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The relationships of phytosterols and oxyphytosterols in plasma and aortic valve cusps in patients with severe aortic stenosis

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ABSTRACT

Phytosterols such as campesterol and sitosterol are susceptible to oxidation by reactive oxygen species. We hypothesize that the plant sterols (PS) campesterol and sitosterol and their 7-oxygenated metabolites (POPs) correlate within and between human plasma and aortic valve cusps tissues. Plasma and tissue concentrations of PS and POPs were analyzed by gas chromatography–mass spectrometry-selected ion monitoring. Prior to analysis valve cusps tissue was mechanically separated from the calcified parts. PS and POP levels per dry cusps tissue weight were significantly higher compared with the concentrations in the calcified part. Against our hypothesis we found that despite the fact that there is a high correlation between plant sterols in and between plasma and valves cusps tissue, as well as a high correlation between plant sterols and oxyphytosterols and oxyphytosterols themselves within the valve cusps tissue, there was hardly any correlation in the amount of oxyphytosterols in plasma and between plasma and valves. Because plasma samples are easily accessible for large scale population based studies, we have to understand in more detail what the analysis of POPs implies in terms of CVD risk for the future.

1. Introduction

Inflammatory processes triggered by calcification and sterol deposits are key players in the initiation and progress of atherosclerosis [1]. This results in atherosclerotic arterial calcification and calcific aortic valve disease [2]. Next to cholesterol, also plant sterols such as campesterol and sitosterol are found in these lesions [3,4]. Surgical valve replacement is at present the only effective treatment of calcific valve disease [5].

Just like for cholesterol, phytosterols such as campesterol and sitosterol are susceptible to oxidation by reactive oxygen species, leading to the formation of phytosterol oxidation products (POPs) either in food products or endogenously in mammalian tissues and blood (for a general review see [6–9]). Studies on the physiological distribution within the human body and its relationship to its substrate campesterol or sitosterol are scarce. Only a few studies were performed on the presence of POPs in blood from healthy volunteers using highly specific, sensitive, and selective gas chromatography–mass spectrometry, in part as isotope dilution methodology [10–16], or by the use of time-of-flight mass spectrometry [17]. Semi-quantitative determination of 7-oxygenated campesterol and sitosterol in microscopic slices from the aortic lesions in female LDLR ($^{+}/-$) knockout mice fed atherogenic control diet without or together with oxysterols or oxyphytosterols showed a significant increase of the corresponding oxyphytosterols during the feeding period [18]. Unfortunately, in that study POPs were not measured in serum, which hampered the comparison between concentrations in serum and aortic lesions. Moreover, thus far no study measured the concentrations of oxyphytosterols in plasma and cardiovascular tissue from humans simultaneously.

The main purpose of the present study was to evaluate the correlation between the plant sterols campesterol and sitosterol and their 7α- and 7β-hydroxylated metabolites as well as 7-keto-campesterol/-sitosterol within and between plasma and aortic valve cusps. For this purpose the calcificated part of the valve cusps was mechanically separated from the tissue part.

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2. Materials and methods

2.1. Human subjects

The protocol was approved by the local medical ethics committee according to the declaration of the World Medical Association of Helsinki and the institutional and governmental guidelines of the University Hospital of Saarland, Homburg, Germany. We included 104 consecutive patients (36 females/68 males) between 40 to 87 years of age who were admitted to our hospital for elective aortic valve replacement due to severe aortic stenosis. During a structured interview, study participants were assessed for established cardiovascular risk factors and concomitant medication. Demographic data of the study participants are given in Table 1. Sixty-eight patients were treated with simvastatin. Venous blood samples were drawn on the day before the scheduled valve replacement. Aortic cusps were removed from aortic rings in the operation room and kept frozen at −80 °C until work-up.

2.2. Blood plasma preparation

Blood was centrifuged for 5 min at 4000 rpm and 0.25 mg butylated hydroxytoluene (BHT) was added as antioxidant to one mL plasma. The plasma samples were kept at −20 °C until analysis.

2.3. Sterol and oxyphytosterol extraction from valve cusps

Excised valve cusps were dried in a Savant™ SpeedVac™ concentrator (Thermo Fisher Scientific, Schwerte, Germany) for 24 h and the calcified parts were mechanically sorted from valve cusps tissues (Fig. 1).

