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Endostatin and osteopontin are elevated in patients with both coronary artery disease and aortic valve calcification

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ABSTRACT

Background: The angiostatic factor endostatin (ES) plays an important role as mediator of angiogenesis. Elevated osteopontin (OPN) was associated with valve calcification in healthy individuals. The present study aimed to investigate ES and OPN levels in patients with both coronary artery disease (CAD) and aortic valve calcification (AVC).

Methods and results: In total 224 non- or ex-smoking patients (161 male, mean age: 61.09 ± 11.02 years; 63 female: mean age: 67.49 ± 7.87 years) with angiographically verified and quantified CAD were recruited. Serum ES and plasma OPN levels were measured by ELISA and AVC was evaluated by a parasternal short axis view and quantified as non-, mild or moderate/severe. There was a stepwise increase of ES measurable with increasing severity of AVC, independent from age, BMI and CAD-severity ($p = 0.018$; $F = 4.09$). OPN also increased significantly with the grade of AVC severity ($p = 0.029$; $F = 3.61$) but was no longer significant when the co-variables ($p = 0.31$; $F = 1.18$) were inserted.

Conclusions: This is the first study showing an association of ES with AVC in CAD-patients independent from age, BMI and CAD-severity which seems to be of distinct interest when trying to understand the process of heart valve calcification. OPN also correlates with AVC-severity but is mostly dependent on the age of the patients.

1. Introduction

The balance between angiogenic and angiostatic processes, which is controlled by numerous factors, is of particular importance for a healthy vascular system. However, angiogenesis also plays an important role in pathological angiogenesis and calcification of cardiac valves although it is rarely subject of investigation [1]. Calcification of aortic valves is the most frequent valvular disease with a prevalence of about 3–9% [2]. Furthermore, it was shown that stenotic aortic valves contain 3 types of neovessels: small and medium microvessels and organized arterioles. In stenotic valves, the distribution of neovessels is significantly higher and correlates with valvular calcification grade and mast cells in the stenotic area were shown to be activated and to contain VEGF [3].

ES is a component of nearly all endothelial basement membranes in the human body and turned out to be, in the long run, a strong angiostatic factor by inhibiting proliferation [4] of endothelial cells and tube formation [5] whereby it might also have angiogenic effects [6] depending on its concentration and on the type of cell it interacts with [7]. Its circulating amounts are influenced by several secondary circumstances such as the presence of diabetes [8] or physical activity [9]. Chalajour et al. investigated the angiogenic response of valvular endothelial cells to aortic valve stenosis in an ex vivo model of aortic leaflets and showed that sprouts from stenotic valves exhibited endostatin [10]. However, whereas recombinant murine endostatin was shown to inhibit microvessel formation in rat aortic rings in an ex vivo model, human endostatin did not [11].

Although OPN is not counted among "classic" angiogenesis-factors such as ES, it was suggested as a kind of "survival factor" for different types of cells (e.g. vascular smooth muscle cells [12]) and has angiogenic potential due to activation of PI3K/AKT- and ERK pathways through VEGF in endothelial cells [13]. OPN was shown to be absent in native non-calcified human aortic valves but present in minimally and highly calcified ones [14]. Similar results were obtained for rheumatic and non-rheumatic mitral valves [15,16]. A further study revealed that OPN is not only present in living aortic valve tissue but also in calcified areas of bioprosthetic heart valves [17]. It gets synthesized mainly by macrophages (and to a small amount by endothelial and smooth muscle cells) [18] and is localized at the surface of calcified deposits [19]. A correlation of elevated plasma levels of OPN and AVC was also found in healthy elderly subjects [20] and dephosphorylation of OPN

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correlates with severe calcification [21]. On the one hand OPN is involved in the process of calcification in bones [22] but on the other hand it was also shown to stimulate bone resorption [23]. Concerning vascular calcification, Wada et al. showed in a cell culture system that exogenous OPN potently inhibited calcification by inhibition of apatite growth [24].

