Distinct Downregulation of C-Type Natriuretic Peptide System in Human Aortic Valve Stenosis

Tuomas O. Peltonen, MD; Panu Taskinen, MD, PhD; Ylermi Soini, MD, PhD; Jaana Rysä, MSc; Jarkko Ronkainen, MD; Pasi Ohtonen, MSc; Jari Satta, MD, PhD; Tatu Juvonen, MD, PhD; Heikki Ruskoaho, MD, PhD; Hanna Leskinen, MD, PhD

Background—Aortic valve calcification is an actively regulated process that displays hallmarks of atherosclerosis. Natriuretic peptides (A-, B-, and C-type natriuretic peptides [ANP, BNP, and CNP]) have been reported to have a role in the pathogenesis of vascular atherosclerosis, but their expression in aortic valves is not known. Here, we characterized and compared expression of natriuretic peptide system in aortic valves of patients with normal valves (n=4), aortic regurgitation (n=11), regurgitation and fibrosis (n=6), and aortic valve stenosis (n=21).

Methods and Results—By reverse-transcription polymerase chain reaction, all 3 natriuretic peptides were found to be expressed in aortic valves. CNP mRNA levels were 92% lower (P<0.001) in stenotic valves, whereas no significant changes in the expression of ANP and BNP genes were found compared with valves obtained from patients with aortic regurgitation. CNP was localized by immunohistochemistry with specific CNP (32-53) antibody to valvular endothelial cells and myofibroblasts. Gene expression of furin, which proteolytically cleaves proCNP into active CNP, was 54% lower in aortic valve stenosis (P=0.04). Moreover, natriuretic peptide receptor-A and natriuretic peptide receptor-B mRNA levels were 78% and 76% lower, respectively, in stenotic valves. In contrast, gene expression of corin, a proANP- and proBNP-converting enzyme, and natriuretic peptide receptor-C did not differ between groups.

Conclusions—We show that natriuretic peptides, their processing enzymes, and their receptors are expressed in human aortic valves. Aortic valve stenosis is characterized by distinct downregulation of gene expression of CNP, its processing enzyme furin, and the target receptors natriuretic peptide receptor-B and natriuretic peptide receptor-A, which suggests that CNP acts as a paracrine regulator of the aortic valve calcification process. (Circulation. 2007;116:1283-1289.)

Key Words: natriuretic peptides • furin • valves • natriuretic peptide receptors • calcification

Aortic valve calcification is the most common valve disease, and it has been shown to be associated with an increased risk of cardiovascular death.1,2 Calcified aortic valve disease represents a spectrum of disease that ranges from mild aortic valve sclerosis to severe aortic valve stenosis. Recent evidence suggests that aortic valve calcification is an actively regulated and cell-mediated process that displays hallmarks of atherosclerosis.2–5 The calcified aortic valve lesion develops endothelial injury and inflammation and is characterized by lipid accumulation, matrix metalloproteinase activation, and activation of the renin-angiotensin system, together with formation of an osteoblast-like phenotype.2,4–7 In addition, numerous factors that control valvulogenesis and angiogenesis, such as vascular endothelial growth factor-A and fibroblast growth factor-4, are involved in the pathogenesis of nonrheumatic aortic valve stenosis.8,9

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The natriuretic peptide family consists of 3 members: atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). Whereas ANP and BNP are secreted mainly by cardiomyocytes, CNP is a local endothelium-derived factor found in vasculature.10 ANP, BNP, and CNP are produced as proforms, and they are converted into mature peptides by proteolytic processing of the respective precursor molecules. Recently, corin, a membrane-bound type II serine protease, has been identified as a proANP- and proBNP-converting enzyme,11 whereas the intracellular endopeptidase furin processes proCNP to the mature 53-amino acid peptide.12 Mouse corin cDNA was originally identified as a novel member of the LDL receptor (LDLR) family and was therefore named LDLR-related protein 4 (LRP4).13 LRP4 transcripts were detected almost exclusively in heart in mouse and humans.14 The biological

