carried plasma cholesterol levels (4).

Although a lack of effect of phytosterols on lipid levels has recently been reported (5), long term studies have demonstrated the efficacy of phytosterols in reducing LDL-Chol levels with a decrease ranging from 3% to 25% (6,7) and their use has also been recommended (8).

A beneficial effect of plant sterol ingestion on LDL particle characteristics has also been reported (9). LDL particles undergo physical-chemical alterations during their life-cycle in plasma and, instead of normal LDL-receptor uptake, these modified LDL are catabolized by CD36, a class B scavenger receptor involved in atherosclerosis (10).

The aim of this study was to examine the effect of the oral administration of single-dose phytosterol-enriched yoghurt on lipoprotein metabolism in 15 non-familial hyper-cholesterolemic patients. Lipoprotein levels, LDL-receptor activity, LDL receptor affinity, and CD36 expression were evaluated before and after treatment.

MATERIALS

Fetal calf serum and RPMI 1640 with L-glutamine were obtained from GibcoBRL (Life Technologies, Milan, Italy). Bovine serum albumin (BSA), 1,1'-Dioctadecyl-3,3,3',3'-tetramethyl-

indocarbocyanine perchlorate (DiI), dimethyl-sulfoxide (DMSO), penicillin, streptomycin and lipoprotein deficient serum (LPDS) were obtained from Sigma (St. Louis, USA). Lymphoprep was from Nycomed Pharma AS (Oslo, Norway). Millex-GV filters (pore diameter 0.22µm) were obtained from Millipore S.A. (Molsheim, France). Fifty mL and 15 mL conical tubes, 50 mL and 100 mL flasks were obtained from Falcon, Becton Dickinson Labware (NY, USA). All commercially available materials were of the highest grade. OptiPrep™ was a kind gift from Axis-Shield (Sentinel, Milan, Italy). Fluorescent-labeled monoclonal antibodies PE-CD14 and FITC-CD36, nonspecific isotype matched control antibodies (PE-conjugated rat IgG2α and FITC-conjugated rat IgG1) were from BD Pharmingen (Oxford, UK). QIAamp Blood Kit was from Qiagen-Geneco M-Medical s.r.l. (Florence, Italy). Taq DNA polymerase was obtained from Roche Diagnostic (Milan, Italy). HhaI restriction endonuclease was from New England Biolabs (Ipswich, USA). Silver staining was from Biorad (Milan, Italy).

METHODS

Healthy Volunteers

Healthy unrelated volunteers were enrolled as lymphocyte and LDL donors from the staff and students of the Department of Internal Medicine, University of Turin, Italy.

Subjects and study design

Study subjects were 15 outpatients (10 males, 5 females), attending the Lipid Clinic of the Department of Internal Medicine at the University of Turin, affected by polygenic hypercholesterolemia. The diagnosis was made on the basis of triglycerides levels <200 mg/dL, LDL-Cholesterol >160 mg/dL and absence of elevated blood cholesterol in first degree relatives. The patients were previously treated with statins but had discontinued the therapy due to appearance of clinical and hematological side effects.

Treatment protocol was designed to allocate patients to one of the two 4-week treatment sequences: diet + placebo or diet + phytosterols using a randomized, single-blind, crossover design.

Each phase was followed by a three weeks-washout period during which the subjects consumed the diet, administered during the first phase.

A diet was designed based on the recommended nutrient intakes for Europeans to provide 2250 Kcal per 70-kg individual daily (males) and 1800 Kcal per 60-kg individual daily (females). The Harris-Benedict equation was used to estimate each patient's basal energy requirement (11), which was then multiplied for an activity factor of 1.4 to compensate for the additional energy needs of mildly to moderately active healthy adults. The nutrient content of the diet was calculated by using Winfood (Medimatica, Te, Italy). Dietary carbohydrate, fat and protein made up 55%, 30% and 15% of ingested energy respectively; with a saturated fat less than 7% of total calories as suggested (8). The diet was consumed by the patients at home without supervision. However, the patients were asked to keep an alimentary diary which was subsequently examined by the dietician during the patients visit.

Phytosterols were given as a commercially available yoghurt drink. This is semi-skimmed milk fermented with lactic acid bacteria, commonly used in the manufacture of yoghurt, with a caloric value of 69 Kcal per 100g of product and a Protein/Carbohydrate/Fat content of 3.2, 11.5 and 1.1 g, respectively. The fat content is 0.1g of saturated fat, 0.68g of monounsaturated fat and 0.32g of polyunsaturated fat. The yoghurt is supplemented with 1.6g esterefied phytosterols (equivalent to 1.0 g free phytosterols) obtained from tall oil, a by-product of wood processing. The placebo drink was fermented pasteurized milk with a caloric value of 48 Kcal per 100g of product and a fat content of 1.5g.