ES and OPN doubtlessly play important roles in CAD but at present their role in AVC is not clear.

2. Material and methods

2.1. Population

In total 161 male (mean age: 61.09 ± 11.02 years) and 63 female (mean age: 67.49 ± 7.87 years) patients, never-smoker or ex-smoker for at least 7 years, with angiographically verified CAD of different severity were recruited. The protocol was approved by the Ethical Committee of the Medical University of Vienna and informed written consent was obtained from patients.

2.2. Definition of CAD

All patients underwent a coronary angiography for diagnostic and/or therapeutic reasons on grounds of their underlying disease. The coronary artery system was divided into 17 segments and stenosis grade for each segment was measured. A simple 3-point-grading system ("Coronary Score" [25]) was developed considering both frequency and severity of CAD. The patients received 0 points for non-stenosed or only calcified segments, 1 point for each stenosis from <30–<50%, 2 points for each stenosis from 50–<70% and 3 points for each stenosis >70%.

2.3. Echocardiographic analysis

Echocardiographic data were obtained with the use of the commercially available ultrasound systems (GE Medical Systems Vivid 7 Dimensions, Horton, Norway). Transthoracic echocardiography was performed by experienced echocardiographer without knowing levels of ES or OPN and was therefore "blinded". The severity of AVC was assessed by two-dimensional echocardiography in a parasternal short axis view. Patients with bicuspid aortic valves were excluded from the evaluation of AVC because bicuspid aortic valves are associated with valvular calcifications per se.

The degree of calcification of the aortic valve (Fig. 1) was scored as follows: 1: no calcification; 2: mild calcification (small isolated spots); and 3: moderate/severe calcification (multiple larger spots or extensive thickening and calcification of all cusps).

2.4. Endostatin and osteopontin analysis

ES was analyzed in serum, OPN in plasma by Enzyme-linked Immunosorbent Assay (ELISA) according to the instructions of the manufacturer.

2.5. Statistical analysis

Statistical analysis was done with SPSS 20.0. Continuous and normally distributed data is described by means ± standard deviation (SD) and group differences were tested by independent sample t-test. Univariate analysis of variance (ANOVA) was used to test the influence of AVC severity on ES and OPN. In a next step variables significantly related to AVC were entered as covariates. Ordinal logistic regression was used to model significant outcome variables onto AVC.

All tests are performed two-sided and p-values ≤0.05 were considered significant.

3. Results

Important anthropometric data, blood pressure and results from ECG are shown in Table 1. Female patients were about 6.5 years older, had a significantly lower BMI ($p = 0.017$) and diastolic blood pressure ($p < 0.001$) and were less commonly ex-smokers ($p = 0.003$) compared to men. No further significant sex-specific differences occurred. Atrial fibrillation was observed in 23 patients, consequently, the PQ-interval refers to the remaining 221 patients.

The coronary score representing the severity of CAD was significantly higher in male compared to female patients ($p = 0.017$). The most frequently affected coronary segments were the medial and proximal