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effects of natriuretic peptides are mediated by specific cell-surface guanylate cyclase–linked receptors. ANP and BNP are selective ligands of natriuretic peptide receptor (NPR)-A, whereas CNP is a selective agonist of NPR-B.14 NPR-C is the most abundant NPR and is thought to act as a clearance receptor for natriuretic peptides.14

All natriuretic peptides function as autocrine and paracrine factors both in the heart and in the vasculature by regulating cardiac myocyte and smooth muscle cell growth.15,16 They all also inhibit fibroblast proliferation and extracellular matrix deposition,14,17 which suggests that natriuretic peptides may play a major role in the pathogenesis of atherosclerosis. In support of this, increased vascular atherosclerosis and smooth muscle cell hypertrophy has been reported in mice lacking the NPR-A receptor,18 and expression of CNP and its receptors (NPR-B and NPR-C) in human coronary arteries correlates with severity of atherosclerotic lesions.19

In the present study, we tested the hypothesis that the natriuretic peptide system is involved in the regulation of aortic valve calcification. We measured the gene expression of natriuretic peptides (ANP, BNP, and CNP) in human aortic valves and compared expression of natriuretic peptide genes in patients with aortic valve stenosis to that of patients with normal aortic valves. Furthermore, we evaluated mRNA levels of processing enzymes (corin and furin) and natriuretic receptors (NPR-A, NPR-B, and NPR-C) in diseased human aortic valves. The present study shows that the CNP system is distinctly downregulated in human aortic valve stenosis.

Methods

Patients

Aortic valves were obtained from 42 patients (33 men and 9 women, mean age 57.7 ± 17.0 years) at the time of aortic valve surgery (Table 1). The study protocol was approved by the Research Ethics Committee of Oulu University Hospital and conformed to the principles outlined in the Declaration of Helsinki. All operations were performed after normal surgical procedures. Aortic valve cusps were immersed in liquid nitrogen immediately after removal and stored at −70°C until analyzed. For histological evaluation and reverse-transcription polymerase chain reaction (RT-PCR), one leaflet of the valve was taken and divided into halves. One half of the leaflet was used for histological evaluation and the other for mRNA measurements. The part taken for histological analysis was cut into pieces vertically through the leaflet, fixed, and embedded in paraffin. The area of the leaflet was estimated from these sections.

Patients were divided into 4 groups. The control group consisted of 4 patients who underwent surgery because of root pathology of ascending aorta with normal aortic annulus diameter—ie, the aortic valve leaflets themselves were normal and nonregurgitant. The aortic valve cusps were noncalcified, smooth, and pliable. The aortic regurgitation (AR) group consisted of 11 patients with noncalcified, smooth, and regurgitant pliable valve cusps. The third group, the AR+fibrosis group, consisted of 6 patients who underwent surgery because of AR but who were identified as having macroscopic thickening of aortic valve cusps, which were microscopically identified mainly as fibrotic lesions. These patients had no significant transvalvar pressure gradient. The aortic stenosis (AS) group consisted of 21 patients who had nonrheumatic, severe aortic valve sclerosis. The mean peak-to-peak transvalvar pressure gradient was 85±47 mm Hg in the AS group.

Patients’ demographics are listed in Table 1. Distribution of bicuspid aortic valves was similar in all groups. There were no significant differences in left ventricular ejection fraction, comorbidities, or drugs used between the groups; however, patients in the AS group were significantly older than patients in the control and AR groups (Table 1). End-diastolic left ventricular diameter was increased in patients with AR (Table 1).

