The effect of a low-fat spread with added plant sterols on vascular function markers: results of the Investigating Vascular Function Effects of Plant Sterols (INVEST) study¹⁻⁵

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ABSTRACT

Background: Plant sterols (PSs) lower LDL cholesterol, an established risk factor for coronary artery disease (CAD). No direct evidence is available supporting a reduced risk of CAD for foods with added PSs. Endothelial dysfunction is seen as an early indicator of atherosclerotic damage.

Objectives: This study was primarily designed to investigate the effect of a low-fat spread with added PSs on brachial artery endothelial function as measured by flow-mediated dilation (FMD). Second, effects on arterial stiffness, blood pressure, serum lipids, and plasma PS concentrations were investigated. We hypothesized that PSs would not worsen FMD but would rather modestly improve FMD.

Design: This study had a double-blind, randomized, placebo-controlled, parallel design. After a 4-wk run-in period, 240 hypercholesterolemic but otherwise healthy men and women consumed 20 g/d of low-fat spread without (control) or with added PSs (3 g/d) during 12 wk. Pre- and postintervention, vascular function measurements and blood sampling were performed.

Results: In total, 232 participants completed the study period. For the primary endpoint FMD, 199 participants were included in the statistical analysis. PS intake did not affect FMD (+0.01 percentage points; 95% CI: −0.73, 0.75) compared with control. Measures of arterial stiffness (pulse wave velocity and augmentation index) and blood pressure were also not significantly changed compared with control. After PS intervention, LDL cholesterol significantly decreased on average by 0.26 mmol/L (95% CI: −0.40, −0.12) or 6.7% compared with control. Plant sitosterol and campesterol concentrations significantly increased in the PS group up to on average 11.5 μmol/L and 13.9 μmol/L (expressed as geometric means), respectively.

Conclusions: The intake of a low-fat spread with added PSs neither improved nor worsened FMD or other vascular function markers in hypercholesterolemic men and women. As expected, serum LDL cholesterol decreased, whereas plasma PSs increased after PS intake. This study was registered at clinicaltrials.gov as NCT01803178. Am J Clin Nutr 2015;101:733–41.

Keywords: cholesterol, flow-mediated dilation, plant sterols, randomized controlled trial, vascular function

INTRODUCTION

Phytosterols are lipid-like compounds that occur in foods of plant origin. Comprising both plant sterols (PSs)⁶ and their saturated counterparts plant stanols, phytosterols have been shown to lower LDL cholesterol concentrations through partial inhibition of intestinal cholesterol absorption. An average intake of 2 g phytosterols/d lowers LDL cholesterol by 0.31–0.34 mmol/L or 8–10% (1, 2). Elevated blood LDL cholesterol is an established risk factor in the development of atherosclerosis and coronary artery disease (CAD) (3, 4).

Direct evidence supporting an LDL cholesterol-mediated reduction in CAD risk has so far not been generated for foods with added PSs. Considering the difficulties of performing an endpoint trial with PSs (5), endothelial function is seen as a viable option to investigate effects of PSs beyond cholesterol lowering. Endothelial dysfunction is a key aspect in the initiation and progression of atherosclerosis and is partly determined by the burden of CAD risk factors, including hypercholesterolemia (6–8). Brachial artery flow-mediated dilation (FMD) is a measure of large artery endothelial function and has been shown to be associated with cardiovascular risk (9). So far, 6 studies have investigated the effect of PSs (10–12) and/or plant stanols (11–15) on FMD. Although 5 of 6 studies showed effects on FMD in the positive direction, they all failed to show significant improvements in FMD after phytosterol-enriched food intake despite significant reductions in LDL cholesterol.

Despite their established LDL cholesterol–lowering effect, there is some concern about the benefits of foods with added PSs...
in the prevention of CAD risk because supplemental intake of PSs increases plasma PS concentrations (16). In patients with homozygous phytosterolemia, a rare genetic disease, the excretion of phytosterols from the body is hampered due to a loss of function of the ATP-binding cassette transporters ABCG5 and ABCG8, caused by genetic mutations. This leads to very high plasma concentrations of phytosterols in patients with this disease. These patients often (17) but not always (18) display premature atherosclerosis and CAD. In addition, elevated plasma PS concentrations have been associated with increased CAD risk in some (19, 20) but not all (21, 22) epidemiologic studies. Whether this association really exists (23) and, if so, whether this association is explained by the PSs themselves or perhaps by plasma PSs being a surrogate marker of increased cholesterol absorption (24) is still a matter of debate.

In the current large-sample Investigating Vascular Function Effects of Plant Sterols (INVEST) study, the primary aim was to better estimate the size and variability of the effect of a low-fat spread with added PSs on FMD. This would allow investigating whether consumption of PSs might negatively affect endothelial function, as suggested in an animal study that showed that plasma PS concentrations after feeding very high PS doses were correlated with impaired endothelial-dependent vasorelaxation (25), and exploring a presumed small beneficial effect of PSs on FMD, which is expected based on the available evidence (26). Second, the effect of PS intake on arterial stiffness, blood pressure (BP), serum lipids, and plasma PS concentrations was investigated.

METHODS

This study was conducted from January 2013 through August 2013 at the Charité Research Organization, in Berlin, Germany. The study was conducted in accordance with applicable laws and regulations and with the ethical principles that have their origin in the Declaration of Helsinki, Finland. The protocol, informed consent, and advertisements were approved by the ethical committee of Charité Hospital. Written informed consent was obtained from all study participants. The study was registered at clinicaltrials.gov as NCT01803178.

