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Abstract: Extracts from pine tree bark containing a variety of flavonoids have been used in traditional medicine. Pycnogenol is a proprietary bark extract of the French maritime pine tree (*Pinus pinaster* ssp. *atlantica*) that exerts antioxidative, anti-inflammatory, and anti-platelet effects. However, the effects of Pycnogenol on endothelial dysfunction, a precursor of atherosclerosis and cardiovascular events, remain still elusive. **Methods and results** Twenty-three patients with coronary artery disease (CAD) completed this randomized, double-blind, placebo-controlled cross-over study. Patients received Pycnogenol (200 mg/day) for 8 weeks followed by placebo or vice versa on top of standard cardiovascular therapy. Between the two treatment periods, a 2-week washout period was scheduled. At baseline and after each treatment period, endothelial function, non-invasively assessed by flow-mediated dilatation (FMD) of the brachial artery using high-resolution ultrasound, biomarkers of oxidative stress and inflammation, platelet adhesion, and 24 h blood pressure monitoring were evaluated. In CAD patients, Pycnogenol treatment was associated with an improvement of FMD from 5.3 ± 2.6 to 7.0 ± 3.1 ($P < 0.0001$), while no change was observed with placebo (5.4 ± 2.4 to 4.7 ± 2.0 ; $P = 0.051$). This difference between study groups was significant [estimated treatment effect 2.75; 95% confidence interval (CI): 1.75, 3.75, $P < 0.0001$]. 15-F(2t)-Isoprostane, an index of oxidative stress, significantly decreased from 0.71 ± 0.09 to 0.66 ± 0.13 after Pycnogenol treatment, while no change was observed in the placebo group (mean difference 0.06 pg/mL with an associated 95% CI (0.01, 0.11), $P = 0.012$). Inflammation markers, platelet adhesion, and blood pressure did not change after treatment with Pycnogenol or placebo. **Conclusion** This study provides the first evidence that the antioxidant Pycnogenol improves endothelial function in patients with CAD by reducing oxidative stress. **Clinical Trial Registration:** ClinicalTrials.gov identifier: NCT00641758.

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Effects of Pycnogenol on Endothelial Function in Patients with Stable Coronary Artery Disease - A Double-blind, Randomized, Placebo- controlled, Crossover Study

Pycnogenol improves endothelial function

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Abstract

Objective and Background: Extracts from pine tree bark containing a variety of flavonoids have been used in traditional medicine. Pycnogenol is a proprietary bark extract of the French maritime pine tree (*Pinus pinaster ssp. atlantica*) that exerts antioxidative, anti-inflammatory and anti-platelet effects. However, the effects of Pycnogenol on endothelial dysfunction, a precursor of atherosclerosis and cardiovascular events remain still elusive.

Methods and Results: 23 patients with coronary artery disease (CAD) were completed this randomized, double-blind, placebo-controlled crossover study. Patients received Pycnogenol (200mg/d) for 8 weeks followed by placebo or vice versa on top of standard cardiovascular therapy. Between the 2 treatment periods a 2-week washout period was scheduled. At baseline and after each treatment period, endothelial function, non-invasively assessed by flow-mediated dilatation (FMD) of the brachial artery using high-resolution ultrasound, biomarkers of oxidative stress and inflammation, platelet adhesion and 24-hour blood pressure monitoring were evaluated. In CAD patients FMD improved from 5.3 ± 2.6 to 7.0 ± 3.1 ($p<0.0001$) by Pycnogenol treatment; while no change was observed with placebo (5.4 ± 2.4 to 4.7 ± 2.0 ; $p=0.051$). This difference between study groups was significant (estimated treatment effect 2.75; 95%CI 1.75-3.75, $p<0.0001$). 15-F_{2t}-Isoprostane, an index of oxidative stress, significantly decreased from 0.71 ± 0.09 to 0.66 ± 0.13 after Pycnogenol treatment, while no change was observed in the placebo group (mean difference 0.06pg/ml with an associated 95% confidence interval [0.01, 0.11], $p=0.012$). Inflammation markers, platelet adhesion and blood pressure did not change after treatment with Pycnogenol or placebo.

Conclusions: This study provides the first evidence that the antioxidant Pycnogenol improves endothelial function in patients with coronary artery disease by reducing oxidative stress.

Keywords: Pycnogenol; coronary artery disease; clinical trial; endothelial function; oxidative stress

Clinical Trial Registration: ClinicalTrials.gov identifier: NCT00641758

Introduction

Extracts from pine tree bark have been used in traditional medicine. Pycnogenol is a bark extract of the French maritime pine tree (*Pinus pinaster ssp. atlantica*). The extract is prepared using a standardized procedure that includes an extraction of fresh pine bark with ethanol and water to the requirements published by the United States Pharmacopoeia (USP).¹ The resulting product is a mixture of flavonoids as monomers, i.e. catechin and taxifolin, and condensed polymers (85%), as well as dodeoameric flavonols, designated as procyanidins, differing in structure and chain length. Procyanidins are biopolymers composed of catechin and epicatechin, widely distributed in the plant kingdom. Many edible fruits contain procyanidins, for example apples, berries, peanuts and grapes. Additionally, the extract contains phenolic acids as gallic, caffeic, and ferulic acid as minor constituents. Pycnogenol also contains glycosylation products, i.e., sugar derivatives of phenolcarboxylic acids and taxifolin.² These molecules have antioxidant properties and may act as modulators of metabolic enzymes and other cellular functions.²