Extraction of RNA and Real-Time Quantitative PCR

Total RNA was isolated from aortic valve cusps by the guanidine thiocyanate–CsCl method.20 For RT-PCR, first-strand cDNA was synthesized from 0.5 μg of RNA (First Prime Kit, Amersham, Uppsala, Sweden). Human natriuretic peptides (ANP, BNP, and CNP), processing enzymes (corin and furin), NPRs (NPR-A, NPR-B, and NPR-C), and 18S mRNA levels were measured by real-time quantitative RT-PCR analysis with TaqMan chemistry on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif) as described previously.21 The sequences of the forward and reverse primers and fluorogenic probes for RNA detection are provided in Table 2. The results were normalized to 18S RNA quantified from the same samples.

Histological Analysis

For histological analysis, the aortic valve samples were fixed in 10% buffered formalin solution and embedded in paraffin. Sections 5 μm thick were cut and stained with hematoxylin and eosin for quantification of calcified area. When necessary, EDTA treatment was used to decalcify the sections. The cusp area was determined morphometrically with a grid (with a known area) on the slides, and the

<table>
<thead>
<tr>
<th>TABLE 1. Patient Characteristics</th>
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<tbody>
<tr>
<td>No. of patients</td>
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<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Male, n (%)</td>
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<tr>
<td>Bicuspid valve, n (%)</td>
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<tr>
<td>Age, y (range)*</td>
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<tr>
<td>Left ventricular EF, %</td>
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<tr>
<td>LVEDD, mm*</td>
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<td>DM, n (%)</td>
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<td>Coronary disease, n (%)</td>
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<td>ASO, n (%)</td>
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<td>COPD, n (%)</td>
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EF indicates ejection fraction; LVEDD, left ventricular end-diastolic volume; DM, diabetes mellitus; ASO, peripheral atherosclerosis; and COPD, chronic obstructive pulmonary disease.

Values are mean±SD unless other stated. *P<0.01 (ANOVA).

Pronounced differences were seen in patients with AR (Table 1). End-diastolic left ventricular diameter was increased in patients with AR (Table 1).
were made in a blinded manner by an experienced pathologist. Stain CNP-positive cells in aortic valves. All histological analyses

Bachem, King of Prussia, Pa) in a dilution of 1:2000 was used to histochemical staining. Specific CNP (32-53) antibody (T-4223, purchased from Dako (Glostrup, Denmark). The primary factor VIII rabbit anti-human antibody to factor VIII–related antigen was to visualize density of blood vessels in aortic valves. A polyclonal antibody was applied to the slides for 1 hour at a dilution of 1:50. Localization of CNP in aortic valve cusps was made by immuno- 

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Quantification of Calcified Area

There were no signs of calcification in aortic valve cusps in the control or AR groups, and only 1 patient had a measurable calcified lesion in the AR+ fibrosis group. The proportion of calcified area to total area of aortic valve in the AS group was 25±15% (Figure 1A). When factor VIII staining was used to visualize density of vessels in aortic valves, a trend for increasing density of vessel formation in the AS group was observed (Figure 1B). There were no differences in histological findings between bicuspid and trileaflet valves (data not shown).

Expression of Natriuretic Peptide Genes in Aortic Valve Cusps

We measured mRNA levels of natriuretic peptides in human aortic valve cusps by RT-PCR. All 3 natriuretic peptides were