Study population

Individuals were recruited among inhabitants of Berlin and surroundings by using advertisements. Interested persons were invited for an information session where full details of the study protocol were shared. In total, 662 persons joined the screening procedure. During 2 screening visits, several evaluations were performed, including medical history, medication use, physical examination, electrocardiogram, height, weight, vital signs, fasted blood sampling for hematology, clinical chemistry, and blood lipids and a cotinine test. Individuals were eligible when they met the predefined selection criteria, including the following: being apparently healthy men and postmenopausal women, aged 40–65 y; having borderline-high to high LDL cholesterol at screening (130–190 mg/dL or 3.4–4.9 mmol/L); having a BMI (in kg/m²) between 18 and 30; having no occurrence of cardiovascular disease, systemic inflammatory conditions, or diabetes mellitus; not using lipid-lowering foods, lipid-lowering drugs, or other drugs that may interfere with the study measurements; not smoking; being willing to comply with the study protocol; and having signed the informed consent.

Study design

This study was designed as a single-center, randomized, double-blind, placebo-controlled, parallel study with 240 study participants and 2 treatments: a spread with added PSs in the form of PS esters and a control spread without added PSs. The intervention period lasted 12 wk and was preceded by a run-in period of 4 wk for stabilizing blood lipid concentrations and allowing the participants to get familiarized with the study regimen. A statistician (UG) randomized the participants by using permuted block randomization with stratification for age, sex, and screening LDL cholesterol concentration. Before and after the intervention, vascular function measurements were performed and fasted blood samples were drawn for measuring serum lipids and plasma PS concentrations. Health and well-being, use of concomitant medication, and adverse events (AEs) were monitored throughout the study.

Test products and dietary and lifestyle instructions

During the run-in period, participants were provided with control spread. During the intervention period, participants were provided with low-fat spread with added PS esters or control spread. Each day, participants consumed two 10-g portions of test spread with main meals. The PS spread was produced with 22.8% PS esters. The amount of PSs expressed as free equivalents was 15% (i.e., 3 g PSs in 20 g of spread). The PS esters were sourced from BASF. In the control spread, PS esters were replaced by vegetable oil. Total fat content of both test spreads was ~40%. The proportion of SFAs, MUFAs, and PUFAs was ~25%, ~25%, and ~50% of total fat, respectively. Detailed information on the composition of the test spreads is provided in Table 1. The test spreads were produced at Unilever Research and Development Vlaardingen, The Netherlands. Equal amounts of flavors and colorants (β-carotene) were added to ensure that the 2 test spreads were as similar as possible with respect to taste and appearance. The participants and all staff involved in the conduct of the study were blinded for the treatments. Concentrations of PSs were measured in a random selection of the test spreads across all production batches to check correct production of the spreads; the amount of PSs in the PS spread was on average 14.4%, so 2.9 g PSs per 20 g test spread.

Study participants received detailed information on how to consume and store the test spreads. After each 4-wk period, participants returned all opened and unopened tubs to the test facility for a compliance check. Noncompliance with test product intake was defined as having consumed <90% of total spread intake and/or missing more than one intake in the 3 d preceding the study visits. Dietary intake as such was not assessed.

During the entire study period, participants were encouraged to minimize changes in their habitual diet and lifestyle. Study participants were instructed to refrain from consuming phytosterol-enriched foods or supplements or other products claiming to lower blood cholesterol. Concomitant medication that could interfere with the study outcomes (i.e., use of statins, ezetimibe, fibrates, diabetic drugs, triglyceride-reducing drugs, angiotensin II receptor blockers, and angiotensin-converting enzyme inhibitors) or antibiotics were not allowed. Strenuous exercise was not allowed for at least 48 h before the test days. Furthermore, participants were requested to refrain from taking anti-inflammatory drugs, stimulants, and/or...
8% sitostanol, 3% brassicasterol, and some other phytosterols.

chial artery (we obtained an optimal longitudinal B-mode scan of the bra-

duration of the study. By using high-resolution ultrasound with
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tection and wall-tracking system (FMD studio; Quipu SRL).

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ated for 4 min after occlusion. Brachial artery diameter

suprasystolic pressure for 5 min and then deflated to induce

room. Participants and sonographers were matched for the

current guidelines (27). Measurements were performed by well-

trained sonographers with participants in the supine position after

and received breakfast after all measurements were performed.

Study measurements

Endothelial function was assessed as FMD of the brachial

artery, which was our primary outcome, in accordance with

current guidelines (27). Measurements were performed by well-

trained sonographers with participants in the supine position after

a rest of at least 10 min in a quiet, temperature-controlled (22–

4°C) room. Participants and sonographers were matched for the
duration of the study. By using high-resolution ultrasound with a
15-MHz linear array transducer (VIVID E9; General Electric),
we obtained an optimal longitudinal B-mode scan of the bra-

chial artery (~5 cm above the elbow crease) with the probe held
by a stereotactic clamp to ensure steady image recordings. After
1 min of baseline acquisition, a forearm cuff was inflated to
suprasystolic pressure for 5 min and then deflated to induce
reactive hyperaemia. Recordings of the brachial artery were
continued for 4 min after occlusion. Brachial artery diameter
was measured on acquired frames by a computerized edge de-
tection and wall-tracking system (FMD studio; Quipu SRL).
FMD was calculated as the difference between the maximum
diameter after occlusion and the mean baseline diameter divided
by the mean baseline diameter and expressed in percentage
points (pp). All recorded scans were analyzed at a core labo-
ratory by a single independent operator who was blind to the
study’s participants and phase. Directly after the FMD mea-
surement, aortic pulse wave velocity (PWV), augmentation in-
dex (AIx), and central BP (CBP) were assessed by noninvasive
oscillometry by using an Arteriograph (TensoMed). The surro-
gate carotid-femoral distance was measured between the sternal
notch and the pubic symphysis with a tape measure. At least 2
Arteriograph measurements were performed. If the 2 PWV
values differed by >0.5 m/s, a third measurement was performed.
The PWV, AIx, and CBP values were then determined as the
median of the measurements. For office BP, the nondominant
arm was used, which was supported at heart level. Three BP
measurements were taken with an oscillometric device at 2-min
intervals, and the last 2 readings were used to calculate the
mean resting systolic and diastolic BP.

