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ENDOTHELIAL FACTORS IN THE PATHOGENESIS OF AORTIC VALVE STENOSIS

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ACTA UNIVERSITATIS OULUENSIS
D Medica 997

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**ENDOTHELIAL FACTORS
IN THE PATHOGENESIS OF
AORTIC VALVE STENOSIS**

Academic dissertation to be presented, with the assent of the Faculty of Medicine of the University of Oulu, for public defence in Auditorium 101 A of the Faculty of Medicine (Aapistie 5 A), on December 19th, 2008, at 12 noon

OULUN YLIOPISTO, OULU 2008

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Acta Univ. Oul. D 997, 2008

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ISBN 978-951-42-8987-3 (Paperback)
ISBN 978-951-42-8988-0 (PDF)
<http://herkules oulu fi/isbn9789514289880/>
ISSN 0355-3221 (Printed)
ISSN 1796-2234 (Online)
<http://herkules oulu fi/issn03553221/>

Cover design
Raimo Ahonen

OULU UNIVERSITY PRESS
OULU 2008

Peltonen, Tuomas, Endothelial factors in the pathogenesis of aortic valve stenosis

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Acta Univ. Oul. D 997, 2008

Oulu, Finland

Abstract

Calcified aortic valve disease represents a spectrum of disease spanning from mild aortic valve sclerosis to severe aortic valve stenosis (AS), being an actively regulated disease process and showing some hallmarks of atherosclerosis. The calcified aortic valve lesion develops endothelial injury and is characterized by inflammation, lipid accumulation, renin-angiotensin system activation and fibrosis. There is no approved pharmacological treatment available in AS.

This study was aimed to characterize gene expression of endothelial factors in aortic valves in patients representing different stages of calcified aortic valve disease to reveal new targets for pharmacological interventions in AS. Aortic valves obtained from 75 patients undergoing valve replacement surgery were studied. Expression of natriuretic peptides (ANP, BNP and CNP), their processing enzymes (corin and furin), natriuretic receptors (NPR-A, NPR-B and NPR-C), endothelin-1 (ET-1), endothelin converting enzyme-1 (ECE-1), endothelin receptors A and B (ET_A and ET_B), and apelin pathway (apelin and its receptor APJ) was characterized by reverse-transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry.

AS was characterized by distinct downregulation of gene expression of CNP, its processing enzyme furin and the target receptor NPR-B. Furthermore, increased amount of ET-1 and its target receptor ET_A as well as imbalance between ET_A and ET_B receptors and downregulated endothelial nitric oxide synthase (eNOS) gene expression were observed. Finally, gene expression of apelin and APJ receptor were significantly upregulated in stenotic valves when compared to controls in combination with disequilibrium between expression of angiotensin II receptors AT₁ and AT₂. The study provides a better understanding of molecular mechanisms associated with calcific aortic valve disease and suggest potential targets for novel therapeutic interventions.

Keywords: aortic valve stenosis, apelin, APJ, C-type natriuretic peptide, endothelin receptors, endothelin-1, furin, natriuretic peptide receptors

To Eevi, Severi and Jenni

Acknowledgements

This work was carried out at the department of Pharmacology and Toxicology, Institution of Biomedicine, University of Oulu, during years 2000–2008. I am grateful to Professor Olavi Pelkonen, the head of the Department of Pharmacology and Toxicology, for providing optimal conditions for research work in his department. His leadership, encouraging attitude and wide knowledge of science created a perfect atmosphere for scientific work.

I owe my deepest gratitude to my supervisor Professor Heikki Ruskoaho, whose guidance has created the basis of this thesis. His broad knowledge of the cardiovascular system and pharmacology combined with the enthusiasm and optimistic attitude towards science and life in general has inspired me throughout these years. The research environment for preparing this thesis in the research group, having a status of center of excellence of Finnish Academy, has been excellent.

I am very grateful to Docent Ken Lindstedt and Docent Timo Savunen for their careful review of this thesis and for constructive criticism. I would like to thank Lauri Gardner, M.Phil., for his assistance in proofreading the text.