Cholesterol, campesterol and sitosterol, and oxyphytosterols (7α- and 7β-hydroxy- and 7-keto-campesterol/-sitosterol) were extracted from a cusps tissue aliquot (dry weight) with one mL Folch reagent (chloroform/methanol; 2:1; (v:v); with 0.25 mg BHT added per mL solvent) per 10 mg dried valve cusps tissue. Extraction was performed for 48 h at 4 °C in a dark cold room. The extracts were kept at −20 °C until analysis. The extraction of cholesterol and non cholesterol sterols in calcified valve cusps plaque was similarly performed as described for the tissue.

Table 1
Patients characteristics.

Characteristic	Total (n = 104)
Age (years)	69.8 ± 10.3
Gender: female/male	36/68
Body mass index (kg/m^2)	28.4 ± 6.2
Aortic valve area index (cm^2)	0.77 ± 0.22
Smoking: no/yes	56/48
Hypertension: no/yes	19/85
Diabetes typ I/II/no	2/26/76
Statin/non-statin user	68/36

2.4. Sterol and oxyphytosterol analyses

One mL plasma and two mL of the Folch extract of valve cusps tissue or calcified valve cusps plaque underwent alkaline hydrolysis, extraction of the free sterols and oxyphytosterols, silylation to their corresponding trimethylsilyl ethers prior to gas chromatographic separation and detection either by flame ionization detection (for cholesterol using 5α-cholestane as internal standard) or by mass selective detection (for plant sterols using epicoprostanol and for oxyphytosterols using the corresponding deuterium labeled oxyphytosterols as internal standards, respectively) as described in detail previously [12,19].

2.5. Statistical analyses

Data were tested for normal and Gaussian distribution. Differences for plant sterols and POPs between statin and non-statin users were tested by two-tailed Student t-test. Correlations between absolute and cholesterol corrected sterols and oxyphytosterols within and between plasma and valve cusps tissues were calculated with Pearson correlation equation. P-values <0.05 were considered statistically significant. All statistical tests were performed with SPSS 21 (Chicago, Illinois, U.S.A.) software.

3. Results

3.1. Sterol concentrations are significantly lower in calcified parts than in tissue parts of the valve cusps

Concentrations of sterols or oxyphytosterols in tissues are usually given in mass units per wet or dry weight. Calcified plaque is characterized by a higher weight as compared with pure endothelial tissue. As expected, the concentrations (per mg dry weight) of cholesterol and non-cholesterol sterols found in the calcified part of the valve cusps were significantly lower compared to concentrations in the non-calcified valve cusp tissue (Table 2). The coefficients of variation indicate variations for the concentrations of different sterols in plaque and tissues.

3.2. Correlations of plant sterols in plasma and aortic valve cusps

As no significant differences in the plasma or cusps tissue levels of plant sterols or their 7-oxygenated metabolites could be found between the simvastatin treated patients versus non-statin users all data were included for further correlation analysis. Absolute and cholesterol corrected levels of campesterol and sitosterol show a strong correlation in plasma and in aortic valve cusps and

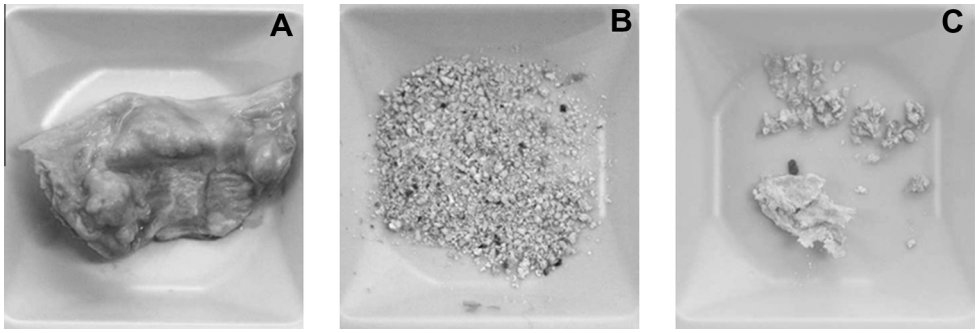

Fig. 1. To sort the "wheat from the chaff", valve cusp (A), calcified material (B) and valve cusp tissue (C).