At recruitment, the patients had a session with a dietician who recorded their anthropometric characteristics, and gave a controlled diet. A blood sample was drawn for plasma lipid measurement. The patients' anthropometric and biochemical characteristics are listed in **Table 1**. Patients were scheduled for a second visit 4-6 weeks later, when they were interviewed again by the dietician and a blood sample was drawn to measure their plasma lipid and lipoprotein values.

The patients were instructed to drink the placebo or the sterol-enriched yoghurt in the morning and all the patients completed the protocol. At the end of each treatment period blood samples were collected from all patients. Patients gave their informed consent and the protocol was approved by the local Ethical Commission.

To evaluate LDL metabolism, LDL-receptor activity was measured on patients' lymphocytes and LDL receptor affinity was determined, by competitive assay, on LDL-receptors expressed by lymphocytes obtained from healthy control subjects. The receptor affinity of patients' LDL was measured after LDL isolation by self-generating gradient. LDL isolated from healthy volunteers and labeled with DiI were used to measure patients LDL-receptor activity and LDL receptor affinity. The expression of CD36 was measured on patient CD14+ peripheral mononuclear cells isolated in the same analytical session.

Biochemical measurements

Plasma total cholesterol, triglycerides (Tg), ApoAI and B were analyzed using a Hitachi 911 Automatic Autoanalyzer and commercial enzymatic kits (Sentinel, Milan, Italy). HDL-2 and HDL3-Chol were determined after precipitation of Apo B-rich lipoproteins. Plasma HDL2- and HDL3-Chol levels were determined according to Gidez et al.: HDL2 and HDL3 were separated after precipitation of ApoB-containing lipoproteins with heparin and manganese chloride, and HDL2 were further precipitated with dextran sulphate (12).

Apolipoprotein E genotype

All the patients were typed for Apo E genotype, assessed by polymerase chain reaction (PCR) amplification of genomic DNA using specific oligonucleotide primers. Genomic DNA was isolated from frozen EDTA whole blood using the QIAamp Blood Kit. Genomic DNA (0.5 µg) was amplified in 25 µl reaction mixture containing 10 mmol/L Tris-HCl (pH 8.3); 50 mmol/L KCl; 200 µmol each of dATP, dCTP, dGTP and dTTP; 1.5 mmol/L MgCl₂; 200 pmol of each primer, and 1.8 U Taq DNA polymerase.

The amplification cycle was performed in a gene Gene Cycler (Biorad, Milan, Italy). Five minutes of denaturation at 94°C was followed by 30 cycles of 1 min at 90°C, 1 min at 60°C and 2 min at 70°C. PCR products were cleaved with 4 U of HhaI restriction endonuclease, as recommended by the manufacturer. Subsequently, the samples were electrophoresed through a 10% nondenaturing polyacrylamide gel and visualized by silver staining. The sizes of HhaI fragment were estimated by comparison with known size markers (MspI-digested pUC18 DNA).

Lymphocytes isolation and culture

Lymphocytes were prepared under sterile conditions, using a modified version of the method of Böyum (13) as previously described (14). To obtain maximal receptor expression, lymphocytes were incubated for 72h at 37°C in a humidified carbon dioxide incubator (CO₂ 5.0%) in sterile RPMI with 10% human lipoprotein-depleted serum.

Briefly, diluted blood was layered upon lymphoprep (2:1 v/v) and centrifuged at 400g for 30 min at 20°C. The interface containing the lymphocytes was isolated, the cells were washed three times with sterile phosphate-buffered saline (PBS) and finally with RPMI enriched with L-glutamine (290 mg/L), penicillin (100,000 U/L), streptomycin (100 mg/L), and 10% fetal calf serum (FCS). After sedimentation the cell pellet was resuspended in the above solution in a 50 mL flask. Non-adherent cells were harvested and washed twice with cold RPMI/BSA (2 g/L). Cells were adjusted to obtain a final concentration of $0.8-1 \times 10^6$ cells/mL in each tube and used directly in the assay. Viability of the cells was > 95 % as assessed by trypan blue exclusion test.