In a pharmacokinetic study healthy volunteers ingested a single dose of 300 mg and repeated doses of 200 mg Pycnogenol.³ In blood samples catechin, taxifolin, ferulic acid, caffeic acid and the metabolite 5-(3',4'-dihydroxyphenyl)- γ -valerolactone (M1) were detected in concentrations up to 100 ng/mL. While the first four compounds are genuinely present in the maritime pine bark extract the metabolite M1 is formed *in vivo* from catechin polymers by gut microbiota.^{4, 5} This metabolite had been previously also found in urine samples after Pycnogenol intake.⁶ Additionally to these known constituents and metabolite ten yet unknown compounds were detected in human plasma samples.⁷

The effects of the identified compounds have been investigated *in vitro* with the metabolite M1 revealing the highest activity. On a $\mu\text{g/mL}$ basis M1 was more potent

than the original maritime pine bark extract in inhibiting the activity of matrix metalloproteinases (MMP) 1, 2 and 9.³ M1 was also about twice as potent as hydrocortisone in inhibiting the release of MMP-9 from freshly isolated human monocytes after stimulation with bacterial lipopolysaccharide (LPS). Thus, it appears very likely that M1 plays a major role in mediating the biological effects of Pycnogenol.

However, it cannot be excluded that other yet unknown components or metabolites of Pycnogenol also contribute to its biologic activity. Plasma samples of volunteers who ingested Pycnogenol contained bioactive compounds which in addition to the inhibition of MMP-9 release statistically significantly inhibited NF-kB activation⁸ as well as cyclooxygenase (COX) 1 and 2 activity.⁹

Pycnogenol has been shown to exert anti-inflammatory effects and to inhibit platelet aggregation, both risk factors for cardiovascular disease.² *In vitro*, Pycnogenol prolonged the ascorbate radical lifetime to the greatest extent by regenerating ascorbic acid.¹⁰ Similarly, Pycnogenol inhibits LDL peroxidation, lipid peroxidation in phospholipid liposomes,² and lipid peroxidation caused by t-butylhydroperoxide. Furthermore, Pycnogenol has antioxidant activity.¹⁰ Finally, in isolated blood vessels, Pycnogenol inhibits vasoconstriction caused by adrenalin via the production of nitric oxide.¹¹ The present study was designed to evaluate the effect of Pycnogenol on endothelial function in patients with coronary artery disease (CAD).

Methods

Study population

Patients with CAD, documented by coronary angiography, nuclear imaging or a positive stress test, on stable cardiovascular medication for at least one month and ≥ 18 years of age, who gave written informed consent, were recruited at the Clinic of Cardiology, Cardiovascular Center, University Hospital Zurich, Switzerland.

Exclusion criteria were acute myocardial infarction, unstable angina, stroke, or coronary intervention / revascularization procedure within three months prior to study entry; uncontrolled symptomatic congestive heart failure (New York Heart Association functional class $>II$) in the last 4 weeks prior to study entry; smoking, alcohol or illicit substance abuse; uncontrolled blood pressure despite adequate therapy ($\geq 160/100$ mmHg); symptomatic hypotension; ventricular tachyarrhythmias; any cardiomyopathy, untreated thyroid dysfunction or adrenal insufficiency; renal failure (creatinine clearance using the MDRD-formula¹² < 50 ml/min); liver disease; chronic use of long-acting nitrates; oral or intravenous steroid therapy; insulin-dependent diabetes mellitus; anaemia (Hb < 10 g/dl); known hypersensitivity to Pycnogenol; systemic inflammatory diseases (e.g. rheumatoid arthritis, Crohn's disease); known human immunodeficiency virus (HIV) infection or active virus – hepatitis; pregnancy or breast-feeding, women with childbearing potential without adequate contraception; malignancy (unless healed or in remission > 5 years); recipient of any major organ transplant (e.g. lung, liver, heart) or renal replacement therapy and the participation in another study within the last month.

Study design and protocol

In this single-centre, prospective, randomized, double - blind, placebo - controlled study in cross over fashion we analyzed the impact Pycnogenol on endothelium-

dependent and -independent vasodilatation in patients with stable CAD receiving optimal standard therapy. Prespecified secondary endpoints were change in high sensitive CRP and CD-40 Ligand, change in total antioxidative capacity, oxidized LDL and 8-isoprostanes, change in ambulatory blood pressure (ABP) and change in shear-stress dependent platelet function. The protocol was approved by the Institutional Review Board (Ethics Committee of the Canton Zurich) and the Swiss Agency for Therapeutic Products (Swissmedic, Bern, Switzerland). The study was registered at ClinicalTrials.gov (Identifier: NCT00641758).