After completion of the vascular function measurements, fasted
blood samples were drawn from the antecubital vein by using
tubes for serum or plasma. Serum lipids (LDL cholesterol, total
cholesterol, HDL cholesterol, and triglycerides) were analyzed
by colorimetry on a Beckman Coulter AU analyzer at Synlab,
Germany. Plasma concentrations of PSs were measured by using
gas chromatography–mass spectrometry with flame ionization
detection at Unilever Research and Development, Vlaardingen,
The Netherlands. All samples of each participant were analyzed
within the same assay.

Table 1: Nutritional composition of the test spreads

| Nutrition values$^a$ | PS spread | Control spread |
|----------------------|-----------|----------------|
| Energy, kcal         | 1505.7    | 1492.6         |
| Energy, kJ           |           |                |
| Total protein, g     | 0.0       | 0.0            |
| Total carbohydrates, g | 0.0     | 0.0            |
| Sugar, g             | 0.0       | 0.0            |
| Fat total, g         | 39.4      | 40.3           |
| SFAs, g              | 9.3       | 9.5            |
| MUFA s, g            | 10.0      | 10.3           |
| UFAs, g              | 19.6      | 20.2           |
| Total n-3 UFAs, g    | 3.6       | 4.0            |
| ALA, g               | 3.6       | 4.0            |
| Total n-6 UFAs, g    | 16.0      | 16.2           |
| TFAs, g              | 0.5       | 0.4            |
| Cholesterol, mg      | 0.7       | 0.9            |
| PS ester, g          | 22.8      | 0.0            |
| PSA, g               | 14.4      | 0.0            |
| Sodium, mg           | 6.9       | 9.0            |
| Vitamin A, µg        | 610.0     | 610.0          |
| Vitamin E, mg        | 9.8       | 12.9           |
| Fiber, g             | 0.0       | 0.0            |
| Water, g             | 45.6      | 59.6           |

$^a$ALA, α-linolenic acid; PS, plant sterol; TFA, trans fatty acid.

This study was powered to be able to differentiate with good
certainty a potential minimal negative effect (the noninferiority
margin) from that of the assumed positive effect. A true-positive
effect size of +0.5 pp was assumed based on the pooled mean of
5 previous studies (10–14, 26). As the noninferiority margin, its
negative counterpart of −0.5 pp was chosen. The necessary
sample size was calculated in the familiar setting of a 2-sample
t test with a difference of 1 pp (+0.5 pp to −0.5 pp), an SD of 2.5
pp, a 2-sided α of 0.05 and a power of 0.8. This would require
199 participants in total. Accounting for an overall dropout rate of
20%, 240 participants were included in the current study.

Agreement on protocol deviations and quality of FMD data points
was obtained during the blind review meeting through expert
consensus. For each parameter, statistical analysis was performed
for the intention-to-treat population (including all available data points
of all participants included in the study) and the per protocol
population (including all biochemical/biological/physiologic plau-
sible data points of all participants who correctly followed the
protocol). Here, the results of the per protocol population are
reported. The results of the intention-to-treat population were similar.

The primary endpoint was the change from baseline in FMD
after intervention with PSs compared with control. This was es-
timated in an ANCOVA model with change from baseline in FMD
as outcome, baseline FMD value as covariate, and treatment and
FMD operator as fixed effects. Effects are reported as least squares
means and 2-sided 95% CIs. All other parameters (PWV, AIx,
CBP, office BP, blood lipids, and plasma PSs) were statistically
analyzed in a similar way. Treatment effects on FMD were
interpreted based on their 95% CIs according to the noninferiority
and equivalence testing principles as outlined in the Consolidated
Standards of Reporting Trials statement (28). Correlation analysis
was performed to investigate the relation between changes in LDL
cholesterol and changes in FMD, as well as between changes in
plasma PSs and changes in FMD in the participants who received
PS treatment. All analyses were performed with the statistical
software package SAS version 9.4 (SAS Institute).
RESULTS

Overview of study population

A total of 150 men (62.5%) and 90 women (37.5%) were included in the study, almost all Caucasian. An overview of the participants’ characteristics at baseline is provided in Table 2. In total, 8 participants dropped out prematurely. Eight participants violated the protocol (i.e., not being weight stable, not being fasted, or having used prohibited medication). Ultrasound scans of 24 participants were rejected because of poor quality and/or instability of the images caused by inconsistency of clear artery borders. One subject missed the last FMD visit. For FMD (i.e., the primary endpoint), 199 participants were included in the analysis (Figure 1). Compliance with test product intake was high (>90%).

Vascular function

Baseline FMD of the included participants (n = 199) was on average 5.1 ± 2.6 pp (i.e., CV was 0.5). Neither age nor sex significantly affected the change from baseline in FMD. The effect of PSs on FMD was +0.01 pp (95% CI: −0.73, 0.75) compared with control and not significant (Table 3). The 95% CI includes +0.5 pp, which was the predefined noninferiority margin. In relative terms, the change in FMD on PS intake was 0.1% compared with control. Descriptive statistics of the baseline artery diameter, the maximal diameter after hyperemia, and the shear rate before and after intervention are provided in Supplementary Table 1. Because the FMD at baseline was significantly different between the 2 treatment groups (control group: 4.7 ± 2.6 pp and PS group: 5.5 ± 2.6 pp), a simulation analysis was performed to assess the effect of PSs on FMD based on repeated (n = 100) random subsets of the population with appropriate weights to achieve balanced FMD values at baseline. This analysis did not show different estimated effects of PSs on FMD (data not shown). Measures of arterial stiffness (PWV and AIx), CBP, and office systolic BP were not significantly changed after PS intake compared with control (Table 3). Only office diastolic BP was significantly lowered by 1.4 mm Hg (95% CI: −2.7, −0.1) after PS intake compared with control.