Isolation and collection of patients LDL with self-generating gradient

LDL was isolated by a single-step centrifugation method using OptiPrepTM, an iodixanol self-generating gradient, as previously described (15) and outlined by the supplier (Axis Shield Reference Manual M10). In brief, a solution of plasma/OptiPrep (4:1 v/v), lower phase, was mixed in a plastic conical tube. The upper phase was made up of 0.75 parts of OptiPrep mixed with 4.25 parts of 0.14M sodium chloride buffered to pH 7.4 with HEPES (HBS). From the upper phase, 1.4mL was transferred to Beckman Optiseal tubes for the TLN100 rotor; 1.4 mL of the lower phase

was transferred to the bottom of the tube below the upper phase using a syringe with a long metal cannula. HBS (~0.1 mL) was carefully layered on top of the upper phase to fill the tube. The tubes were capped and centrifuged at 100,000rpm (350,000g) for 2 h 30 min at 16°C in the TL100 Beckman ultracentrifuge. LDL appeared as a distinct ring in the middle of the tube and was collected by piercing the tube using a syringe fitted with a large gauge needle. Purity was checked by agarose gel electrophoresis.

Isolation and labeling of normal LDL

Low density lipoprotein (density 1.019-1.063 g/mL) and lipoprotein deficient serum (density >1.210 g/mL) were isolated and purified by sequential preparative ultracentrifugation as described by Havel (16) and LDL was labeled with DiI (DiI-LDL) as described (17). LDL specific activity was measured with a spectrofluorimeter. The difference in specific activity between different preparations of LDL was less than 14%.

LDL-receptor assay

LDL-receptor activity was determined on patient's lymphocytes incubated with normal DiI labeled LDL as described (17) with at a final concentration of 10 µg/mL LDL protein for 2 hours at 37°C on a rotary shaker in a humidified carbon dioxide incubator (5.0% CO₂). Cells were then washed twice, resuspended in PBS and directly analyzed by flow cytometry. Final cell concentration was 0.4–0.6 x 10⁶ cells/mL. Each experiment was performed in duplicate.

LDL binding assay

To assess binding, LDL from each subject was tested in a competition assay using DiI-labeled pooled human LDL as tracer. Whereas incubation at 37°C allows binding and internalization of LDL through its own receptor, incubation at 4°C reflects binding only (18). Lymphocytes obtained from healthy subjects were incubated in the dark with increasing concentrations of patient's LDL and fixed dose of pooled DiI-LDL. The patient's LDL concentration which inhibited 50% of the

binding of pooled LDL was determined by flow cytometry. The value obtained was defined as binding or affinity of patient's LDL, after normalization with a normal LDL competition curve and expressed as %. Each experiment was performed in duplicate.

CD36 expression

Mononuclear cells ($0.5 - 0.6 \times 10^6$ cells/mL) were isolated as above and incubated with 10 μ L of fluorescent-labeled monoclonal antibodies PE-CD14 and FITC-CD36 for 20 minutes at room temperature in the dark. Staining with nonspecific isotype matched control antibodies (PE-conjugated rat IgG2 α and FITC-conjugated rat IgG1) was performed in each experiment. Cells were washed twice with PBS and finally resuspended in 500 μ L of PBS. Analysis of stained monocytes was performed on a FACScan flow cytometer using the Cell Quest Software (Becton-Dickinson). For each sample 10000 events were collected.

During analysis, monocytes were identified by their reactivity with CD14-FITC and their distinctive forward and orthogonal light scatter profile. Mean fluorescence intensity (MFI) values were used as indirect measures of antigen density after conversion from the logarithmic scale used for data acquisition to a linear scale for data analysis. MFI values derived for cells stained with isotype control antibodies were taken as indicators of autofluorescence, non-specific antibody binding or instrument noise and were subtracted from MFI values obtained by staining for CD14 or CD36.

Flow cytometry measurements

The uptake of DiI-LDL was measured on a FACScan flow cytometer (Becton-Dickinson, Mountainview, USA). A morphological scatter, forward-scatter FSC (cell size) and side-scatter SSC (cell granularity), for each lymphocyte or monocyte preparation was performed. DiI emission (FL2) was measured at 585 ± 21 nm using a bandpass filter from gated cell population. The fluorescence signals from 10,000 cells were routinely collected. The number of receptor-positive cells was determined setting the marker at the highest range of the unstained lymphocytes (autofluorescence) in the acquisition plot (SSC/FL2); in the following acquisition of stained

lymphocytes, the fluorescence signals over the marker were expressed as percentage of positive cells out of total cells in gated region. The % value obtained (measure in duplicate) reflects the degree of DiI-LDL uptake (binding and internalization) by lymphocytes through the LDL-receptor. The mean intra-assay coefficient value was below 2.0 %.