After screening and recruiting, the patients were randomly assigned into two groups. For randomization an unpredictable allocation sequence was provided by external institutions (InterCorNet Zurich, Switzerland), which were responsible for the blinding and labelling of the drugs. The study drug and placebo were prepared in identical capsules to ensure uniform appearance of both formulations by the manufacturer Horphag Research (UK) Ltd, London, United Kingdom. All investigators including the ultrasonographers were unaware of the allocation procedure at any time. The patients were randomized to receive either Pycnogenol 200mg/day or matching placebo in the first part or *vice versa* in the second part with a washout period of two weeks in-between.

At each visit (baseline and after 8, 10, and 18 weeks) endothelial function and ABP were measured, blood samples were drawn, the clinical status assessed and adverse events recorded. At each visit a safety analysis was performed including assessment of electrolytes, of liver and kidney function, as well as a white and red blood cell count. Pregnancy testing in women with childbearing potential was performed only at the first visit. The patients were advised not to take their usual drugs in the morning of the examination day (all examinations and measurements were performed in the morning), moreover, the medical therapy was unchanged

throughout the study. Blood samples and the evaluation of flow-mediated dilatation were performed before the patients took their medications. The regular medications and the study drug were taken thereafter and before the 24-hour ABPM was placed.

Endothelium-dependent and -independent vasodilatation

Flow-mediated dilatation (FMD) was performed according to the current guidelines^{13, 14} and as previously described.¹⁵ In brief, a B-mode high-resolution ultrasound scan of the left brachial artery was obtained by highly trained and experienced sonographers in a longitudinal section between 2 and 10 cm above the elbow, using a high-resolution 10 MHz linear array transducer and a high-resolution ultrasound system Siemens X300 (Siemens Switzerland AG, Zurich, Switzerland). The analogue video signal was acquired with a video processing system that computed the artery diameter in real-time (FMD Studio¹⁶, a system for real-time measurement, Institute of Clinical Physiology, Pisa, Italy). The high reproducibility of the method has been demonstrated recently.^{17, 18} Baseline vessel size was considered as the mean of the measures obtained during the first minute. FMD was calculated as the maximal percent increase in diameter above baseline. Endothelium-independent dilatation was measured after sublingual glycerol trinitrate (GTN; 0.4mg, Nitrolingual Spray, Pohl-Boskamp, Hohenlockstedt, Germany) application by recording arterial diameter continuously for at least six minutes. The response to GTN is calculated as the maximum percent increase in vessel size above the baseline. The reproducibility of our laboratory was previously published.¹⁵

Special laboratory analysis

Oxidative stress markers: 8-epi-PGF₂alpha (15-F_{2t}-Isoprostane) was measured in

the plasma with an 8-Isoprostane Enzyme Immunoassay (8-isoprostane Express EIA Kit, Cayman Chemicals, Ann Arbor, MI, USA; intra-assay CV 7.2%, inter-assay CV 15.5%).

High Sensitivity C-reactive protein (hs-CRP): A high sensitive immunoluminometric assay (Imulite 2002, DCP, Los Angeles USA) was used to measure hsCRP levels as described by Wood et al.¹⁹

Soluble Intercellular Adhesion Molecule-1 (sICAM-1), soluble Vascular Cell Adhesion Molecule-1 (sVCAM-1), soluble CD40 Ligand (sCD40L), oxidized LDL (oxLDL): EDTA plasma was drawn from each patient and immediately frozen at -70°C. Plasma samples were thawed only once for aliquotation before they were subjected to analyses. Quantitative detection of soluble Intercellular Adhesion Molecule-1 (sICAM-1), soluble Vascular Cell Adhesion Molecule-1 (sVCAM-1), soluble CD40 Ligand (sCD40L) (all Bender Med Systems, Vienna, Austria), oxidized LDL (oxLDL) (Immundiagnostik, Bernsheim, Germany) and of Lipoprotein-associated phospholipase A₂ (Lp-PLA₂) (Uscn Life Sciences Inc., Wuhan, China) was performed using enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions. Briefly, pre-coated microwell plates absorbed with specific capture antibodies directed against the respective molecule were washed thoroughly with the washing buffers provided. Plasma samples were diluted where indicated and applied in duplicates before a 1 to 2 hour incubation at room temperature. Detection of the target molecules was performed using an HRP-conjugated detection antibody specific for the respective molecule, and visualized during a colour-giving reaction using the provided TMB substrate solution. Absorption was measured at 450nm and values were normalized to the respective internal standards supplied by the manufacturer.

Total antioxidant capacity: Total antioxidant capacity of plasma was measured using the total antioxidant capacity kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. Briefly, plasma was allowed to reduce Cu^{++} for 1.5 hours at room temperature. Reduced Cu^+ was chelated with a colorimetric probe and absorbance was measured at 570nm. Results were expressed as trolox equivalent according to a trolox standard curve.