On the basis of correlation analysis in the group that received PSs, it appeared that changes in plasma PS concentrations were not related to changes in FMD (partial correlation = −0.09; P > 0.05), whereas a reduction in LDL cholesterol was modestly but significantly related to an increase in FMD (partial correlation = −0.20; P < 0.05) (Figure 2).

Blood lipid and PS concentrations

At baseline, serum total and LDL cholesterol concentrations were on average 5.77 ± 0.92 and 3.91 ± 0.60 mmol/L, respectively. Total and LDL cholesterol concentrations were significantly reduced on average by 0.26 mmol/L (4.5%) and 0.26 mmol/L (6.7%), respectively, after PS intake compared with control (Table 4). No significant changes were observed in HDL cholesterol (+0.6%) and triglyceride (−2.2%) concentrations compared with control. Plasma sitosterol concentrations significantly increased in the PS group from 6.7 to 11.5 μmol/L and campesterol from 11.4 to 13.9 μmol/L. These values are based on back-transformed data and represent geometric means. No obvious changes were observed in the control group. Compared with control, plasma sitosterol and campesterol concentrations increased on average by 77.9% and 32.6%, respectively. The sum of 6 major phytosterols (i.e., sitosterol, campesterol, brassicasterol, stigmastanol, sitostanol, and campestanol) was significantly increased by 41.6% compared with control (Table 4).

Adverse events

A total of 85 participants experienced one or more AEs (209 in total) during the intervention period. Overall, the incidence of AEs was mild to moderate and all not related to the study procedures. Three participants experienced a serious AE during the intervention period (thermal burn, gastroenteritis, or depression

### Table 2

| Characteristic | PS group (n = 126) | Control group (n = 114) | Overall (N = 240) |
|---------------|-------------------|------------------------|-------------------|
| Male (% male) | 77 (61.1)         | 73 (64.0)              | 150 (62.5)        |
| Female (% female) | 49 (38.9) | 41 (36.0)              | 90 (37.5)         |
| Age, y | 53.4 ± 6.7 | 53.1 ± 6.9 | 53.2 ± 6.8 |
| Weight, kg | 78.0 ± 13.0 | 77.4 ± 13.1 | 77.7 ± 13.0 |
| Height, cm | 174.6 ± 8.6 | 174.8 ± 9.5 | 174.7 ± 9.0 |
| BMI, kg/m² | 25.5 ± 2.8 | 25.2 ± 2.7 | 25.3 ± 2.8 |
| Waist circumference, cm | 88.9 ± 10.6 | 89.3 ± 10.4 | 89.1 ± 10.5 |
| Hip circumference, cm | 102.0 ± 6.9 | 101.9 ± 6.0 | 101.9 ± 6.5 |
| Total cholesterol, mmol/L | 5.65 ± 1.09 | 5.74 ± 1.01 | 5.69 ± 1.05 |
| LDL cholesterol, mmol/L | 3.83 ± 0.73 | 3.90 ± 0.63 | 3.86 ± 0.68 |
| HDL cholesterol, mmol/L | 1.39 ± 0.46 | 1.35 ± 0.41 | 1.37 ± 0.44 |
| Triglycerides, mmol/L | 0.95 (0.74, 1.39) | 1.10 (0.80, 1.47) | 1.05 (0.76, 1.43) |
| FMD, pp | 5.4 ± 2.8 | 4.6 ± 2.7 | 5.0 ± 2.8 |
| SBP, mm Hg | 122.2 ± 12.5 | 123.3 ± 12.1 | 122.7 ± 12.3 |
| DBP, mm Hg | 74.5 ± 8.0 | 74.6 ± 8.5 | 74.5 ± 8.2 |

1DBP, diastolic blood pressure; FMD, flow-mediated dilation; pp, percentage points; PS, plant sterol; Q, quartile; SBP, systolic blood pressure.

2Mean ± SD (all such values).

3Triglyceride values were not normally distributed and are therefore reported as medians (Q1, Q3).
followed by weight loss). These serious AEs were not related to the test product intake, and were all resolved.

**DISCUSSION**

The present study showed that the regular intake of a low-fat spread with added PSs over 12 wk neither improved nor worsened FMD in hypercholesterolemic but otherwise healthy men and women. Measures of arterial stiffness and BP were also not affected. The PS intake led to a significant reduction in total and LDL cholesterol concentrations, although the effect on LDL cholesterol was smaller (~7%) than anticipated (~12%) for the dose of PSs provided (3 g/d) (1, 2). Plasma PS concentrations were significantly increased with PS intake as expected based on previous investigation (16).

So far, only a few studies have been performed that investigated the effect of phytosterols on FMD. Three of these studies were performed in hypercholesterolemic adults who consumed spreads enriched with PSs or plant stanols at doses of ~2 g/d (11–13). In these studies, FMD was not significantly changed; placebo-corrected FMD effect sizes ranged between 0.37 and 1.02 pp for PSs and between 0.12 and 0.91 pp for plant stanols. LDL cholesterol was significantly reduced in these studies by 9–16%. Two other studies were performed in prepubescent familial hypercholesterolemic children who were provided with spreads or yogurts enriched with ~2 g/d PSs (10) or plant stanols (14). These studies also found no significant changes in FMD (effects ranged between 0.05 and 0.50 pp), despite significant reductions of 9–14% in LDL cholesterol. In a study with patients with type 1 diabetes (15), plant stanol...
intake showed a tendency for a worsening in FMD (−2.47 pp); this effect, however, was not significant and mainly driven by an improvement in FMD in the control group.