In the LDL binding assay, performed at 4°C, the fluorescence signals over the marker reflect the concentration of patients LDL which inhibited the normal DiI-LDL binding to cell surface. The concentration value which inhibited 50% binding of normal LDL was normalized to the normal LDL competition curve and expressed as %.

Statistics

The effects of the two treatments on each variable were measured as percent change (after treatment value / before treatment value × 100) and were compared by paired-samples *t*-test. Results were also compared by repeated measures covariance analysis (ANCOVA), including the treatment sequence in the model. All tests were two-sided and a P value below 0.05 was considered as statistically significant. All analyses were performed using the SAS statistical package v. 8.2 (SAS Institute, Cary, NC, USA). All data, if not specified otherwise, are mean ± SD.

RESULTS

All the patients enrolled completed the study and no adverse events were reported. The baseline anthropometric characteristics of the study subjects are presented in **Table 1**. During the control diet and the treatment period with phytosterols and placebo body mass index (BMI), blood pressure and fasting blood glucose levels showed no significant changes (data not shown).

The lipid parameters were analyzed over the four-week phytosterol oral administration and over the placebo period, measured as percent change before/after treatment and compared by paired-sample *t*-test. Analysis revealed the absence of significant changes after placebo treatment. By contrast, phytosterol consumption caused a reduction in LDL-Chol plasma levels (2.4%, $p < 0.05$ and 4.3%, $p < 0.01$, respectively) with a non-significant reduction in ApoB levels. In spite of a 4.3%

increase of HDL-Chol ($p < 0.05$), a significant increase in HDL3-Chol ($p < 0.01$) with a non-significant reduction in HDL2-Chol levels was observed.

When the effect of the two treatments was compared by covariance analysis for repeated measures including the treatment sequence, total cholesterol became non-significant while the difference in LDL-Chol ($p = 0.03$), HDL-Chol ($p = 0.01$) and HDL3-Chol ($p = 0.01$) remained significant. Also ApoA1 levels showed a significant increase ($p = 0.03$) (**Table 2**).

Although the four-week phytosterol consumption had no significant effect on LDL-receptor activity, LDL binding to LDL-receptors expressed by control lymphocytes in comparison to placebo was significantly increased (9.7%, $p = 0.01$) (**Table 3**).

CD36 expression measured as mean fluorescence intensity of CD14+ cells, showed a marked decrease after 4 weeks phytosterol treatment in all patients (18%, $p = 0.01$). These differences retained their significance also when the treatment sequence was included in the analysis by ANCOVA.

All the patients were typed for Apo E genotype and were all homozygous for the most common $\epsilon 3$ allele excluding one patient, heterozygous for the $\epsilon 4$ allele. Due to the low number of subjects in each Apo E genotype groups no comparison was made between the different Apo ϵ alleles.

DISCUSSION

The study was designed to evaluate the effect of a controlled diet supplemented with a fixed dose of phytosterols, provided as a yogurt drink, on lipoprotein metabolism in a group of patients with polygenic hypercholesterolemia. Polygenic hypercholesterolemia is the most common form of hypercholesterolemia caused by a susceptible genotype, still unknown, aggravated by excessive saturated fat, trans fatty acid and cholesterol intake. The patients did not exhibit any LDL-receptor defect and manifested a moderate hypercholesterolemia (240-350 mg/dL) with serum triglyceride concentrations within the reference range.

Phytosterol-consuming patients showed a statistically significant reduction in total cholesterol and LDL-Chol levels, a significant increase in ApoA1, HDL-Chol and HDL3-Chol and a non-significant decrease in ApoB when measured as percent change before/after treatment and compared by paired-sample *t*-test. No statistically significant difference was observed after placebo treatment. When data were compared by covariance analysis the reduction of LDL-Chol remained significant ($p=0.03$), as did the changes in HDL-Chol ($p=0.01$), HDL3-Chol ($p=0.001$) and ApoA1 levels ($p=0.03$).