<table>
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<th>Gene</th>
<th>Sequence</th>
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<tr>
<td>ANP</td>
<td>Forward: GCTTGTACCCCTCTAAAGACAGA &lt;br&gt;Reverse: ATCCGGCAGGCTCGCGA &lt;br&gt;Probe: CGAGAGGTCGCTGCTACACTG</td>
</tr>
<tr>
<td>BNP</td>
<td>Forward: CAGGACAGCAGCGAAGACAGA &lt;br&gt;Reverse: CAGGATGCTCGCTCCACTG &lt;br&gt;Probe: TGGAGAGGCGCTGCTACACTG</td>
</tr>
<tr>
<td>NPR-A</td>
<td>Forward: GCTGCTCTCCTTGAAGAAGGCA &lt;br&gt;Reverse: ATCAGACAGCAAGCAGAACACAG &lt;br&gt;Probe: GAGGAGAAGGACGATCGTGA</td>
</tr>
<tr>
<td>Furin</td>
<td>Forward: CCAAGCTTTCTTGACACACA &lt;br&gt;Reverse: GCCATCTCCGGCCGAC &lt;br&gt;Probe: TGGAGAGGTCGCTGCTACACTG</td>
</tr>
<tr>
<td>NPR-B</td>
<td>Forward: GCTGCTCTCCTTGAAGAAGGCA &lt;br&gt;Reverse: ATCAGACAGCAAGCAGAACACAG &lt;br&gt;Probe: CAGGGTGGTGCCCTTCCGTCA</td>
</tr>
<tr>
<td>NPR-C</td>
<td>Forward: ACCGACTGTGATCGTCACTG &lt;br&gt;Reverse: ATCAGACAGCAAGCAGAACACAG &lt;br&gt;Probe: TGGAGAGGTCGCTGCTACACTG</td>
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<tr>
<td>18S</td>
<td>Forward: TGGGTGGTGCTGCCTTCCTG &lt;br&gt;Reverse: AGTAAATATATTCGGCACAGG &lt;br&gt;Probe: CAGGGTGGTGCCCTTCCGTCA</td>
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Gene Sequence

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<td>Sequences used for mRNA quantification</td>
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<tr>
<td>Gene</td>
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<tr>
<td>ANP</td>
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<td>BNP</td>
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<td>NPR-B</td>
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<td>NPR-C</td>
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<td>18S</td>
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Statistical Analysis

Results are expressed as mean and SD. To minimize type II error (ie, risk of a false-negative result), we first compared patients with normal valves (control group, n=4) with the AR group (n=18) by Mann-Whitney U test. Because there were no significant differences in gene expression of natriuretic peptides, their processing enzymes, or target receptors between the control group with normal aortic valves and the AR group, the 3 groups (AR, AR+ fibrosis, and AS) were compared in the main statistical analysis. Because groups differed by age and sex (Table 1), these variables were set as covariates in the ANCOVA to control their influence on measurements. Neither of these covariates was significant in any of the ANCOVA models. If ANCOVA showed a significant group effect, the adjusted means between groups were compared in a pairwise manner. Sidak adjustment with 3 replications was used to control multiple-comparison problems. Probability values for group effect ($) and adjusted probability values ($) according to Sidak adjustment are reported. If the dependent variable was heavily right-skewed, then logarithmic transformation was used. Spearman correlation coefficients ($) were determined. Analyses were performed by SPSS for Windows (version 14.0, SPSS Inc, Chicago, Ill). Two-tailed probability values <0.05 were considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Figure 1. A, Proportion of area of calcification in aortic valve cusps to total area of aortic valve cusps. B, Area of angiogenesis in aortic valve cusps, in square millimeters. Results are mean±SD. **P<0.05 vs AR group. C indicates control group (n=4); AR, AR group (n=11); AR+ Fibr, AR+ fibrosis group (n=6); and AS, AS group (n=21).
found to be expressed in aortic valves. There were no significant differences in the gene expression of natriuretic peptides between the control group with normal aortic valves and the AR group (Figure 2). Remarkably, CNP mRNA levels were 92% lower ($P<0.001$, $P<0.001$) in stenotic valves than in valves obtained from patients with AR (Figure 2A). In addition, CNP mRNA levels were lower (83%, $P=0.002$) in the AS group than in the AR+fibrosis group. There was also a tendency for mRNA levels of ANP to decrease in patients with valvular stenosis, but this change in mRNA levels was not statistically significant (Figure 2B). However, ANP mRNA levels were lower (42%, $P=0.023$, $P=0.048$) in the AS group than in the AR+fibrosis group. There were no statistically significant changes in expression of the BNP gene between groups (Figure 2C). To evaluate the localization of CNP in aortic valves, we used immunohistochemistry. CNP-positive staining was found in valvular endothelial cells, myofibroblasts, and stromal cells (Figure 3). The levels of CNP mRNA, as measured by RT-PCR, did not differ significantly between the bicuspid and trileaflet valves in patients with AS (data not shown).