I would also wish also express my sincere thanks to Panu Taskinen, MD, Ph.D.. His role was crucial when being the main surgeon of the project in the Oulu University Hospital collecting aortic valves used in this research. I also appreciate Panu's demanding attitude to push forward the project.

I want to thank “secret ace” of our aortic valve project, Pasi Ohtonen, M.Sc., showing the meaning and significance of our results with the language of biostatistics to the referees confronting us. Methodological guidance received from Hanna Leskinen, MD Ph.D., Professor Ylermi Soini, Professor Olli Vuolteenaho and Juha Näpänkangas, MD, were of a great value.

I wish to thank my co-authors, Jaana Rysä, Jarkko Ronkainen, Docent Jari Satta and Professor Tatu Juvonen for the collaboration.

I sincerely thank my closest colleagues Sini Rautio, Jani Aro, Annina Kelloniemi, Anna-Maria Kubin, Hanne Luosujärvi, Anne-Mari Moilanen, Elina Koivisto, Leena Kaikkonen, Pauli Ohukainen, Harri Pennanen, Virva Pohjolainen, Jenni Tikkanen and Marja Tölli, for the numerous unforgettable moments in the laboratory as well as during congress trips. Thanks belong to all the old and new colleagues in the “Natriuretic Peptide Team of Ruskoaho” – it has been a privilege to work with you all.

Conducting research can be considered as a state of mind rather than active work. This state of mind can be most probably reached in Sikaosasto, where ideas are ennobled to become the articles published in world leading cardiovascular journals.

I am indebted to the staff of the Department of Pharmacology and Toxicology and the Department of Pathology for their skilful technical assistance, which has been essential for this work. I owe my particular debt of gratitude to Marja Arbelius, Pirjo Korpi, Tuulikki Kärnä, Manu Tuovinen, Kaisa Penttilä, Kati Viitala, Sirpa Rutanen, Esa Kerttula, Terttu Keränen and Marja Räninä; as well as to Raija Hanni for being the mother of the department

My thanks belong to my friends Kalevi and Sylvi Ahde, Juha and Sanna Hagman, Samppa Hakkarainen, Perttu Hiisivuori, Jukka Himmanen, Mika Hämäläinen, Essi and Risto Ilomäki, Hannu Juuti, Arto and Ritva-Liisa Kahlos, Jukka-Pekka Karjalainen, Hannu Koistinen, Antto Kulla, Kai Laatikainen, Johan and Eeva Löfgren, Jari Markkanen and Eva Schromm, Tuomo Mehtälä, Jussi Näyhä, Mika Pohjonen, Heidi Salmi, Olli-Pekka and Tuija Seppänen, Jussi and Sanni Pietilä, Laura Ristimäki, Timo Toivonen, Karoliina Tuppurainen, Mikko and Reetta Vahteri, Jussi Virolainen and Vesa Vähäkangas for a good time also outside of the world of science.

I want to thank my brother Jaakko and my sister Johanna for being there and being the godfather and godmother to Severi and Eevi. I thank my parents Raili and Tuomas Peltonen for giving me a good basis for my life as well as being role models for me in bravely ploughing my own furrow in science. I also wish to thank Marketta and Jouko Hakkarainen for their support and pleasant moments in every day life as well as during holidays.

Finally, I want to express my dearest thanks to my family for your endless love; Eevi and Severi, you have taught me more than I have ever learned before. The time spent with you has been the rays of sunshine claring up my coulds of doubt. My beloved wife Jenni, without you this thesis would not have been finished. You are the light of my every day life.

This research project have been supported financially by the Academy of Finland, the Finnish Medical Foundation, the Aarne Koskelo Foundation, the Finnish Foundation for cardiovascular research (three (3) grants), Biocenter Oulu, Einar and Karin Stroems Foundation, Emil Aaltonen Foundation, Finnish Society of Angiology, Ida Montin Foundation, Maud Kuistila's Memory Foundation, Paavo Ahvenainen Foundation, the Research and Science Foundation of Farnos and Sigrid Juselius Foundation, which I gratefully acknowledge.