The decrease in LDL-Chol reported here (4.3%) agrees with the results presented in the literature. Some trials failed to lower plasma lipid levels (5, 19), but in a meta-analysis consisting of 41 stanol/sterol ester trials with the dose varying from 0.8 to 4.2 g of stanol/sterol/day, the majority of studies demonstrated an LDL-Chol decrease, although with a wide range (3% to 25%) (6). As other authors (5,19) observed, the reason for the slight changes of LDL-Chol levels in our patients may be due to the product formula and to the timing of consumption. Actually, the more significant reduction of lipid values was obtained using sterol-enriched solid food products, while recent studies on the effect of low-fat milk products on lipid profile showed a pooled 4.9% LDL-Chol difference after treatment, with a difference meal-time dependent (20). The food product we used is a 1.6 g phytosterol-enriched yogurt and was consumed by our patients, in the morning outside mealtimes. As our patients were not $\epsilon 4$ carriers, a determinant in cholesterol absorption (21), we speculate that the LDL-Chol decrease we obtained may be related to our study design with a reduced efficacy of phytosterol consumption as liquid products, out of mealtimes. Thus, a reduction of LDL-Chol levels associated to an increase in HDL-Chol and HDL3-Chol represent a positive event due to phytosterol ingestion in regards to protection against atherosclerosis.

Moreover, HDL3-Chol increase shows a general positive effect of phytosterols on lipid metabolism: HDL3 particles exert an anti-atherogenic activity by way of antioxidative, anti-inflammatory, anti-thrombotic and anti-apoptotic actions (22).

The LDL-receptor pathway represents a key determinant in cholesterol homeostasis and normal LDL-receptor activity is a pre-requisite for normal cholesterol levels as LDL carry 60 to 80% of plasma cholesterol. Functional tests of LDL-receptor activity and LDL ligand function can theoretically identify defects in LDL catabolism, distinguish between receptor and ligand defects and provide useful information about pharmacological/dietetic intervention. These studies have been based classically on the use of ¹²⁵I-labeled LDL on cultured human fibroblast (23). Fluorescence flow cytometry represents a simple and fast technique allowing the quantification of LDL-receptor expression and LDL ligand function, also on patient's peripheral blood mononuclear cells. Human lymphocytes, lacking scavenger receptors are a suitable and widely used model to investigate LDL metabolism both in normal and pathological states (24, 25).

The method used to measure LDL binding (which involved the use of patient's LDL in competition with normal DiI-labelled LDL for normally expressed LDL-receptor) gave information about the qualitative abnormalities in patient's LDL particle.

The reduction of LDL-Chol we observed was not associated with an increase in LDL-receptor activity, measured as LDL uptake by patient lymphocytes. Other authors reported plant stanol esters consumption a reduction in LDL-Chol linked to increased LDL-receptor activity (26). Changes in LDL-receptor activity are dependent on cholesterol cell concentration, in turn to LDL cholesterol plasma levels and cell synthesis.

Our inability to document an increase in LDL-receptor activity may be due to the small reduction of LDL-Chol level. By contrast, LDL affinity increased significantly, with a near 10% increase after sterols consumption. LDL particle's content and size is a major determinant in their recognition and catabolism through LDL-receptor and the role of small dense LDL in the development of atherosclerotic lesions is well known (27) and a decrease of smaller LDL particles by sterol-enriched diet was recently observed (9).

We can speculate that the increased affinity of LDL observed is related to a reduction of smaller LDL particle and, because of this, to a change in the LDL particle number, as stated by

LDL Chol and Apo B decrease. Consequently, the beneficial effect related to phytosterols results in a better catabolism of LDL.

The beneficial effect of a phytosterol supplemented diet is also the significant reduction (-18%) in CD36 expression after treatment. Class B scavenger receptor CD36 is a complex multifunctional protein that plays a critical role in the initiation of atherosclerotic lesions through its ability to bind and internalize modified LDL, facilitating the formation of lipid-engorged macrophage foam cells (10,28,29). CD36 expression on cell surface is up-regulated by oxidized LDL with a dose dependent increase (30,31) and, as previously cited, a reduction in CD36 expression may indicate a decrease in oxidated LDL related to the phytosterol supplemented diet.

Our results confirm previous reports showing a significant decrease of plasma concentrations of oxidated LDL after plant sterols enriched diet (32,33).

In conclusion, our data indicate a positive effect of single-dose phytosterol-enriched yogurt on lipoprotein metabolism with a slight improvement in common lipid parameters in comparison to LDL behavior and cellular receptor expression.

Our data agree with the general consensus regarding the role of plant sterols in human cholesterol metabolism and may provide a further explanation of the mechanism of the action of phytosterols in hypercholesterolemic patients.

ACKNOWLEDGMENTS:

We thank Franco De Michieli for helpful cooperation in collection of data.

Contribution of each author: Design Study: Ruiu G, Pinach S, Marena S; Practical performance:

Pinach S, Ruiu G, Uberti B, Alemanno N; Data Analysis: Gambino R, Veglia F; Preparation

Manuscript: Ruiu G, Pinach S, Burt D.