In the present study, we observed no change in FMD after PS intake compared with control, whereas we anticipated a modest improvement of 0.5 pp based on the combined evidence from FMD studies with phytosterols (26). It could be speculated that the LDL cholesterol–lowering effect observed in our study (−7%) was not sufficient to mediate an improvement in endothelial function. It could also be speculated that changes in LDL

**TABLE 3**

Vascular function and blood pressure in hypercholesterolemic men and women who consumed a low-fat spread enriched with plant sterols or a low-fat control spread

| Outcome/treatment | n  | Baseline, mean ± SD | End of intervention, mean ± SD | Absolute change 2 | 95% CI |
|-------------------|----|---------------------|-------------------------------|-------------------|------|
| FMD, pp           |    |                     |                               |                   |      |
| Control           | 98 | 4.70 ± 2.61         | 4.73 ± 2.60                   | −0.027            | −0.80, 0.25 |
| PSs               | 101| 5.53 ± 2.56         | 5.10 ± 3.05                   | −0.27             | −0.79, 0.26 |
| ∆                 | 199| 0.01                | −0.73, 0.75                   |                   |      |
| PWV, m/s          |    |                     |                               |                   |      |
| Control           | 97 | 8.35 ± 1.86         | 8.12 ± 1.68                   | −0.18             | −0.35, 0.00 |
| PSs               | 109| 8.08 ± 1.38         | 7.81 ± 1.08                   | −0.32*            | −0.49, −0.15 |
| ∆                 | 206| −0.14               | −0.38, 0.10                   |                   |      |
| AIX, %            |    |                     |                               |                   |      |
| Control           | 99 | 30.65 ± 16.62       | 29.46 ± 16.01                 | −1.11             | −2.55, 0.33 |
| PSs               | 109| 29.48 ± 13.92       | 27.44 ± 14.01                 | −2.11*            | −3.49, −0.74 |
| ∆                 | 208| −1.01               | −3.00, 0.99                   |                   |      |
| Central SBP, mm Hg|    |                     |                               |                   |      |
| Control           | 98 | 125.8 ± 17.9        | 120.6 ± 17.7                  | −5.2*             | −7.1, −3.4 |
| PSs               | 109| 126.7 ± 17.6        | 119.4 ± 15.1                  | −7.2*             | −8.9, −5.4 |
| ∆                 | 207| −1.9                | −4.4, 0.6                     |                   |      |
| Central DBP, mm Hg|    |                     |                               |                   |      |
| Control           | 98 | 80.0 ± 9.6          | 77.0 ± 10.3                   | −3.2*             | −4.4, −1.9 |
| PSs               | 109| 81.3 ± 9.5          | 77.0 ± 8.9                    | −4.2*             | −5.3, −3.0 |
| ∆                 | 207| −1.0                | −2.7, 0.7                     |                   |      |
| Office SBP, mm Hg |    |                     |                               |                   |      |
| Control           | 107| 123.5 ± 12.3        | 119.4 ± 13.5                  | −3.9*             | −5.3, −2.6 |
| PSs               | 117| 122.1 ± 12.8        | 116.9 ± 10.9                  | −5.4*             | −6.7, −4.1 |
| ∆                 | 224| −1.5                | −3.4, 0.4                     |                   |      |
| Office DBP, mm Hg |    |                     |                               |                   |      |
| Control           | 107| 74.6 ± 8.6          | 72.6 ± 8.8                    | −2.1*             | −3.0, −1.2 |
| PSs               | 117| 74.5 ± 8.1          | 71.1 ± 7.8                    | −3.5*             | −4.3, −2.6 |
| ∆                 | 224| −1.4*               | −2.7, −0.1                    |                   |      |

1*Significant at \( P < 0.05 \). AIX, augmentation index; DBP, diastolic blood pressure; FMD, flow-mediated dilation; pp, percentage points; PS, plant sterol; PWV, pulse wave velocity; SBP, systolic blood pressure.

2Absolute changes from baseline are expressed as least squares means and 95% CIs after correction for baseline (and FMD operator in case of FMD results). Treatment effects were estimated in an ANCOVA model.

3FMD at baseline was significantly different between the 2 treatment groups.

**FIGURE 2** Correlation between changes in LDL cholesterol and plasma phytosterols and changes in FMD. The participants in the plant sterol group were included for this correlation analysis. FMD, flow-mediated dilation; pp, percentage points.
cholesterol do not per definition result in changes in endothelial function. Studies with ezetimibe monotherapy have overall not shown clear correlations between changes in LDL cholesterol and changes in FMD (29–31). Furthermore, in a community study with 5000 individuals, classic risk factors, including dyslipidemia, explained only 15.4% of the variation in FMD (32). On the other hand, in patients who underwent LDL apheresis and thereby acutely reduced their LDL cholesterol by 76.5%, endothelium-dependent vasodilation was significantly improved (33). Furthermore, in familial hypercholesterolemia, a disease associated with lifelong elevations in plasma cholesterol concentrations (~3 mmol/L higher compared with healthy controls) due to inherited mutations in LDL receptor genes, FMD is clearly impaired (3–4 pp lower compared with healthy controls) (10, 34). In an attempt to explore the relation between changes in LDL cholesterol and changes in endothelial function on PS intervention, we plotted the individual changes in LDL cholesterol against those in FMD of the participants who received the PS spread and observed a small but significant correlation (partial correlation = −0.20; P < 0.05; Figure 2). Because the placebo-controlled effect of PSs on FMD was zero, it could be hypothesized that a certain minimal reduction in LDL cholesterol (>0.26 mmol/L) is required to improve endothelial function. This theory requires further investigation.