**Selective Decrease in Furin Gene Expression in Aortic Valve Stenosis**

Because CNP gene expression was lower in patients with severe AS, we next measured mRNA levels of furin, which proteolytically cleaves proCNP into active peptide in aortic valves. mRNA levels of furin were measurable in all types of aortic valves. The expression of furin was markedly lower (54%, $P=0.045$, $P=0.04$) in stenotic aortic valves than in the AR group (Figure 4A), whereas mRNA levels of corin, identified as a proANP- and proBNP-converting enzyme, did not differ between the groups (Figure 4B).

**Downregulation of NPR-A and NPR-B in Stenotic Valves**

To further evaluate the functional role of the natriuretic peptide system in human aortic valve stenosis, we studied the expression of NPR genes in aortic valves. CNP acts in an autocrine/paracrine fashion to induce vasorelaxation and vascular remodeling mainly through its cognate receptor NPR-B, whereas NPR-A mediates the effects of ANP and BNP. All 3 types of NPRs were present in aortic valves. NPR-A and NPR-B mRNA levels were 78% ($P=0.004$, $P=0.005$) and 76% ($P=0.001$, $P=0.002$) lower, respectively, in patients with aortic valve stenosis than in the AR group (Figures 5A and 5B). A significant correlation between patient age and NPR-B mRNA levels was observed in the AS group (Spearman $\rho=0.48$, $P=0.026$). In contrast, there was no significant difference between groups in the expression of NPR-C, which does not possess any known intrinsic enzymatic activity.

**Discussion**

Aortic valve sclerosis is a common problem in developed countries, with a prevalence of $\approx25\%$ in people 65 to 75
years of age. Although only a small percentage of patients with aortic valve calcification progress to aortic valve stenosis, the prevalence of severe aortic obstruction increases with age, being present in up to 4% of adults >65 years of age.

Therefore, pathophysiological mechanisms regulating aortic valve calcification have been intensively characterized to reveal novel targets for pharmacotherapy to slow the progression of the disease. Here, we show for the first time that all components of the natriuretic peptide system are expressed in human aortic valves. Remarkably, aortic valve stenosis was characterized by distinct downregulation of gene expression of CNP, its processing enzyme furin, and target receptors NPR-B and NPR-A, which suggests that CNP may be an important regulator of the calcification process in aortic valves. The results should be interpreted with caution, however, because the control group, although unique, was small. Moreover, the groups differed by age, with patients in the AS group being older than patients in the control and AR groups. Nevertheless, the only significant correlation in the AS group between age and the measured parameters was for NPR-B mRNA levels. Although groups also differed by sex, it is unlikely that sex confounded the results, because there were fewer men in the AS group (62%) than in the other groups (75% to 100%), and male gender has been associated with calcific aortic valve disease.

All natriuretic peptides have been shown to be expressed in vascular wall. They mediate beneficial effects on vasculature by decreasing vascular smooth muscle and endothelial cell growth. In the present study, we observed that CNP is mainly localized in the endothelial cells and myofibroblasts of aortic valves. Previously, it has been shown that vascular endothelium and valve endothelium have functional differences. In proliferation analysis, porcine valve endothelial cells grew more rapidly than aortic endothelial cells and had differential transcriptional and proliferative profiles. Given that pathophysiological processes in vascular endothelium and valve endothelium may differ, the present finding of the existence of natriuretic peptides and their proteolytic enzymes and receptors in human aortic valve cusps represents a new possible regulatory system involved in valve pathophysiology.