Table 1

**Anthropometric and plasma lipid baseline characteristics of the patients
(Values are means \pm SD)**

| | |
|---------------------------------|------------------|
| Age (yrs) | 54.2 \pm 7.3 |
| Sex (M/F) | 10/5 |
| BMI (Kg/m ²) | 24.3 \pm 1.7 |
| Systolic Blood Pressure (mmHg) | 131.5 \pm 10.3 |
| Diastolic Blood Pressure (mmHg) | 75.5 \pm 4.9 |
| Smoke (Y/N) | 2/13 |
| Total Cholesterol (mg/dl) | 308.2 \pm 19.6 |
| HDL Cholesterol (mg/dl) | 47.2 \pm 6.9 |
| HDL2 Cholesterol (mg/dl) | 19.5 \pm 5.3 |
| HDL3 Cholesterol (mg/dl) | 28.5 \pm 4.5 |
| LDL Cholesterol (mg/dl) | 230.6 \pm 26.7 |
| Triglycerides (mg/dl) | 152.2 \pm 23.9 |
| Blood Glucose (mg/dl) | 79.4 \pm 13.1 |
| HbA1c (%) | 5.3 \pm 0.6 |
| Apolipoprotein B (mg/dl) | 172.2 \pm 32.0 |
| Apolipoprotein A1 (mg/dl) | 134.7 \pm 9.3 |

Table 2

Effects of placebo and phytosterol-enriched yoghurt drink on plasma lipid, lipoprotein and apolipoprotein values

| Variable | Placebo | Δ% | Yoghurt | Δ% | P |
|--------------------------|----------------|-----------|----------------|-----------|----------|
| Triglycerides | | | | | |
| before treatment | 133.9±26.8 | 1.5 | 133.3±25.7 | 4.0 | ns |
| after treatment | 135.0±21.9 | | 139.6±25.5 | | |
| Total Cholesterol | | | | | |
| before treatment | 309.9±21.4 | 0.9 | 315.3±28.3 | 2.4* | ns |
| after treatment | 312.7±24.1 | | 307.1±21.0 | | |
| HDL Cholesterol | | | | | |
| before treatment | 47.8±8.4 | 1.4 | 49.5±10.4 | 4.3* | 0.01 |
| after treatment | 47.2±9.4 | | 51.6±11.1 | | |
| HDL2 Cholesterol | | | | | |
| before treatment | 19.4±5.8 | 0.7 | 19.5±5.8 | 3.0 | ns |
| after treatment | 19.3±6.1 | | 18.4±5.0 | | |
| HDL3 Cholesterol | | | | | |
| before treatment | 28.4±4.4 | 0.7 | 28.2±4.3 | 17.1§ | 0.001 |
| after treatment | 28.2±4.4 | | 33.2±7.5 | | |
| LDL Cholesterol | | | | | |
| before treatment | 235.3±27.5 | 1.2 | 239.1±38.1 | 4.3§ | 0.03 |
| after treatment | 238.5±33.2 | | 227.6±31.6 | | |
| Apolipoprotein B | | | | | |
| before treatment | 174.6±34.7 | 0.5 | 172.6±35.8 | 3.3 | ns |
| after treatment | 173.6±34.2 | | 166.4±36.0 | | |
| Apolipoprotein A1 | | 1.1 | | 3.2 | 0.03 |

| | | | | | |
|------------------|------------|--|------------|--|--|
| before treatment | 137.6±9.3 | | 138.6±9.3 | | |
| after treatment | 135.9±10.8 | | 141.9±10.0 | | |

Values, mg/dl, are means \pm SD. *P* indicate statistical difference evaluated by repeated measures covariance analysis, including treatment sequence in the model.

* $p < 0.05$, § $p < 0.01$ indicate difference before/after treatment evaluated by paired *t*-test.

Table3

LDL-receptor activity, LDL binding and CD36 expression

| Variable | Placebo | $\Delta\%$ | Yoghurt | $\Delta\%$ | <i>P</i> |
|------------------------|----------------|------------------------------|----------------|------------------------------|-----------------|
| LDL uptake (%) | | | | | |
| before treatment | 68.6±7.3 | 1.7* | 76.7±9.0 | 3.7* | ns |
| after treatment | 69.9±8.4 | | 72.6±9.1 | | |
| LDL binding (%) | | | | | |
| before treatment | 71.6±6.7 | 1.6 | 72.1±11.6 | 9.7* | 0.01 |
| after treatment | 72.6±6.6 | | 78.8±13.7 | | |
| CD36 (MIF #) | | | | | |
| before treatment | 1904.1±411.6 | 2.6 | 2286.5±476 | 18.2§ | 0.01 |
| after treatment | 1849.5±410.1 | | 1806.3±246 | | |

LDL uptake (%) by patients' lymphocytes is expression of LDL-receptor activity. Through the paper the terms are used as synonymous. LDL binding (%) of the patients is the affinity of the particles to LDL receptors

expressed by normal lymphocytes. Through the paper the terms are used as synonymous.