The observation that the PS-induced change in FMD was 0.01 pp demonstrates that endothelial function was not impaired. Also, there was no correlation found between changes in plasma PSs and changes in FMD. This suggests that an increase in plasma PSs is unlikely to counteract beneficial vascular effects of PSs, which are expected based on their LDL cholesterol–lowering properties. Indeed, plant stanols, which are known to lower plasma PS concentrations, do not affect FMD (11–14) differently than PSs do (10, 11, 12), whereas they are equally effective in lowering LDL cholesterol (35). In an animal study with normal, wild-type mice, it was speculated that elevated plasma PS concentrations after PS feeding could be atherogenic because these concentrations were correlated with impaired endothelial vasorelaxation in situ (25). However, in this mouse model, cholesterol concentrations were unaffected, suggesting that this model was probably not appropriate for studying the effects of PSs. Also, the dose of PSs used in these mice was ~100 times higher than the amount of PSs that is recommended for lowering

### TABLE 4

| Outcome/treatment | n  | Baseline | End of intervention | Absolute change | Relative change, % |
|-------------------|----|----------|---------------------|-----------------|--------------------|
| **Total cholesterol, mmol/L** | | | | | |
| Control           | 105| 5.80 ± 0.91 | 5.45 ± 1.01         | −0.34* (−0.48, −0.20) | −5.8 |
| PSs               | 113| 5.75 ± 0.93 | 5.16 ± 0.90         | −0.60* (−0.73, −0.46) | −10.4 |
|                  | 218|           |                     | −0.26* (−0.46, −0.07) | −4.5 |
| **LDL cholesterol, mmol/L** | | | | | |
| Control           | 105| 3.94 ± 0.59 | 3.71 ± 0.69         | −0.22* (−0.32, −0.12) | −5.5 |
| PSs               | 113| 3.89 ± 0.62 | 3.42 ± 0.64         | −0.48* (−0.58, −0.38) | −12.3 |
|                  | 218|           |                     | −0.26* (−0.40, −0.12) | −6.7 |
| **HDL cholesterol, mmol/L** | | | | | |
| Control           | 105| 1.36 ± 0.40 | 1.29 ± 0.39         | −0.07* (−0.11, −0.03) | −5.0 |
| PSs               | 113| 1.42 ± 0.45 | 1.35 ± 0.43         | −0.06* (−0.10, −0.02) | −4.3 |
|                  | 218|           |                     | 0.01 (−0.04, 0.06)   | 0.6 |
| **log(triglycerides), mmol/L** | | | | | |
| Control           | 105| 0.11 ± 0.50 | 0.08 ± 0.49         | −0.02 (−0.08, 0.04)  | −2.1 |
| PSs               | 113| 0.04 ± 0.43 | 0.00 ± 0.47         | −0.04 (−0.10, 0.02)  | −4.2 |
|                  | 218|           |                     | −0.02 (−0.11, 0.07)  | −2.2 |
| **log(sum of major phytosterols), µmol/L** | | | | | |
| Control           | 104| 2.91 ± 0.37 | 2.90 ± 0.38         | −0.04 (−0.08, 0.01)  | −3.5 |
| PSs               | 109| 3.07 ± 0.37 | 3.37 ± 0.36         | 0.31* (0.27, 0.36)   | 36.6 |
|                  | 213|           |                     | 0.35* (0.28, 0.41)   | 41.6 |
| **log(sitosterol), µmol/L** | | | | | |
| Control           | 107| 1.74 ± 0.38 | 1.74 ± 0.39         | −0.02 (−0.07, 0.03)  | −1.8 |
| PSs               | 114| 1.90 ± 0.40 | 2.44 ± 0.41         | 0.56* (0.51, 0.61)   | 74.7 |
|                  | 221|           |                     | 0.58* (0.51, 0.65)   | 77.9 |
| **log(campesterol), µmol/L** | | | | | |
| Control           | 107| 2.24 ± 0.41 | 2.20 ± 0.44         | −0.06* (−0.11, −0.02) | −6.0 |
| PSs               | 115| 2.43 ± 0.42 | 2.63 ± 0.38         | 0.22* (0.18, 0.26)   | 24.7 |
|                  | 222|           |                     | 0.28* (0.22, 0.35)   | 32.6 |

*Significant at P < 0.05. PS, plant sterol.

*All values are means ± SDs.

Absolute changes from baseline are expressed as least squares means and 95% CIs after correction for baseline. Treatment effects were estimated in an ANCOVA model.

Serum triglyceride and plasma PS values were not normally distributed and were log-transformed to allow statistical analysis. Relative changes from baseline in serum triglyceride and plasma PSs are expressed on the original scale.

The major phytosterols included sitosterol, campesterol, brassicasterol, stigmasterol, sitostanol, and campestanol.
LDL cholesterol in humans (2 g/d). Furthermore, it was recently demonstrated that, in hamsters fed a high-cholesterol diet, endothelial function was improved after intake of sitosterol and stigmasterol compared with control and compared with their oxidized counterparts (36).

