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Increased plant sterol deposition in vascular tissue characterizes patients with severe aortic stenosis and concomitant coronary artery disease

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ABSTRACT

The aim of the study was to evaluate the relationship between phytosterols, oxyphytosterols, and other markers of cholesterol metabolism and concomitant coronary artery disease (CAD) in patients with severe aortic stenosis who were scheduled for elective aortic valve replacement.

Markers of cholesterol metabolism (plant sterols and cholestanol as markers of cholesterol absorption and lathosterol as an indicator of cholesterol synthesis) and oxyphytosterols were determined in plasma and aortic valve tissue from 104 consecutive patients with severe aortic stenosis ($n = 68$ statin treatment; $n = 36$ no statin treatment) using gas chromatography-flame ionization and mass spectrometry. The extent of CAD was determined by coronary angiography prior to aortic valve replacement.

Patients treated with statins were characterized by lower plasma cholesterol, cholestanol, and lathosterol concentrations. However, statin treatment did not affect the sterol concentrations in cardiovascular tissue. The ratio of campesterol-to-cholesterol was increased by 0.46 ± 0.34 μg/mg (26.0%) in plasma of patients with CAD. The absolute values for the cholesterol absorption markers sitosterol and campesterol were increased by 18.18 ± 11.59 ng/mg (38.8%) and 11.40 ± 8.69 ng/mg (30.4%) in the tissues from patients with documented CAD compared to those without concomitant CAD. Campesterol oxides were increased by 0.06 ± 0.02 ng/mg (17.1%) in the aortic valve cusps and oxidized sitosterol-to-cholesterol ratios were up-regulated by 0.35 ± 0.2 ng/mg (22.7%) in the plasma of patients with CAD. Of note, neither cholestanol nor the ratio of cholestanol-to-cholesterol was associated with CAD.

Patients with concomitant CAD are characterized by increased deposition of plant sterols, but not cholestanol in aortic valve tissue. Moreover, patients with concomitant CAD were characterized by increased oxyphytosterol concentrations in plasma and aortic valve cusps.

1. Introduction

Atherosclerosis is a dynamic process, with interactions between inflammatory, vascular, and cellular components [1], that is characterized by concomitant calcific aortic valve disease and atherosclerotic arterial calcification [2]. High serum cholesterol concentrations are an independent risk factor for coronary artery disease (CAD) [3,4]. Large prospective randomized studies have

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shown that lowering total cholesterol and low-density lipoprotein (LDL-) cholesterol with statins for primary and secondary prevention is associated with reduced cardiovascular morbidity and mortality [5,6]. Therefore, statins, which are competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme-A-reductase (HMG-CoA reductase), are the first line treatment for medical cholesterol reduction [7]. Surgical aortic valve replacement is the gold standard for symptomatic aortic stenosis [8]. In addition to cholesterol, phytosterols such as campesterol and sitosterol are detected in atherosclerotic lesions [9–11]. Due to their chemical structure, phytosterols are susceptible to oxidation in various areas of their sterol structure, resulting in several different phytosterol oxidation products (POPs) [12]. Variable mechanisms are assumed to be responsible for the origin of oxyphytosterols in the human body, including intestinal absorption of oxyphytosterols in foods, autoxidation or ROS mediated oxidation after intake of phytosterols, and UV light-catalyzed conversion of phytosterols in the skin [13]. The distribution of oxidized plant sterols in the human body and their influence on cholesterol metabolism and atherosclerosis are only partly understood. Due to their structural similarities, a great part of the evidence regarding phytosterol oxidation processes is based on studies investigating oxysterols [12]. The cytotoxic effect of oxidized cholesterol and their likely role in the pathogenesis of cardiovascular disease has been reported in a number of studies [14–16]. In recent years, various possible biological effects, including modulation of cholesterol homeostasis, anti-inflammatory and anti-carcinogenic activities, and anti-diabetic properties, have been associated with specific oxyphytosterols [13,17]. However, there are also indications for potential harmful effects of plant oxidation products, such as atherogenicity and toxicity [18]. Plat et al. have recently observed in female LDLR ± mice that the consumption of high fat diets enriched with oxysterols or oxyphytosterols increased the extent of atherosclerotic lesions [19]. Moreover, we found that plasma oxyphytosterols do not correlate with oxyphytosterols in aortic valve tissue [20]. However, thus far, no study has analyzed the relationship between phytosterols and oxyphytosterols measured simultaneously in plasma and cardiovascular tissue with regard to CAD. Therefore, the aim of the present study was to assess the relationships between phytosterols and their corresponding oxidation products in the plasma and aortic valve cusps with concomitant CAD in patients with severe aortic stenosis.