Endothelin-1 (ET-1): The ET-1 sandwich ELISA-system from Assay Designs (Ann Arbor, MI, USA) was used according to the manufacturer's instructions to measure ET-1 protein concentrations in EDTA plasma samples. Briefly, 500 μL of each plasma sample was extracted with 650 μL extraction solvent (acetone: 1 N HCl: water (40:1:5)) and dried under nitrogen streaming. Reconstituted in 120 μL assay buffer samples were employed for testing. The sensitivity of the assay was determined to be 0.41pg/mL, the intra-assay coefficients of variation were 6.7% at 35.9pg/mL, 8.9% at 2.3pg/mL, and 8.8% at 1.1pg/mL; cross reactivity with human Big ET-1, ET-2 and ET-3 was low (human Big ET-1 <0.1 %, ET-2 21%, ET-3 3.6%).

Asymmetric dimethylarginine (ADMA): ADMA concentration was measured from plasma samples by a competitive ELISA-system including an acylation step as pre-treatment (DLD Diagnostika GmbH, Hamburg, Germany), with a standard range from 0.1-5.0 $\mu\text{mol/L}$. 20 μL of each plasma sample were employed for testing. The detection limit of the system was 0.05 $\mu\text{mol/L}$. As described previously,²⁰ the intraassay coefficients of variation were 5.7% at 0.66 $\mu\text{mol/L}$ and 6.4% at 1.01 $\mu\text{mol/L}$; the interassay coefficient of variation ranged from 8.3% to 10.3%. Cross-reactivity with arginine and other methylarginines was low (arginine <0.02%; NG-monomethyl-L-arginine 1.0%; symmetric dimethylarginine 1.2%). The correlation coefficients with liquid chromatography–mass spectrometry and gas chromatography–mass

spectrometry were high (0.991; 0.984), with a good linearity between 0.1 and 5.0mol/L.

Symmetric dimethylarginine (SDMA): SDMA was measured from serum samples by a competitive ELISA-system including an acylation step as pre-treatment (DLD Diagnostika GmbH, Hamburg, Germany) with a standard range from 0.08-3.0µmol/L. 20µL of each plasma sample were employed for testing. The detection limit of the system was 0.05 µmol/L. The intraassay coefficients of variation were 5.7% at 0.52µmol/L, 6.1% at 0.75µmol/L and 4.7% at 1.72µmol/L; cross-reactivity with arginine and other methylarginines were low (arginine <0.01%; NG-monomethyl-L-arginine 0.70%; asymmetric dimethylarginine 0.44%).

Ambulatory blood pressure measurement

ABP measurements were obtained over 24 hours using the “Tracker NIBP 2” (Delmar, Del Mar Reynolds Medical, Hertford, UK) before and after the active treatment phase according to recent guidelines.²¹ Patients were asked to keep their arm calm while the cuff was inflating and to avoid excessive physical exertion during monitoring. The monitors were programmed to take readings every 15 minutes during daytime and every 30 minutes during nighttime.

Shear Stress–dependent platelet function

Shear stress–dependent platelet function was assessed with a cone and platelet analyzer as described previously.^{15, 22}

Statistical analysis

The primary endpoint was the change in FMD after eight weeks of treatment with Pycnogenol compared to placebo. Based on previous studies,²³ the estimated

sample size for this study is 25 (to show a difference of 1.3% in FMD when the standard deviation of 1.5%, giving 5% two-sided significance level and 85% power when using a t-test). Analysis was performed using Wilcoxon Mann-Whitney U-tests (to account for possible non-normality of the endpoints), using methods discussed by Senn.²⁴ That is, we considered two distinct groups of patients: Group 1 who received Pycnogenol followed by placebo, and Group 2 who received placebo followed by Pycnogenol. Within each group, data are summarized by examining within patients changes between Periods 1 and 2 (for Group 1, this is the change from baseline while on placebo minus the change in baseline while on Pycnogenol; for Group 2, the change from baseline while on Pycnogenol minus the change in baseline while on placebo). These unpaired change scores are then analyzed using an unpaired Wilcoxon Mann-Whitney U-Test, as proposed by Hill and Armitage²⁵, and later discussed by Senn.²⁴ Statistical tests were performed two-sided. The effect of Pycnogenol is estimated as the average of the two group-specific mean change scores. The period effect is estimated using the difference of the two group-specific mean change scores (divided by 2). The carry-over effect was excluded using an unpaired Wilcoxon test of within patient change from baseline including only the first period of treatment. A Bonferroni-Holm²⁶ correction was made if necessary. For the “responder analysis”, patients were divided evenly into groups based on their FMD to Pycnogenol and placebo. Those with better responses were considered “responders”, the others, “non-responders”. The analyses for C-reactive protein and 15-F_{2t}-Isoprostane were then performed again separately for the 2 groups. Results are presented as mean \pm SD or SEM as described. Analysis of the primary end-point was performed in the R programming language (R Development Core Team, 2009). The statistical software package SPSS 17 (SPSS Inc., Chicago, IL, USA) was used

to evaluate difference in the clinical characteristics. Statistical significance was accepted at $p < 0.05$.