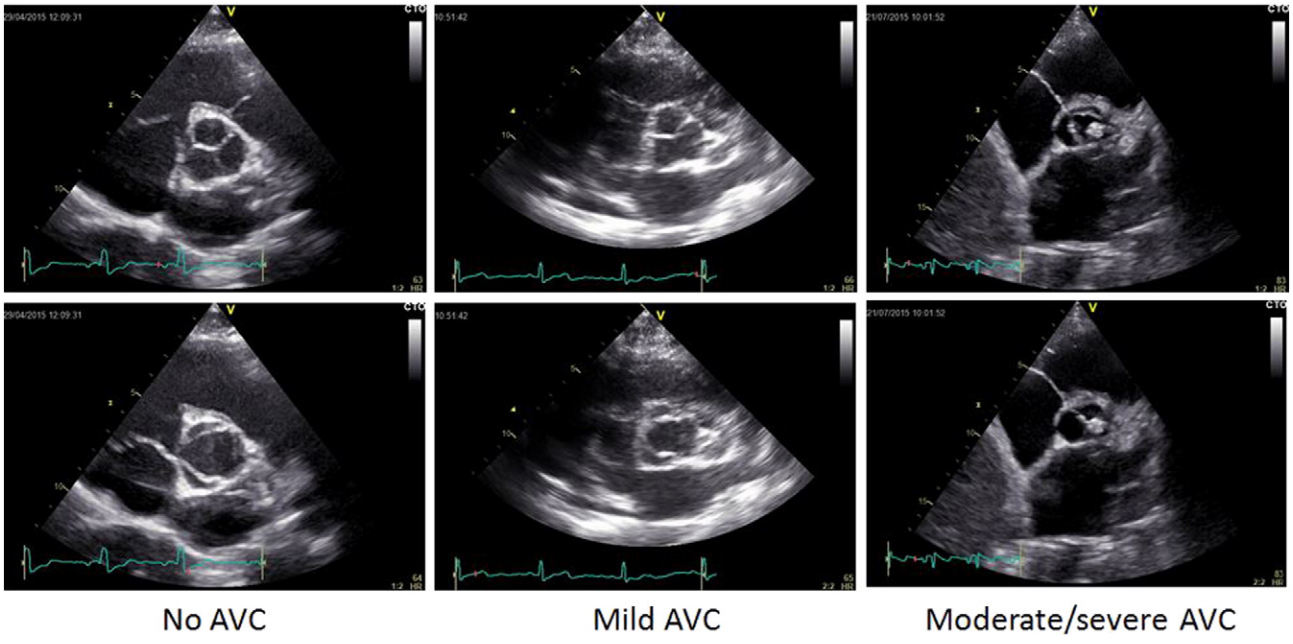

Fig. 1. Severity of AVC.

Table 1
Patient description.

	Female	Male	Total	p-Value
Age (years)	67.49 ± 7.87	61.09 ± 11.02	62.89 ± 10.61	<0.001
BMI (kg/m^2)	26.80 ± 5.40	28.71 ± 4.90	28.17 ± 5.11	0.017
SBP (mm Hg)	131.82 ± 17.04	129.84 ± 16.15	130.39 ± 16.39	0.431
DBP (mm Hg)	72.30 ± 8.77	77.68 ± 11.77	76.16 ± 11.25	<0.001
HR (bpm)	69.95 ± 13.05	70.78 ± 13.31	70.54 ± 13.21	0.676
PQ (ms)	156.14 ± 32.02	165.65 ± 30.17	162.91 ± 30.94	0.055
QTc (ms)	452.14 ± 42.68	443.87 ± 35.45	446.23 ± 37.77	0.176
QRS (ms)	99.40 ± 23.08	103.99 ± 24.73	102.68 ± 24.31	0.206
Coronary score (points)	6.08 ± 5.63	8.02 ± 5.37	7.47 ± 5.50	0.017
Ex-smokers (%/n)	47.6/30	68.9/111	62.9/141	0.003
Diabetes mellitus (%/n)	34.9/22	26.1/42	28.6/64	0.189
Hypertension (%/n)	95.2/60	91.9/148	92.9/208	0.388
Hyperlipidemia (%/n)	88.9/56	91.9/148	91.1/204	0.475

Patient description: anthropometric data, systolic and diastolic blood pressure (SBP, DBP), heart rate (HR), data from ECG and prevalence of ex-smoking, diabetes mellitus, hypertension and hyperlipidemia. Data is given as mean ± SD or %/n.

LAD (55.4 and 52.7%), the proximal, medial and distal RCA (47.3; 42.0 and 30.8%) the proximal and medial LCX (29.5 and 21.0%) and the distal LAD (19.6%). The other segments (left main, intermediate branch, 1st and 2nd marginal and diagonal branches, posterolateral branch, distal LCX and ramus interventricularis posterior) were less commonly affected.