Values are means \pm SD. *P* indicate statistical difference evaluated by repeated measures covariance analysis, including treatment sequence in the model.

* $p < 0.05$, § $p < 0.01$ indicate difference before/after treatment evaluated by paired *t*-test.

#MIF indicates mean intensity of fluorescence, expressed in arbitrary unit.

REFERENCES

1. Moreau R A, Whitaker M B, Hicks K B. Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. *Progress in Lipid Research* 2002; 41: 457–500. [doi:10.1016/S0163-7827\(02\)00006-1](https://doi.org/10.1016/S0163-7827(02)00006-1)
2. Best M M, Duncan C H, Van Loon E J, Wathen J D. Lowering of serum cholesterol by the administration of a plant sterol. *Circulation* 1954; 10: 201-206.
3. Heinemann T, Kullak-Ublick A, Pietruck B, von Bergman K. Mechanism of action of plant sterols on inhibition of cholesterol absorption. *Eur J Clin Pharmacol* 1991; 40 (suppl 1): 59-63S.
4. Plat J, Mensink R P. Plant Stanol and Sterol Esters in the Control of Blood Cholesterol Levels: Mechanism and Safety Aspects. *Am J Cardiol* 2005; 96(suppl): 15D–22D. [doi:10.1016/j.amjcard.2005.03.015](https://doi.org/10.1016/j.amjcard.2005.03.015)
5. AbuMweis S S, Vanstone C A, Ebine N, Kassis A, Ausman L M, Jones P J H, et al. Intake of a Single Morning Dose of Standard and Novel Plant Sterol Preparations for 4 Weeks Does Not Dramatically Affect Plasma Lipid Concentrations in Humans. *J Nutr* 2006;136: 1012–1016.
6. Katan M B, Grundy S M, Jones P, Law M, Miettinen T, Paoletti R, for the Stresa Workshop Participants. Efficacy and safety of plant stanols and sterols in the management of blood cholesterol levels. *Mayo Clin Proc* 2003; 78: 965–978.
7. Normen L, Frohlich J, Trautwein E. Role of plant sterols in cholesterol lowering. In: Dutta PC, Ed. *Phytosterols as Functional Food Components and Nutraceuticals*. New York, Basel: Marcel-Dekker, Inc, 2004: 243–315
8. National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation,

- and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation* 2002; 106: 3143–3421.
9. Shrestha S, Freake HC, McGrane MM, Volek JS, Fernandez ML. A combination of psyllium and plant sterols alters lipoprotein metabolism in hypercholesterolemic subjects by modifying the intravascular processing of lipoproteins and increasing LDL uptake. *J Nutr.* 2007 May;137 (5):1165-70.
 10. Febbraio M, Silverstein RL. CD36: Implications in cardiovascular disease. *International Journal of Biochemistry and Cell Biology* (2007); 39: 2012-2030. [doi:10.1016/j.biocel.2007.03.012](https://doi.org/10.1016/j.biocel.2007.03.012)
 11. Harris J A, Benedict F G. *A Biometric Study Of Basal Metabolism In Man.* Washington Dc: Carnegia Institution Of Washington, 1919.
 12. Gidez LI, Miller GJ, Burstein M, Slagle S, Eder HA. Separation and quantitation of subclasses of human plasma high density lipoproteins were performed by a simple precipitation procedure. *J Lipid Res.* 1982; 23(8):1206-23.
 13. Böyum A. Isolation of mononuclear cells and granulocytes from human blood. *Scand J Clin Lab Invest* 1968; 97: 77- 89.
 14. Ruiu G, Pinach S, Gambino R, Uberti B, Alemanno N, Pagano G, et al. Influence of Cyclosporine on Low-density Lipoprotein Uptake in Human Lymphocytes. *Metabolism* 2005; 54: 1620-1625. [doi:10.1016/j.metabol.2005.06.010](https://doi.org/10.1016/j.metabol.2005.06.010)
 15. Sawle A, Higgins M K, Olivant M P, Higgins J A. A rapid single-step centrifugation method for determination of HDL, LDL, and VLDL cholesterol, and TG, and identification of predominant LDL subclass. *J Lipid Res* 2002; 43: 335-343.
 16. Havel R J, Eder H A, Bragdon J H. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest* 1955; 34: 1345-1353.