Results

Study population

A total of 28 patients were enrolled, 5 patients discontinued the study prematurely, 3 patients withdrew consent and 2 patient were excluded for adverse events: On patient in the Pycnogenol group was hospitalised for acute decompensated heart failure; one patient in the placebo group was excluded after development of an itching rash. Therefore, 23 patients (19 males and 4 females) with a mean age of 63.1 ± 7.1 years (range: 49-73 years) and a body mass index of 27.3 ± 3.3 kg/m² completed the study protocol and were included into the analysis. Mean left ventricular ejection fraction was $62 \pm 10\%$. With the included 23 patients, the statistical power of 80%, is sufficient to detect differences of 1.3% in FMD between the two treatments. All patients had stable coronary artery disease, 11 patients with 1-vessel disease, 1 patient with 2-vessel disease and 11 patients with 3-vessel disease. Mean left ventricular ejection fraction was $62 \pm 10\%$ in these patients.

Effect of Pycnogenol on endothelium-dependent and -independent vasodilatation

After 8 weeks treatment with Pycnogenol, FMD increased from $5.3 \pm 2.6\%$ to $7.0 \pm 3.1\%$, while it remained unchanged in the placebo group ($5.4 \pm 2.4\%$ to $4.7 \pm 2.0\%$; see **Table 1** and **Figure 1**). The estimated effect of Pycnogenol on FMD was an increase of 2.75 compared to placebo (mean difference 2.75 with an associated 95% confidence interval [1.75, 3.74], $p < 0.0001$), a statistically significant treatment effect. The period effect for this endpoint was estimated to be -0.32 (95% confidence interval [-1.31, 0.68], $p = 0.51$), and therefore, not statistically significant. The endothelium-independent vasodilatation induced by GTN remained unchanged before and after treatment with Pycnogenol (**Table 1**).

Effect of Pycnogenol on ambulatory blood pressure

Systolic and diastolic ambulatory blood pressure remained unchanged before and after treatment with Pycnogenol (**Table 2**). The estimated effect of Pycnogenol on systolic blood pressure was an increase of 1.17mmHg compared with placebo (mean difference 1.17mmHg with associated 95% confidence interval [-4.71, 7.04], $p=0.68$), a statistically non significant treatment effect. The estimated effect of Pycnogenol on diastolic blood pressure was an increase of 0.06mmHg compared with placebo (mean difference 0.06 mmHg with associated 95% confidence interval [-3.26, 3.37], $p = 0.97$), a statistically non significant treatment effect.

Effect of Pycnogenol on oxidative stress

15-F_{2t}-Isoprostane was reduced significantly after 8 weeks treatment with Pycnogenol compared to baseline and placebo (**Table 2** and **Figure 2**). The estimated effect of Pycnogenol on 15-F_{2t}-Isoprostane was a decrease of 0.06pg/ml compared with placebo (mean difference 0.06pg/ml with an associated 95% confidence interval [0.01, 0.11], $p=0.012$), a statistically significant treatment effect. This effect was even more pronounced, when the patients “responders” and “non-responders” were analysed. The estimated effect of Pycnogenol on 15-F_{2t}-Isoprostane (Responders) was a decrease of 0.1 compared with placebo (mean difference 0.1 with associated 95% confidence interval [0.00, 0.20], $p = 0.045$), a statistically significant treatment effect, while no change was observed in the “non-responders”. The estimated effect of Pycnogenol on 15-F_{2t}-Isoprostane (Non-Responders) was an increase of 0.04 compared with placebo (mean difference 0.04 with associated 95% confidence interval [-0.02, 0.10], $p = 0.15$), which was not statistically significant.

Effect of Pycnogenol on markers of inflammation

The estimated effect of Pycnogenol on C-reactive protein was an increase of 2.18mg/l compared with placebo (mean difference 2.18mg/l with associated 95% confidence interval [-3.35, 7.71], $p=0.42$), a statistically non significant treatment effect. Moreover, all of the following parameters remained unchanged before and after treatment with Pycnogenol or placebo (**Table 2**): Total antioxidant capacity (mean difference $-0.19\mu\text{M}$ with associated 95% confidence interval [-2.20, 1.83], $p=0.85$), sVCAM (mean difference -20.44ng/ml with associated 95% confidence interval [-128.88, 88.00], $p=0.7$), sICAM (mean difference -67.38ng/ml with associated 95% confidence interval [-141.83, 7.07], $p=0.073$), sCD40-ligand (mean difference 0.02ng/ml with associated 95% confidence interval [-0.21, 0.25], $p=0.85$) and OxLDL (mean difference -2.56ng/ml with associated 95% confidence interval [-18.59, 13.47], $p=0.74$), Lp-PLA₂ (mean difference 74.83ng/ml with associated 95% confidence interval [-46.10, 195.76], $p = 0.21$).

Effect of Pycnogenol on SDMA, ADMA and Endothelin-1

The estimated effect of Pycnogenol on SDMA was a decrease of $0.06\mu\text{M}$ compared with placebo (mean difference $-0.06\mu\text{M}$ with associated 95% confidence interval [-0.16, 0.04], $p=0.23$), a statistically non significant treatment effect. The estimated effect of Pycnogenol on ADMA was a decrease of $0.05\mu\text{M}$ compared with placebo (mean difference -0.05 with associated 95% confidence interval [-0.12, 0.03], $p=0.19$). Though 57% of the patients receiving Pycnogenol and only 30% of the patients taking placebo revealed lower ADMA concentrations after the respective treatment period the effect was not statistically significantly different (**Table 2**).