Table 2
Absolute amount of cholesterol and non-cholesterol sterols measured in calcified parts (plaque; $n = 10$) and the concentrations found in corresponding valve cusp tissues (tissue; $n = 10$).

Plaque/tissue	Min.	Mean ± SD	Max.	CV (%)	Min.	Average ± SD	Max.	CV (%)
	Cholesterol (μg/mg)							
Plaque	4.52	8.43 ± 4.59	19.95	54.4				
Tissue	11.47	22.68 ± 7.20	32.44	31.7				
	Campesterol (ng/mg)				*Sitosterol (ng/mg)*			
Plaque	2.92	12.95 ± 15.24	54.50	107.7	4.29	10.31 ± 7.00	28.38	67.9
Tissue	17.17	47.44 ± 48.27	179.61	101.7	13.64	37.80 ± 31.87	116.65	84.3

Table 3
Correlations of plant sterols in plasma and tissues and between both compartments ($n = 104$).

Compartment 1	Sterol	Compartment 2	Sterol	*R*	*p*-Value
Plasma	Campesterol	Plasma	Sitosterol	0.911	<0.001
Plasma	Campesterol	Tissue	Campesterol	0.488	<0.001
Plasma	Campesterol	Tissue	Sitosterol	0.376	<0.001
Plasma	Sitosterol	Tissue	Campesterol	0.371	<0.001
Plasma	Sitosterol	Tissue	Sitosterol	0.350	<0.001
Tissue	Campesterol	Tissue	Sitosterol	0.945	<0.001
Plasma	r_Campesterol	Plasma	r_Sitosterol	0.904	<0.001
Plasma	r_Campesterol	Tissue	r_Campesterol	0.840	<0.001
Plasma	r_Campesterol	Tissue	r_Sitosterol	0.749	<0.001
Plasma	r_Sitosterol	Tissue	r_Campesterol	0.753	<0.001
Plasma	r_Sitosterol	Tissue	r_Sitosterol	0.775	<0.001
Tissue	r_Campesterol	Tissue	r_Sitosterol	0.930	<0.001

between both compartments as shown in Table 3. Interestingly, the correlations between the cholesterol corrected campesterol and sitosterol levels between different compartments are stronger than for the absolute values.

3.3. Correlations of plant sterols and g oxyphytosterols in plasma and in aortic valve tissue

In plasma campesterol shows a weak correlation with its 7β-OH-metabolite ($R = 0.347$; $p < 0.001$), while sitosterol correlates weakly with 7β-OH-sitosterol ($R = 0.211$; $p = 0.032$) and 7-keto-sitosterol ($R = 0.328$; $p = 0.001$). Comparing cholesterol corrected plasma plant sterol and oxyphytosterol levels there were weak correlations of campesterol and 7β-OH-campesterol ($R = 0.283$; $p = 0.004$) and sitosterol and 7-keto-sitosterol ($R = 0.249$; $p = 0.011$). All POP concentrations, except between cholesterol corrected 7α-OH-campesterol and 7β-OH-campesterol and 7-keto-campesterol correlate with each other in plasma (Table 4). Fig. 2A–C as well as Fig. 3A–C show the correlations between absolute oxyphytosterol levels within aortic valve cusps tissues.

Interestingly, all 7-oxygenated metabolites of campesterol and sitosterol show strong correlations with each other in aortic valve cusps (Table 5). Similar strong correlations could be demonstrated for cholesterol corrected oxyphytosterols (data not shown).

3.4. Correlations of oxyphytosterols and cholesterol corrected ratios in plasma and aortic valve cusps

Even though absolute values of plant sterols and their ratios to cholesterol in plasma and aortic valve cusps show strong correlations (Table 3), this could not be demonstrated for oxidized plant sterols. Oxyphytosterol concentrations in plasma and valve cusps showed only weak correlations for 7β-OH-campesterol ($R = 0.204$; $p = 0.039$), 7β-OH-sitosterol ($R = 0.225$; $p = 0.022$) and 7-keto-sitosterol ($R = 0.208$; $p = 0.035$). This restriction cannot be

Table 4
Correlations of absolute and cholesterol corrected oxyphytosterols levels in plasma ($n = 103$).