AVC was present in 26.3% (n = 59) of 224 CAD-patients. As can be seen from Table 2 and Fig. 2 ES and OPN levels increased significantly with a grade of AVC (ES: $F = 7.6$; $p < 0.001$; OPN: $F = 3.6$; $p < 0.03$). ES and OPN levels increased from 196.45 ± 74.12/122.03 ± 65.36 ng/ml in patients without AVC to 217.51 ± 49.69/140.01 ± 65.35 ng/ml in patients with mild AVC ($p = 0.150$/$p = 0.358$) and to 250.22 ± 111.48/152.16 ± 71.71 ng/ml in patients with moderate/severe AVC ($p = 0.004$/$p = 0.015$).

When entering age, BMI and CAD-severity score as covariates in the univariate ANOVA, ES was still significantly dependent on AVC-severity, but not OPN. The ordinal logistic regression model for AVC severity was significant ($\chi^2 = 39.8$, $p < 0.001$) but a moderate predictor ($R^2 = 0.23$). Significant predictors in the model were age ($p < 0.0001$), CAD-severity score ($p < 0.01$), and ES ($p < 0.04$) but neither OPN nor BMI,

4. Discussion

It is well-known that AVC-severity is of high predictive value regarding progression and outcome of aortic valve stenosis. The role of ES and OPN in valve calcification, especially in patients with CAD, is nearly unexplored. In the present study we measured circulating levels of ES and OPN in 224 patients with angiographically verified and quantified CAD whereby 26.3% suffered AVC to gain insight into the role of ES and OPN in AVC.

OPN, a non-collagenous glycosylated 44 kDa-phosphoprotein and component of the mineralized bone, was shown in former studies to be present in both living aortic valve tissue and calcified areas of bioprosthetic heart valves [17] and in plasma of healthy subjects with aortic valve calcification [20]. Concerning OPN, our results confirm previous findings: compared to patients without AVC, patients with mild calcification had about 14.7%, and patients with moderate/severe calcification had about 24.7% higher OPN levels. A stepwise increase in OPN was shown before for the aortic valve [21] but not in patients with per se elevated calcification status such as CAD-patients. However, as our data show, the grade of AVC is rather dependent on the age of the patient than on the circulating OPN amount. Valve calcification is characterized by Ca^{++}-deposition and accumulation resulting from several circumstances (e.g. aging and inflammation) [26]. Our results suggest that bone matrix proteins such as OPN which regulate Ca^{++}-deposition in bone and coronary arteries [27] might also be involved in calcification of aortic valves in patients suffering CAD. All of our patients suffered CAD and were homologous concerning e.g. cardiovascular risk factors and routine laboratory parameters but OPN was higher in patients with moderate and severe AVC compared to a large group of patients without calcifications suggesting a distinct involvement of OPN in valve calcification, despite the presence of CAD. But due to results of a study by Wada et al. [24] who suggested OPN to be a potent preventive factor of pervasive vascular calcification it seems more likely that OPN does not promote but counteracts valve calcification and therefore is elevated in course of a calcification processes.

Table 2
ES and OPN in AVC.

	ES	OPN
No AVC (n = 165)	196.45 ± 74.12	122.03 ± 65.36
Mild AVC (n = 15)	217.51 ± 49.69	140.01 ± 65.35
Mod./sev. AVC (n = 44)	250.22 ± 111.48	152.16 ± 71.71

Serum ES and plasma OPN levels (ng/ml) in dependence of AVC. Data is given as mean ± SD.