17. Løhne K, Urdal P, Leren TP, Tonstad S, Ose L. Standardization of a flow cytometric method for measurement of low density lipoprotein receptor activity on blood mononuclear cells. *Cytometry* 1995; 20: 290-295.
18. Innerarity T L, Pitas R E, Mahley R W. Lipoprotein-receptor interactions. In Albers J J, Segrest J P, Eds. *Methods in Enzymology*, vol 129, Academic Press, 1986, 542-565.
19. Jones P J H, Vanstone C A, Raeini-Sarjaz M, St-Onge M P. Phytosterols in low- and nonfat beverages as part of a controlled diet fail to lower plasma lipid levels. *J Lipid Res* 2003; 44: 1713–1719.
20. Seppo L, Jauhiainen T, Nevala R, Poussa T, Korpela R. Plant stanol esters in low-fat milk products lower serum total and LDL cholesterol. *Eur J Nutr* 2007; 46: 111-117.
21. Vanhanen H T, Blomqvist S, Ehnholm C. Serum cholesterol, cholesterol precursors, and plant sterols in hypercholesterolemic subjects with different apoE phenotypes during dietary sitostanol ester treatment, *J Lipid Res* 1993; 34:1535–1544.
22. Barter P, Kastelein J, Nunn A, Hoobs R. High density lipoproteins (HDLs) and atherosclerosis; the unanswered questions. *Atherosclerosis* 2003; 168:195-211. [doi:10.1016/S0021-9150\(03\)00006-6](https://doi.org/10.1016/S0021-9150(03)00006-6)
23. Goldstein JL, Brown MS: Binding and degradation of low density lipoproteins by cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J Biol Chem* 1976; 249: 5153-5162
24. Terpstra V, van Amersfoort ES, van Velzen AG, Kuiper J, van Berkel TJC. Hepatic and extrahepatic scavenger receptors: function in relation to disease. *Arterioscler Thromb Vasc Biol* 2000; 20; 1860-1876.
25. Duvillard L, Gambert P, Lizard G: Etude du recepteur des lipoproteins de basse densite par cytometrie en flux: interest biologiques et cliniques. *Ann Biol Clin* 2004; 62: 87-91

26. Plat J, Mensink RP. Effects of plant stanol esters on LDL receptor protein expression and on LDL receptor and HMG-CoA reductase mRNA expression in mononuclear blood cells of healthy men and women. *FASEB J* 2002; 16: 258–260.
27. Berneis KK, Krauss RM. Metabolic origins and clinical significance of LDL heterogeneity. *J Lipid Res* 2002; 43: 1363-1379.
28. Campos H, Arnold KS, Balestra ME, Innerarity TL, Krauss RM. Differences in receptor binding of LDL subfractions. *Arterioscler Thromb Vasc Biol* 1996; 16: 794–801.
29. Han J, Hajjar DP, Febbraio M, Nicholson AC. Native and modified low density lipoproteins increase the functional expression of the macrophage class B scavenger receptor, CD36. *J Biol Chem* 1997; 272: 21654 –21659.
30. Viana M, Villacorta L, Bonet B, Indart A, Munteanu A, Sanchezvera I, et al. Effects of aldehydes on CD36 expression. *Free Radical Research* 2005; 39: 973–977.
31. van Bennekum A, Werder M, Thuahnai ST, Han CH, Duong P, Williams DL, et al. Class B Scavenger Receptor-Mediated Intestinal Absorption of Dietary β -Carotene and Cholesterol. *Biochemistry* 2005; 44: 4517-4525.
32. Hansel B, Nicolle C, Lalanne F, Tondu F, Lassel T, Donazzolo Y, et al. Effect of low-fat, fermented milk enriched with plant sterols on serum lipid profile and oxidative stress in moderate hypercholesterolemia. *Am J Clin Nutr* 2007; 86: 790-796.
33. Homma Y, Ikeda I, Ishikawa T, Tateno M, Sugano M, Nakamura H. Decrease in plasma low-density lipoprotein cholesterol, apolipoprotein B, cholesteryl ester transfer protein, and oxidized low-density lipoprotein by plant stanol ester-containing spread: a randomized, placebo-controlled trial. *Nutrition* 2003; 19:369–74.