Parameter	Parameter	*R*	*p*
7α OH-Campesterol	7β OH-Campesterol	**0.224**	**0.023**
	7keto-Campesterol	**0.274**	**0.005**
7β OH-Campesterol	7keto-Campesterol	**0.541**	**<0.001**
7α OH-Sitosterol	7β OH-Sitosterol	**0.493**	**<0.001**
	7keto-Sitosterol	**0.541**	**<0.001**
7β OH-Sitosterol	7keto-Sitosterol	**0.782**	**<0.001**
r_7α OH-Campesterol	r_7β OH-Campesterol	0.153	0.123
	r_7keto-Campesterol	0.164	0.099
r_7β OH-Campesterol	r_7keto-Campesterol	**0.616**	**<0.001**
r_7α OH-Sitosterol	r_7β OH-Sitosterol	**0.452**	**<0.001**
	r_7keto-Sitosterol	**0.478**	**<0.001**
r_7β OH-Sitosterol	r_7keto-Sitosterol	**0.651**	**<0.001**

The bold values indicate the significant correlations.

ascribed to analytical issues since there are good correlations of plant sterols and their oxidized metabolites in plasma as well as in aortic valve cusps. Comparing the sum of all 7-oxygenated phytosterols in plasma with aortic valve cusps tissue only the sum of 7-oxygenated sitosterol showed a weak correlations ($R = 0.215$; $p = 0.029$).

4. Discussion

The aim of our investigation was to investigate the relationships of plant sterols and their respective oxidized forms in plasma and cardiovascular tissue. Therefore, we developed a method to yield homogeneous sample material from entirely heterogeneous aortic valve cusps. Even though there is a strong correlation of plant sterols in plasma, aortic valve cusps and between the two compartments, there was hardly any correlation of oxyphytosterols in

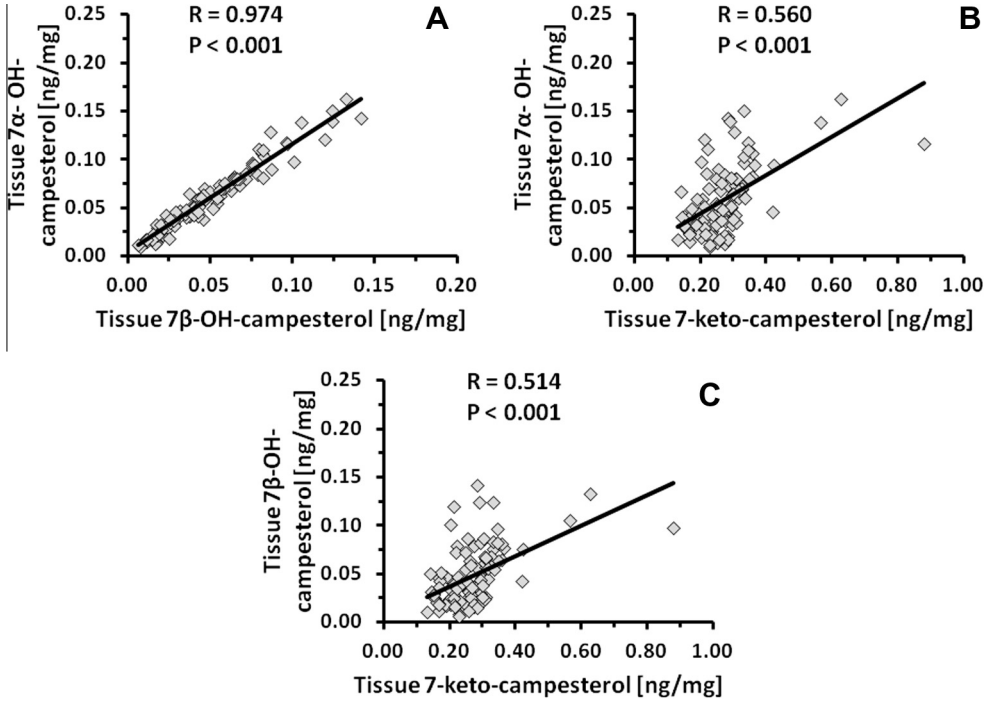

Fig. 2. Correlation between tissue 7α- and 7β-hydroxycampesterol (7α- and 7β-OH-campesterol) (A), tissue 7α-OH- and 7-keto-campesterol (B), and tissue 7β-OH- and 7-keto-campesterol (C).