Our study has several strengths that give support to the conclusions drawn, such as the straightforward and rigid design of the study, inclusion of one of the largest numbers of participants, and the high compliance with test product intake. The latter was reflected in a clear increase in the plasma PS concentrations after PS intervention. Also, vascular ultrasound was performed by well-trained sonographers according to current guidelines (27). FMD was assessed by a single, blinded, independent, and experienced technician with computer-assisted analysis, using edge detection and wall-tracking software, which has been demonstrated to be very reproducible (37). The CV in FMD at baseline was comparable with those reported in other FMD studies (i.e., ~0.5) (37, 38). Furthermore, the PS-induced FMD effect based on FMD assessment by the sonographers at the time of the ultrasound acquisition was comparable with the FMD effect based on centrally assessed FMD by a single, blinded central reader (data not shown).

Some limitations of the study need to be mentioned as well. First, although we assumed that the study participants would have suboptimal FMD because of their elevated prestudy LDL cholesterol concentrations, we cannot exclude that the participants were too healthy (e.g., all nonsmokers, no type 2 diabetes) to allow improvement in endothelial function on treatment. Follow-up studies should preferably select participants with impaired FMD at baseline. Second, LDL cholesterol was significantly changed from baseline (by ~5%) in the control group. Also, central and office BP were significantly changed from baseline. Although the participants were instructed not to change their diet and lifestyle during the study, we cannot rule out that seasonal influences or increased awareness of having elevated blood LDL cholesterol, for example, led to unintended changes in their typical habits, which may have influenced the outcomes of this study. Third, despite stratification for age, sex, and LDL cholesterol, FMD at baseline was significantly different between the 2 intervention groups. The performed ANCOVA analysis with baseline as covariate was one planned safeguard against differences in baseline FMD. Because the difference was larger than expected, we performed simulation analysis with equally balanced baseline FMD. This analysis showed that upfront balancing for baseline FMD would not have changed the outcomes of our study. Last, although changes in endothelial function can occur quickly on intervention [e.g., fat loads can affect FMD within a few hours (39)], it cannot be ruled out that the duration of the current study (3 mo) was insufficient to induce small effects on the vascular system, particularly when assuming that such an effect would be mediated through LDL cholesterol reduction and considering that the observed LDL cholesterol–lowering effect was only rather modest (~7% for a dose of 3 g PS/d).

In summary, endothelial function as measured by brachial artery FMD was neither improved nor worsened with PS intake. The LDL cholesterol–lowering effect of PS intake was confirmed, although the effect was lower than anticipated for the dose of PS tested (3 g/d). LDL cholesterol is an established risk factor in the development of atherosclerosis leading to CAD. Whether reductions in LDL cholesterol due to PS intake would reduce CAD risk via vascular function requires further investigation. Future studies should investigate vascular effects of PS after prolonged intakes of PS with enhanced LDL cholesterol lowering, preferably in participants with compromised vascular function.

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REFERENCES

1. Demonty I, Ras RT, Van der Knaap HCM, Duchateau GSMJ, Meijer L, Zock PL, Geleijse JM, Trautwein EA. Continuous dose-response relationship of the LDL-cholesterol-lowering effect of phytosterol intake. J Nutr 2009;139:271–84.
2. Katan MB, Grundy SM, Jones P, Law M, Miettinen TA, Paolotti R. Efficacy and safety of plant stanols and sterols in the management of blood cholesterol levels. Mayo Clin Proc 2003;78:965–78.
3. Stone NJ, Robinson JG, Lichtenstein AH, Bailey Merz CN, Blum CB, Eckel RH, Goldberg AC, Gordon D, Levy D, Lloyd-Jones DM, et al. 2013 ACC/AHA guideline on the treatment of blood cholesterol to reduce atherosclerotic cardiovascular risk in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. Circulation 2014;129:81–45.
4. Reiner Z, Catapano AL, De Backer G, Graham I, Taskinen MR, Wiklund O, Agewall S, Alegria E, Chapman MJ, Durrington P, et al. ESC/EAS guidelines for the management of dyslipidaemias: the task force for the management of dyslipidaemias of the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS). Eur Heart J 2011;32:1769–818.
5. Gylling H, Plat J, Turley S, Ginsberg HN, Ellegard L, Jessup W, Jones PJ, Lutjohann D, Maerz W, Masana L, et al. Plant sterols and plant stanols in the management of dyslipidaemia and prevention of cardiovascular disease. Atherosclerosis 2014;232:346–60.
6. Vogel RA. Cholesterol lowering and endothelial function. Am J Med 1999;107:479–87.
7. Bonetti PO, Lerman LO, Lerman A. Endothelial dysfunction: a marker of atherosclerotic risk. Arterioscler Thromb Vasc Biol 2003;23:168–75.
8. Widding MY, Gokce N, Keay JA, Vita JA. The clinical implications of endothelial dysfunction. J Am Coll Cardiol 2003;42:1149–60.
9. Ras RT, Streppel MT, Draijer R, Zock PL. Flow-mediated dilation and cardiovascular risk prediction: a systematic review with meta-analysis. Int J Cardiol 2013;168:344–51.
10. de Jongh S, Visser MN, Rol P, Bakker HD, Kastelein JIP, Stroes ESG. Plant sterols lower LDL cholesterol without improving endothelial function in prepubertal children with familial hypercholesterolaemia. J Inherit Metab Dis 2003;26:343–51.
11. Hallikainen M, Lyyra-Laitinen T, Laitinen T, Agren JJ, Pihlajamaki J, Rauramaa R, Miettinen TA, Gylling H. Endothelial function in hypercholesterolemic subjects: effects of plant stanol and sterol esters. Atherosclerosis 2006;188:425–32.
12. Gylling H, Hallikainen M, Raitakari OT, Laakso M, Vartiainen E, Salo P, Korpelainen V, Sundvall J, Miettinen TA. Long-term consumption of plant stanol and sterol esters, vascular function and genetic regulation. Br J Nutr 2009;101:1688–95.
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13. Raitakari OT, Salo P, Gylling H, Miettinen TA. Plant stanol ester consumption and arterial elasticity and endothelial function. Br J Nutr 2008;100:603–8.