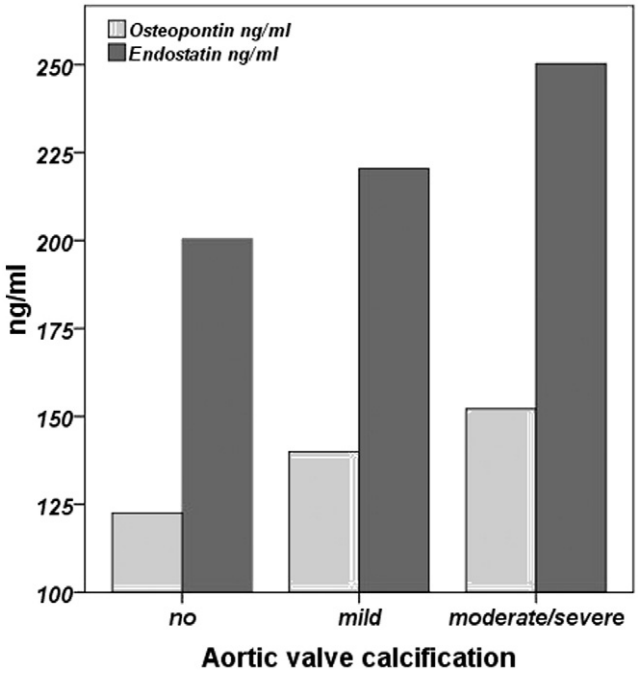

Fig. 2. ES and OPN in AVC.

We furthermore found very similar connection between circulating serum ES levels and AVC, however, its interpretation is much more difficult. Our results showed a strong correlation of ES and the grade of AVC independent from age, BMI and CAD-severity. Whereas literature delivers at least some data and hints for OPN in valve calcification, there is practically nothing available concerning ES. Neovascularization has already been suspected to play a role in aortic stenosis and AVC [28,29]. Healthy human aortic valves are per se avascular structures, however, calcified valves were shown to contain microvessels and arterioles. Compared to patients without AVC patients with mild AVC had about 10.7% and with moderate or severe calcification 27.4% higher serum ES levels. Formerly, valve calcification was supposed to be a passive and degenerative process with deposition of Ca^{++} into valve leaflets as central pathological process. Nowadays, it is assumed that active processes, in particular inflammation, are causal for this group of disease and the deposition of Ca^{++}- is "only" the last step. A mixture of angiogenic and growth factors, inflammatory mediators, enzymes of matrix remodeling, mechanical stimuli and others contribute to the genesis of valve calcification [28,30,31]. However, as the results of the present study show, also the angiostatic factor ES seems to be involved in this process and that is anything but surprising considering that heart valve calcification proceeds very similar to the pathogenesis of atherosclerosis or even the physiological process of bone calcification [32]. Syvaranta et al. [3] raised the possibility that mast cells could be pathogenic players in valve neovascularization: they suspected mast cell-derived tryptase to accelerate valvular neovascularization by degrading ES. Several interactions are thinkable and might explain an ES increase in AVC e.g. to counteract the VEGF-induced neovascularization which is present in aortic stenosis or to counteract inflammatory activity which has also been shown during the process of aortic AVC [26] but due to the lack of data from molecular research and considering the involvement of ES in numerous mechanisms and pathways this remains guesswork.

5. Conclusion

We found a stepwise increase of circulating ES levels in AVC depending on the grade of calcification and independent from age, BMI and CAD-severity in a large study group of patients with angiographically verified CAD suggesting a distinct role and maybe therapeutic possibility of ES in the process of valve calcification. To understand the role of ES and neoangiogenesis in AVC further studies investigating the molecular mechanisms are needed. OPN levels also correlated with AVC-severity but were strongly influenced by the age of the patients.

6. Limitations

Although in total a large number of 224 patients suffering CAD were recruited the number of female patients was low ($n = 63$). A point of criticism might be that angiogenic and angiostatic factors are per se strongly influenced by the presence of CAD. However, for that reason we had a broad number of CAD-patients without AVC delivering reliable baseline levels and considered this aspect in the statistical analysis.

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Disclosures

Conflicts of interest: none.
The authors have no disclosures to make.

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