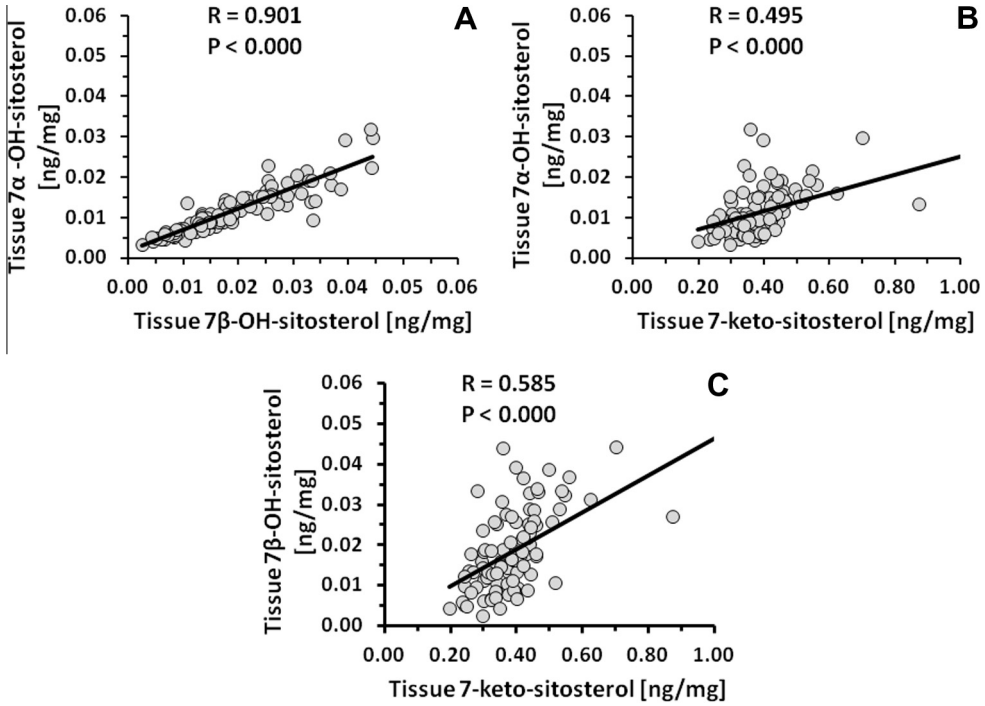

Fig. 3. Correlation between tissue 7α- and 7β-hydroxysitosterol (7α- and 7β-OH-sitosterol) (A), tissue 7α-OH- and 7-keto-sitosterol (B), and tissue 7β-OH- and 7-keto-sitosterol (C).

plasma and between plasma and aortic valve cusps. It could be speculated that higher dynamic metabolic processes for oxidized sterols by radical oxygen species in plasma might be the underlying mechanistic explanation. However, more research in this regard will be need.

Plant sterols in cardiovascular tissue originate from absorbed food sterols. Plant sterols are transported by chylomicrons and LDL-particles in the blood stream and are ultimately deposited in various tissues such as aortic valve cusps [7]. Our data show that campesterol and sitosterol present a strong correlation in plasma, aortic valve tissue as well as between these two compartments. These findings are consistent with previous studies [3,4], and underline the hypothesis that plant sterols behave similar to cholesterol in regard to their distribution in plasma and vascular tissues [20].

Table 5
Correlations of plant sterols and oxyphytosterols in aortic valve cusps tissue (n = 103).

Parameter	Parameter	R	p
Campesterol	7α OH-Campesterol	**0.602**	**<0.001**
	7β OH-Campesterol	**0.629**	**<0.001**
	7keto-Campesterol	**0.637**	**<0.001**
Sitosterol	7α OH-Sitosterol	**0.553**	**<0.001**
	7β OH-Sitosterol	**0.669**	**<0.001**
	7keto-Sitosterol	**0.597**	**<0.001**
r_Campesterol	r_7α OH-Campesterol	**0.539**	**<0.001**
	r_7β OH-Campesterol	**0.560**	**<0.001**
	r_7keto-Campesterol	**0.339**	**<0.001**
r_Sitosterol	r_7α OH-Sitosterol	**0.460**	**<0.001**
	r_7β OH-Sitosterol	**0.520**	**<0.001**
	r_7keto-Sitosterol	**0.234**	**0.017**