The mean endothelin-1 concentrations of the participants were throughout at the lower limit of the reported physiological range between 1.10-2.70ng/l²⁷ and no statistically significant treatment effect was observed (**Table 2**).

Effect of Pycnogenol on shear-stress dependent platelet function

Shear-stress dependent platelet function did not change after treatment with Pycnogenol (**Table 2**). The estimated effect of Pycnogenol on area fraction was a decrease of 0.22 compared with placebo (mean difference -0.22 with associated 95% confidence interval [-1.85, 1.42], $p=0.78$), a statistically non significant treatment effect.

Discussion

This study demonstrates an improvement of endothelial function after 8 weeks treatment with the flavonoid extract Pycnogenol at a dose of 200mg QD as compared to placebo in patients with stable coronary artery disease.

The balance between endothelial function and dysfunction plays an important role in the initiation and progression of atherosclerosis, and in the transition from a stable to an unstable disease state.²⁸ As such the evaluation of endothelial function has emerged as an important endpoint in cardiovascular research. A recent meta-analysis suggests that an impairment of brachial FMD is strongly associated with the occurrence of future cardiovascular events.²⁹ Therefore, preservation or recovery of endothelial function is an important therapeutic aim in the prevention of arteriosclerosis and its clinical complications such as myocardial infarction and stroke. The improvement of endothelial function in our study was associated with a reduction in 8-isoprostanes, a marker for oxidative stress, but not with components of the L-arginine metabolism or markers of inflammation, indicating that antioxidant properties of the compound appear to be involved.

An improvement of endothelial function has been demonstrated by Nishioka *et al.*³⁰ in a randomized, double-blind, placebo-controlled study, including 16 healthy young men who were randomized to receive either Pycnogenol (180mg/d) or placebo for 2 weeks. The investigators measured forearm blood flow (FBF) using strain-gauge plethysmography. They found an augmented FBF response to acetylcholine (ACh) in the Pycnogenol group, while no augmentation was seen in the placebo group. However, sodium-nitroprusside (SNP) response was similar in both treatment groups and the administration of N(G)-monomethyl-L-arginine, a known NO synthase

inhibitor, abolished the Pycnogenol-induced augmentation to FBF response to ACh, suggesting an endothelium-dependent vasodilatation by increased NO production.

Several lines of evidence suggest that oxidative stress may promote endothelial dysfunction through different mechanisms.³¹ First, diverse reactive oxygen species (ROS) are produced at increased levels within the vessel wall and they individually or in combination interfere with the function of endothelial and vascular smooth muscle cells.³² The family of ROS includes highly bioactive, short-living molecules that are derived from reduction of molecular oxygen. Multiple enzyme systems use different substrates as sources of electrons to produce a variety of ROS, the most important of which are nitric oxide (NO), superoxide (O_2^-), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$). Many enzyme systems, including NAD(P)H oxidase, xanthine oxidase and uncoupled nitric oxide synthase (NOS) among others, contribute to production as well as to degradation of ROS.³³ $ONOO^-$ is an important mediator of lipid peroxidation and protein nitration, including oxidation of low density lipoprotein (LDL), which has potent proatherogenic effects.³² NO is a crucial mediator of endothelium-dependent vasodilatation and also plays a role in platelet aggregation and in maintaining the balance between smooth muscle cell growth and differentiation.³⁴ NO is rapidly inactivated by O_2^- to form $ONOO^-$, a known mechanism of endothelial dysfunction.

The short half-life of ROS makes them ideal signalling molecules, but it also confounds their measurement in complex biological systems, particularly in humans *in vivo*.³² On the other hand, F₂-isoprostanes are associated with oxidative stress and are reliable markers of *in vivo* lipid peroxidation.³⁵ Isoprostanes (iPs) are a family of prostaglandin (PG)-like compounds formed non-enzimatically through free radical catalysed attack on esterified arachidonate followed by enzymatic release from cellular or lipoprotein phospholipids.³² The measurement of F₂-isoprostanes (F₂-iPs),

containing F-type ring analogous to prostaglandin (PG) $F_{2\alpha}$, provides a reliable tool for identifying populations with enhanced rates of lipid peroxidation.³² In addition to their potential usefulness as indexes of oxidative stress *in vivo*, 8-iso-PGF $_{2\alpha}$ is a vasoconstrictor and modulates platelet activation in response to other agonists.³² Interestingly, in our study, 8-iso-PGF $_{2\alpha}$ plasma levels decreased significantly by $6.43\pm 16.3\%$ after therapy with Pycnogenol ($p=0.012$) as compared to placebo (increase of $4.54\pm 11.1\%$). Moreover, the pronounced improvement of endothelial function after 8 weeks of treatment with Pycnogenol in the “responder” group was paralleled by an significant decrease in 15-F $_{2t}$ -Isoprostane, while in patients in the “non-responder” group no significant change was detected. These findings are in line with a study performed by Ryan and colleagues³⁶, who examined the effects of Pycnogenol on different cognitive and biochemical parameters in a healthy elderly population (60-85 years old). In this double-blind, placebo-controlled, matched-pair study, 101 participants received Pycnogenol (150mg/d) for three months. The participants in the Pycnogenol group showed an improved working memory and decreased levels of F $_{2}$ -isoprostanes compared to the control group.

