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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

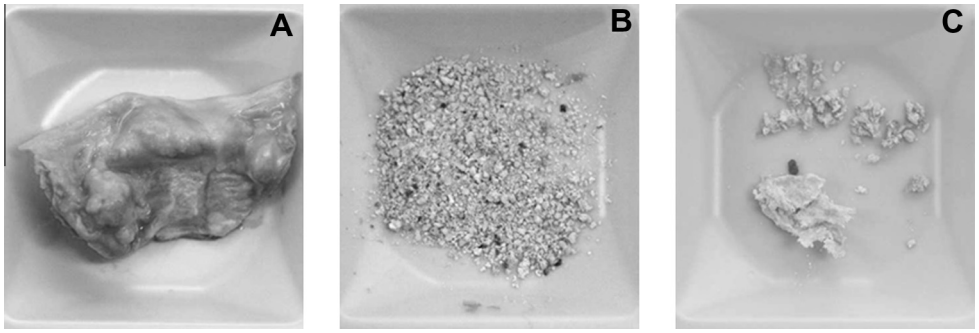

Fig. 1. To sort the "wheat from the chaff", valve cusp (A), calcified material (B) and valve cusp tissue (C).