This new preanalytic step differentiates "the wheat from the chaff" and determines the concentrations of cholesterol, non-cholesterol sterols and oxyphytosterols selectively in tissue of aortic valves. Drying the sample material allows to separate mechanically the demure calcified parts from the flexible and fibrous aortic valve cusps. Following this procedure the tissue of the aortic valve cusps can be easily separated from the remaining calcified plaque material. Using this new calcium separation technique, cholesterol and plant sterol concentrations (per dry weight) in calcified parts of atherosclerotic plaques are significantly lower compared to the concentrations determined in the tissue of the aortic valve cusps. The data presented demonstrate a wide variation of sterol concentration in calcified parts. These individual differences are due to the heterogeneity of the sample material. Therefore, these results underline the importance to use this preanalytic step to yield a homogeneous sample material and repeatability and reliability for comparison of individual results from each patient. Previous data from our group based on tissue analysis including parts with calcifications show a huge variation for individual samples as well as lower sterol concentrations [4]. The results of the present study imply that cholesterol and non-cholesterol sterol concentrations in previously published studies are the sum of the combined concentrations of two different compartments. Accordingly, previously published studies did not differentiate between tissue and plaque concentrations, which makes comparisons in this regard more difficult. For example our group measured 3.6, 2.3, and 3.8-fold higher concentrations of cholesterol, campesterol, and sitosterol, respectively, in tissue of aortic valve cusps compared with the results of Helske et al. who measured in complete atherosclerotic valve cusps [3]. The underestimation in the study by Helske and colleagues is due to increased sample weight through calcified parts containing lower sterol concentrations.

In plasma there were correlations for sitosterol with its respective 7β-hydroxylated and 7-keto-oxidized metabolite and for campesterol with its 7β-OH hydroxylated form. Cholesterol corrected levels only reveal significant correlations for 7β-OH-campesterol and 7-keto-sitosterol. In contrast to plasma, we here show for the first time that plant sterols correlate significantly with their 7-oxidized metabolites within the tissue of aortic valve cusps. In fact, all oxyphytosterols correlate significantly with each other in the aortic valve cusps. This novel finding could relate to local inflammatory processes in atherosclerotic plaques and tissues which generate free radicals and trigger oxidation processes [21,22]. This local inflammation leads to an amplified progress of atherosclerosis. It might be speculated that most of the oxyphytosterols arise from local oxidation of their subsequent substrates campesterol and sitosterol due to these local conditions.

So if indeed oxyphytosterol concentrations in plasma and valve cusps tissue do not correlate very well, one can wonder what do these concentrations tell us. Very recently Baumgartner et al. showed in a well controlled double blind randomized intervention trial that consumption of plant sterol enriched margarines for four weeks increased serum plant sterol concentrations but not oxyphytosterol levels [16]. In contrast, our group has demonstrated previously that plant sterol consumption did increase POP concentrations in plasma [12]. The samples from both studies were determined in the same laboratory using the same method. We do not have an explanation for this apparent inconclusive data. Most important message from both papers is the lack of correlation between the increase in plasma plant sterols and the change in plasma POP concentrations. Thus far we do not know if the serum levels are determined by the uptake of oxyphytosterols from the diet, the conversion from their substrates by radical oxygen species or by a diminished elimination via the bile or a combination of some or all possibilities. Additionally, a disproportion of 7-keto-phytosterols to 7α- and 7β-hydroxyl-campesterol/-sitosterol is a conceivable equilibrium reaction that can influence plasma concentrations of POPs. Besides the possibility that the lack of correlation is due to the fact that plant sterols oxidize locally, another explanation might relate to the type of tissue sampled for this study. The currently reported findings might suggest that these compounds are locally produced in the tissues and do not originate via uptake from circulating oxidized phytosterols. The tissue of aortic valve cusps examined in this study is a highly specialized, robust structure to resist intense mechanical exposure with a low metabolic rate. Therefore, it might be speculated that in atherosclerotic plaque samples which consist of cells with a higher metabolic rate, OPS tissue concentrations would correlate stronger with their respective plasma concentrations. The results of the present study clearly reveal that more research is needed to better understand the relationships of POPS and cardiovascular risk.

Acknowledgments

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