2. Materials and methods

2.1. Study population

Between April 2011 and August 2012, we enrolled 104 consecutive patients with severe aortic stenosis (age 18–90 years) who were scheduled for elective aortic valve replacement. At admission, a detailed medical history was taken with special attention to established cardiovascular risk factors including arterial hypertension, diabetes mellitus, smoking habits, body mass index, age, gender, family history, and statin treatment. The study participants signed a consent form and agreed to provide a blood sample and a portion of the removed aortic valve for sterol determination. The study protocol was approved by the institutional and governmental guidelines of the University Hospital of Saarland, Homburg, Germany. All participants provided informed consent, and the protocol was approved by the ethics committee of the University of the Saarland (number 159/10).

2.2. Blood samples

Venous blood samples were obtained the day before the scheduled aortic valve replacement. Blood samples were centrifuged immediately for 5 min at 4000 rpm. To avoid autoxidation, 0.25 mg butylated hydroxytoluene (BHT) was added as an antioxidant to 1 mL plasma. The plasma samples were stored 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 mechanically sorted from the valve cusps tissue as described in detail [20]. Cholesterol, cholesterol precursors, phytosterols (campesterol, sitosterol), and oxyphytosterols (7α-hydroxy-, 7β-hydroxy-, and 7-keto-campesterol/-sitosterol) were extracted from a cusps tissue aliquot (dry weight) with 1 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 was similarly performed as described for the tissue.

2.4. Sterol and oxyphytosterol analysis

One milliliter of plasma and 2 mL of the Folch extract of valve cusps tissue or calcified valve cusps underwent alkaline hydrolysis, followed by extraction of free sterols and oxyphytosterols and silylation to their corresponding trimethylsilyl ethers prior to gas chromatographic separation and detection by either flame ionization detection (for cholesterol using 5α-cholestane as an internal standard) or mass selective detection (for plant sterols using epicoprostanol and for oxyphytosterols using the corresponding deuterium labeled oxyphytosterols as internal standards, respectively) as described previously [18,21].

One milliliter of plasma and 2 mL of the Folch extract of valve cusps tissue or calcified valve cusps underwent alkaline hydrolysis. Extraction and silylation of free sterols and oxyphytosterols [18] and gas chromatography (GC)-flame ionization of cholesterol and (GC)-mass selective detection (MSD) of non-cholesterol sterols [21] and oxyphytosterols was performed as described in detail previously [18,21].

Briefly, after addition of 50 ng of a mixed deuterium-labeled oxyphytosterol solution containing the internal standards: d_4-7α-OH-Camp, d_4-7α-OH-Sit, d_4-7β-OH-Camp, d_4-7β-OH-Sit, d_5-7-Keto-Camp, d_5-7-Keto-Sit, to 500 µl serum, the samples were exposured for 5 min to a stream of nitrogen to minimize autoxidation of cholesterol and plant sterols. Next, the samples were saponifed for 4 h at room temperature by adding 2 ml of 1 M 90%-ethanolic NaOH solution. The reaction solution was adjusted to pH 7 with 210 µl phosphoric acid in water (1:1, v/v) and 2 ml NaCl solution (9 µg/ml). The sterols (including the oxyphytosterols) were extracted with 2 times 3 ml methylenechloride and the solvent was evaporated under nitrogen at 65 °C. The residue was dissolved in 1 ml n-hexane.

Cholesterol and plant sterols were separated from oxyphytosterols by means of solid-phase-extraction. Silica cartridges (Chromabond, bonded phase SI, 100 mg, 1 ml) were eluted with 3 × 2 ml n-hexane before the sample fraction was loaded. Neutral sterols including cholesterol and phytosterols were washed from the column with 8 × 1 ml 0.5% of 2-propanol in n-hexane (v/v), whereas the absorbed oxyphytosterols were eluted with 4 × 1 ml 30% of 2-propanol in n-hexan (v/v). The oxyphytosterol fraction was dried under nitrogen and silylated by adding 1 ml of silylation reagent (pyridine:hexamethyldisilaze:trimethylchlorosilane, 3:2:1; v/v/v) for 1.5 h at 90 °C. After the evaporation of the solvents, the residue was dissolved in 30 µl n-decane and transferred to an injection vial.

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