14. Jakulj L, Vissers MN, Rodenburg J, Wiegman A, Trip MD, Kastelein JJ. Plant stanol esters do not restore endothelial function in pre-pubertal children with familial hypercholesterolemia despite reduction of low-density lipoprotein cholesterol levels. J Pediatr 2006;148:495–500.

15. Hallikainen M, Lyysa-Laitinen T, Laitinen T, Moilanen L, Miettinen TA, Gylling H. Effects of plant stanol esters on serum cholesterol concentrations, relative markers of cholesterol metabolism and endothelial function in type 1 diabetes. Atherosclerosis 2008;199:432–9.

16. Ras RT, Hiemstra H, Lin Y, Vermeer MA, Duchateau GSMJE, Trautwein EA. Consumption of plant sterol-enriched foods and effects on plasma plant sterol concentrations—a meta-analysis of randomized controlled studies. Atherosclerosis 2013;230:336–46.

17. Sudhop T, Von Bergmann K. Sitosterolemia—a rare disease: are elevated plant sterols an additional risk factor? Z Kardiol 2004;93:921–8.

18. Hansel B, Carrie A, Brun-Druc N, Leclert G, Chantepie S, Coiffard AS, Kahn JF, Chapman MJ, Bruckert E. Premature atherosclerosis is not systematic in phytosterolemia patients: severe hypercholesterolemia as a confounding factor in five subjects. Atherosclerosis 2014;234:162–8.

19. Assmann G, Cullen P, Erbey JR, Ramey DR, Kannenberg F, Schulte H. Plasma sitosterol elevations are associated with an increased incidence of coronary events in men: results of a nested case-control analysis of the Prospective Cardiovascular Munster (PROCAM) study. Nutr Metab Cardiovasc Dis 2006;16:13–21.

20. Rajaratnam RA, Gylling H, Miettinen TA. Independent association of serum squalene and noncholesterol sterols with coronary artery disease in postmenopausal women. J Am Coll Cardiol 2000;35:1185–91.

21. Windler E, Zyrax BC, Kuipers F, Lineisen J, Boehning G, Ritscher M, Grammer TB, Boehm BO, Makela KM, Kahonen M, et al. High intestinal cholesterol absorption is associated with cardiovascular disease and risk alleles in ABCG8 and ABO: evidence from the LURIC and YFS cohorts and from a meta-analysis of randomized controlled studies. J Am Coll Cardiol 2013;62:291–9.

22. Pinedo S, Vo¨ on Bergmann K, Gertz K, Koenig J, Schaefers HJ, et al. Vascular disease and risk alleles in ABCG8 and ABO: evidence from the PROGRESS and prospective of plant sterol and plant stanol research: report of the Maastricht meeting. Atherosclerosis 2012;225:521–33.

23. Thijsse DH, Black MA, Pyke KE, Padilla J, Atkinson G, Harris RA, Parker B, Widesky ME, Tschakovsky ME, Green DJ. Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. Am J Physiol Heart Circ Physiol 2011;300:H2–12.

24. Piaggio G, Elbourne DR, Altman DG, Pocock SJ, Evans SJ. Reporting of noninferiority and equivalence randomized trials: an extension of the CONSORT statement. JAMA 2006;295:1152–60.

25. Krysiak R, Zmuda W, Okopien B. The effect of ezetimibe, administered alone or in combination with simvastatin, on lymphocyte cytokine release in patients with elevated cholesterol levels. J Intern Med 2012;271:32–42.

26. Plat J, Mackay D, Baumgartner S, Clifton PM, Gylling H, Jones PJ. Progress and prospective of plant sterol and plant stanol research: report of the Maastricht meeting. Atherosclerosis 2012;225:521–33.

27. Kurobe H, Aihara K, Higashida M, Hirata Y, Nishiya M, Matsuoka Y, Kanbara T, Nakayama T, Kinoshita H, Sugano M, et al. Ezetimibe monotherapy ameliorates vascular function in patients with hypercholesterolemia through decreasing oxidative stress. J Atheroscler Thromb 2011;18:1080–9.

28. Jakulj L, Vissers MN, Rodenburg J, Wiegman A, Trip MD, Kastelein JJ. Early statin therapy restores endothelial function in children with familial hypercholesterolemia. J Am Coll Cardiol 2002;40:2117–21.

29. Tamai O, Matsuoka H, Itabe H, Wada Y, Kohno K, Imaizumi T. Single LDL apheresis improves endothelium-dependent vasodilatation in hypercholesterolemic humans. Circulation 1997;95:76–82.

30. Liang YT, Wong WT, Guan L, Tian XY, Ma KY, Huang Y, Chen ZY. Effect of phytosterols and their oxidation products on lipoprotein profiles and vascular function in hamster fed a high cholesterol diet. Atherosclerosis 2011;219:124–33.

31. Ghiadoni L, Faita F, Salvetti M, Cordiano C, Biggi A, Puato M, Di Monaco A, De Siati L, Volpe M, Ambrosio G, et al. Assessment of flow-mediated dilation reproducibility: a nationwide multicenter study. J Hypertens 2012;30:1399–405.

32. Lüscher TF, Taddei S, Kaski JC, Jukema JW, Kallend D, Munzel T, Kastelein JJ, Deanfield JE. Vascular effects and safety of dalteparin in patients with or at risk of coronary heart disease: the dal-VESSEL randomized clinical trial. Eur Heart J 2012;33:857–65.

33. Vogel RA, Corretti MC, Plotnick GD. Effect of a single high-fat meal on endothelial function in healthy subjects. Am J Cardiol 1997;79:350–4.