Indeed, Pycnogenol is able to scavenge both hydroxyl radicals and superoxide anions³⁷ and increase the activity of superoxide dismutase, glutathione peroxidase and catalase.³⁸ These *in vitro* effects have been confirmed in healthy subjects, treated with Pycnogenol 150 mg QD.³⁹ In that study oxygen radical absorption capacity increased by 40% during treatment and returned to baseline after wash-out. However, there was no significant change in plasma lipid peroxidation or in LDL-cholesterol oxidation.³⁹ These data are in line with our study, showing no changes in plasma lipids including ox-LDL or in total antioxidative capacity.

Endothelial dysfunction has been linked to elevated blood levels of asymmetric dimethylarginine (ADMA), which is an endogenous inhibitor of NO synthesis.^{40, 41} Even small changes of ADMA concentration alter vascular NO production. Symmetric dimethylarginin (SDMA) correlates with renal function and indicates an increased cardiovascular risk.^{42, 43} It has been shown to increase production of ROS and is associated with atrial fibrillation, endothelial and platelet activation. Although ADMA plasma levels revealed tended to be lower after Pycnogenol treatment neither the effect on ADMA nor SDMA concentrations reached statistical significance. Although the L-arginine / NO metabolism was not studied in particular, our finding strongly suggests that alterations in the L-arginine/NO metabolisms are unlikely involved in the endothelial protective effects of Pycnogenol.

Endothelin-1 (ET-1) is a potent vasoconstrictor modulating the release and action of NO in the vessel wall.⁴⁴ Furthermore, previous studies suggested⁴⁵ that Pycnogenol may lower during the plasma levels of the peptide. However, in the current study no statistically significant effect of the flavonoid extract could be observed. This might be due to the fact that all participants, although CAD patients, exhibited low ET-1 plasma levels at the lower limit of the physiological reference range. Thus, it cannot be excluded that Pycnogenol might affect vascular ET-1 tissue levels.

The anti-inflammatory properties of Pycnogenol have been demonstrated in a large variety of studies, both *in vitro* and *in vivo*.⁴⁶ Pycnogenol is able to inhibit tumour necrosis factor-alpha (TNF- α) induced nuclear factor kappa B (NF- κ B) activation and adhesion molecule expression in human umbilical vein endothelial cells.⁴⁷ In two double-blind, randomized, placebo-controlled trials in patients with asthma, the investigators found improved asthma symptom scores as well as improved pulmonary function, which was paralleled by a reduction in plasma, as well as in urine leukotriene concentrations after Pycnogenol treatment.^{48, 49} While we could

demonstrate a marked improvement in endothelial function in stable CAD patients treated with Pycnogenol for 8 weeks, which was paralleled by a decrease in 15-F_{2t}-Isoprostane, we could not demonstrate an improvement in inflammatory parameters (hs-CRP, sVCAM, sICAM, sCD40L) or total antioxidative capacity. This lack of effect may be due to the sample-size, which of course was calculated for the primary endpoint.

The effects of Pycnogenol on platelet function were investigated by Araghi-Niknam *et al.* in 60 patients with stable coronary artery disease (CAD), using the PFA-100 test with adenosine diphosphate (ADP) or arachidonic acid (AA) induced platelet aggregation.⁵⁰ After 4 weeks of treatment with the flavonoid extract at a dose of 450mg/d a significant reduction of platelet aggregation was observed compared to placebo or baseline values. However, in our study using Pycnogenol 200mg QD we did not find any difference in shear-stress dependent platelet function, which suggests that higher dosages of Pycnogenol are needed to inhibit platelet function.

Recent studies suggested a blood pressure-lowering effect of Pycnogenol. In a double-blind, placebo-controlled cross-over study involving 11 patients, supplementation with Pycnogenol 200 mg QD significantly reduced systolic blood pressure of patients with mild to moderate hypertension from 139.9±3.3 mmHg to 132.7±4.18 mmHg (p<0.05) after eight weeks of therapy, while diastolic blood pressure remained stable (93.8±1.23 vs. 92±1.7 mmHg, p=ns).⁴⁸ In another double-blind, placebo-controlled trial randomizing 58 hypertensive subjects, intake of 100 mg/d Pycnogenol allowed to reduce the dosage of the calcium channel blocker nifedipine required to maintain normal blood pressure levels.⁴⁵ However, we did not find any change in systolic or diastolic blood pressure in our study. This may be explained, at least in part by the fact, that the blood pressure in our study was well controlled at study entry.

Conclusion

This study demonstrates for the first time an improvement of endothelial function after 8 weeks treatment with Pycnogenol at a dose of 200 mg QD in patients with stable coronary artery disease. As Pycnogenol significantly reduced oxidative stress as assessed by plasma levels of 8-isoprostanes, but left ADMA and SDMA as well as plasma endothelin-1 levels unaffected, these protective effects may be related to the antioxidative properties of the flavonoid extract. The clinical implications of these findings need be confirmed in large-scale clinical outcome trials.