In human coronary arteries, all natriuretic peptides have been suggested to be involved in the pathophysiology of intimal plaque formation and vascular remodeling. Previous studies also showed that CNP inhibits neointima formation in balloon-injured rabbit arteries. Compared with ANP, CNP more potently prevents endothelial dysfunction, vascular smooth muscle cell migration and proliferation, and neointima formation in a rabbit model of intimal thickening. Because CNP has been shown to suppress expression of vascular adhesion molecules and plasminogen activator inhibitor-1 activity and to inhibit leukocyte recruitment and platelet-leukocyte interactions, CNP has been considered an antithrombotic factor. Because CNP mRNA levels were markedly lower in stenotic valves, the present findings support the hypothesis that the calcification process may be enhanced by the decreased ability of valve endothelial cells to produce antithrombotic CNP.

Another major finding of the present study is that gene expression of proprotein convertase furin, which modulates formation of biologically active CNP, was downregulated similarly to CNP gene expression during the calcification process. Therefore, impairment of CNP production in aortic valve stenosis occurs both at the level of CNP gene expression and during the posttranslational proteolytic cleavage process that converts precursor molecules to mature, biologically active peptide. However, in addition to CNP, several substrates activated by furin-like proprotein convertases have been described (eg, transforming growth factor-β precursor). Previous immunohistochemical studies have demonstrated the presence of higher levels of transforming growth factor-β1 in calcified human aortic valve cusps than in noncalcified cusps. Therefore, furin may have opposing effects in the calcification process. Increased production of biologically active CNP with antithrombotic properties would be a beneficial process, whereas increased production...
of transforming growth factor-β serves as a proatherogenic mechanism.2

The pathophysiological consequences of reduced CNP gene expression in aortic valve stenosis may be further enhanced by the concomitant downregulation of NPR-B receptors, which mediate the biological effects of CNP. In addition to downregulation of NPR-B, the present study shows that gene expression of NPR-A was lower in stenotic aortic valves. The rank order of activation of NPR-B by natriuretic peptides is CNP>ANP>BNP, and that of NPR-A is ANP>BNP>CNP.33 Thus, although ANP and BNP mRNA levels were not significantly changed in valves of patients with AS compared with valves obtained from control patients or patients with AR, the effects of ANP and BNP may be attenuated by downregulation of their target receptors. Of note, mice lacking NPR-A receptors have been shown to have 64% greater coincidence of atherosclerotic lesions. Of note, mice lacking NPR-A receptors have been shown to have 64% greater coincidence of atherosclerotic lesions and more advanced plaque morphology.18

The molecular mechanisms that mediate downregulation of the CNP system in aortic valve stenosis remain to be studied. Previously, cytokines, such as interleukin-1 and tumor necrosis factor-α, and lipopolysaccharides have been shown to increase CNP secretion from vascular endothelial cells.34 Because inflammation is a well-characterized feature of aortic valve stenosis, with increased expression of interleukin-1β, for example,2,35,36 one would expect stenosis to be associated with increased CNP production rather than downregulation of CNP gene expression. The downregulation of the CNP system could be related to endothelial cell damage in stenotic aortic valve cusps leading to an extensive decrease in the production of a number of endothelium-derived factors, including CNP. However, then one would expect to also observe a decrease in other endothelium-derived components of the natriuretic peptide system, such as NPR-C and corin.

In conclusion, the key finding of the present study is that aortic valve stenosis is characterized by distinct downregulation of gene expression of CNP, its processing enzyme furin, and target receptors NPR-A and NPR-B, which suggests that CNP may be an important regulator of the calcification process in aortic valves. A major question to be studied is whether endothelial dysfunction and endothelial injury during the calcification process leads to decreased CNP production or whether decreased CNP production is an individual risk factor for development of atherosclerotic lesions. Furthermore, it will be of interest to investigate whether CNP has therapeutic potential in aortic valve calcification. To date, only the benefits of statins and ACE inhibitors have been widely studied,2 and the results of these retrospective studies have been controversial.2

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We thank Marja Arbelius, Sirpa Rutanen, Kaisa Penttilä, and Kati Viitala for their expert technical assistance.

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Disclosures
None.

References
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