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Disclosures

The authors report no conflict of interest in connection with this manuscript.

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Legend to the figures

Figure 1: Flow mediated dilatation (FMD, %) of the brachial artery in patients receiving Pycnogenol (black bar) or placebo (white bar). * $p < 0.05$ vs. baseline, § $p < 0.05$ vs. placebo.

Figure 2: 15-F_{2t}-Isoprostane before and after Pycnogenol (black bar) or placebo (white bar); * $p < 0.05$ vs baseline, § $p < 0.05$ vs. placebo.

Table 1: Clinical measures before and after 8-weeks treatment with Pycnogenol or placebo and concomitant medication.

	Baseline for treatment period	Pycnogenol	Baseline for control period	Placebo
Clinical parameters				
FMD (%)	5.3±2.6	7.0±3.1*#	5.4±2.4	4.7±2.0
GTN (%)	18.4±5.4	19.4±4.8	19.5±5.4	19.7±5.7
24-hour SBP (mmHg)	125.8±8.7	125.5±8.1	124.8±9.7	125.7±8.1
24-hour DBP (mmHg)	75.0±7.0	74.3±7.3	73.9±7.5	74.4±6.6
Office SBP (mmHg)	129.9±12.5	131.8±14.5	130.1±12.1	129.8±11.7
Office DBP (mmHg)	77.4±6.8	78.9±7.7	78.9±7.5	79.7±8.0
Office HR (bpm)	58.3±8.3	59.4±9.0	60.3±12.3	57.6±9.9
Concomitant Medication				
Aspirin	23/23 (100%)			
Statin	20/23 (87%)			
ACE-Inhibitor / ARB	18/23 (78%)			
Betablocker	17/23 (74%)			
Diuretics	8/23 (35%)			
Calcium-Antagonist	4/23 (17%)			
Clopidogrel	4/23 (17%)			
Ezetimibe	4/23 (17%)			
Oral anti-diabetics	4/23 (17%)			
Marcoumar	1/23 (4%)			
Alpha-Antagonist	1/23 (4%)			

* p<0.05 vs baseline, # p<0.05 vs placebo

Table 2. Laboratory values before and after treatment with Pycnogenol or placebo.

	Baseline before treatment period	Pycnogenol	Baseline before control period	Placebo
Hb (g/L)	14.5±1.2	14.5±1.2	14.6±1.2	14.7±1.2
Hk (%)	41.4±3.2	41.3±3.8	41.1±3.4	41.6±3.3
Sodium (mmol/L)	141.1±2.3	141.6±2.2	141.4±2.1	141.9±2.1
Potassium (mmol/L)	3.9±0.3	3.8±0.4	3.9±0.4	3.8±0.4
Creatinine (µmol/L)	91.7±13.9	90.6±25	91.6±16.7	93.4±14.1
Glucose (mmol/L)	5.7±1.4	5.6±1.3	5.7±1.1	5.7±1.3
TC (mmol/L)	4.6±0.6	4.6±0.7	4.7±0.7	4.8±0.8
HDL-C (mmol/L)	1.4±0.4	1.4±0.4	1.4±0.4	1.4±0.4
LDL-C (mmol/L)	2.5±0.5	2.5±0.6	2.6±0.6	2.7±0.7
TG (mmol/L)	1.4±0.7	1.5±0.7	1.5±0.9	1.5±0.9
ALT (U/L)	27.7±4.9	30.0±7.0	29.4±6.3	29.2±5.4
AST (U/L)	29.5±8.2	31.6±9.3	92.6±9.7	32.9±9.9
hs-CRP (mg/l)	1.52±1.37	1.52±1.33	1.63±1.71	1.91 ± 3.4
Lp-PLA ₂ (ng/ml)	315.89±23.5	321±27.7	279.4±20.4	368.6±24.8
15-F _{2t} -Isoprostane (pg/ml)	0.71±0.09	0.66±0.13*#	0.72±0.09	0.75±0.10
ADMA (µM)	0.47±0.11	0.49±0.11	0.45±0.12	0.50±0.12
SDMA (µM)	0.47±0.13	0.47±0.13	0.45±0.12	0.51±0.13
Endothelin-1 (ng/l)	1.20±0.36	1.27±0.30	1.23±0.27	1.21±0.30
Platelet Adhesion (%)	3.84±2.0	3.49±1.45	3.2±1.6	3.2±2.0
Total antioxidant capacity (µM)	10.12±1.65	10.1±1.28	9.65±2.72	9.78±2.70
sVCAM (ng/mL)	869±232	791±226	824±265	783±244
sICAM (ng/mL)	284±90	232±77	277±91	274±130
sCD40L (ng/mL)	0.54±1.11	0.50±1.0	0.54±1.10	0.46±1.01
OxLDL (ng/mL)	77.9±119.0	78.9±123.5	85.8±155.4	88.5±154.0

* p<0.05 vs baseline, # p<0.05 vs placebo