Assistance from Boehringer-Ingelheim Finland in form of support in congresses (World Congress of Cardiology (WCC) 2006 and European Society of Cardiology (ESC) 2008 as well as an invoice to Stand Alone 2009) has been accepted.

Oulu, November, 2008

Tuomas O. Peltonen

Abbreviations

ACC	American College of Cardiology
ACE	angiotensin-converting enzyme
ACEI	angiotensin-converting enzyme inhibitor
AHA	American Heart Association
Ang II	angiotensin II
ANOVA	analysis of variance
ANP	atrial natriuretic peptide
AP-1	activator protein-1
APJ	apelin receptor
AR	aortic regurgitation
AS	aortic stenosis
AT ₁ receptor	angiotensin receptor subtype 1
AT ₂ receptor	angiotensin receptor subtype 2
AVA	aortic valve area
BNP	B-type natriuretic peptide
BSA	body surface area
cDNA	complementary deoxyribonucleic acid
CHF	congestive heart failure
CNP	C-type natriuretic peptide
ECE-1	endothelin converting enzyme 1
EF	ejection fraction
EMSA	electrophoretic mobility shift assay
eNOS	endothelin nitric oxide synthase
ESC	European Society of Cardiology
ET-1	endothelin-1
ET _A receptor	endothelin receptor subtype A
ET _B receptor	endothelin receptor subtype B
GPCR	G-protein coupled receptor
IL	interleukin
iNOS	inducible nitric oxide synthase
LVEDD	left ventricular end-diastolic dimension
LVEF	left ventricular ejection fraction
LVH	left ventricular hypertrophy
MCP-1	monocyte chemoattractant protein-1
MMP	matrix metalloproteinase

NPR-A	natriuretic peptide receptor subtype A
NPR-B	natriuretic peptide receptor subtype B
NPR-C	natriuretic peptide receptor subtype C
PCR	polymerase chain reaction
RAS	renin-angiotensin system
RT-PCR	reverse transcription-polymerase chain reaction
TIMP	tissue inhibitor of matrix metalloproteinase
TGF- β	transforming growth factor β
TNF- α	tumour necrosis factor α
VEGF	vascular endothelial growth factor
18S	ribosomal 18S
α -SMA	[alpha] smooth muscle actin
α	alpha
γ	gamma

List of original papers

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

- I Peltonen TO, Taskinen P, Soini Y, Rysä J, Ronkainen J, Ohtonen P, Satta J, Juvonen T, Ruskoaho H & Leskinen H (2007) Distinct down-regulation of C-type natriuretic peptide system in human aortic valve stenosis. *Circulation* 116: 1283–1289.
- II Peltonen TO, Taskinen P, Näpänkangas J, Leskinen H, Ohtonen P, Soini Y, Satta J, Juvonen T, Vuolteenaho O & Ruskoaho H (2008) Increase in tissue endothelin-1 and ET_A receptor levels in human aortic valve stenosis. *Eur J Heart* (in press).
- III Peltonen TO, Näpänkangas J, Vuolteenaho O, Ohtonen P, Soini Y, Juvonen T, Satta J, Ruskoaho H & Taskinen P (2008) Apelin and its receptor APJ in human aortic valve stenosis. Manuscript.

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1 Introduction

Calcific aortic stenosis (AS) is a progressive disease that has, until recently, been considered to be a degenerative and unmodifiable process induced by long-lasting mechanical stress (Otto *et al.* 1997). Calcific aortic valve disease is identified by thickening and calcification of the aortic valve leaflets in the absence of rheumatic heart disease and has been shown to be associated with an increased risk of cardiovascular death. According to population studies, bicuspid aortic valve (i.e. an aortic valve consisting of 2 valve cusps instead of three) predisposes to earlier development and more drastic manifestations of calcified aortic valve disease. Aortic stenosis is present in 2% to 5% of all very elderly patients, is the second most common indication for cardiac surgery, and carries an 80% 5 year risk of progression to heart failure and valve replacement, or, finally, death (Otto *et al.* 1997, Freeman & Otto 2005).

Histopathologic studies have now demonstrated that the development and progression of calcific AS is based on an active process, sharing a number of similarities with atherosclerosis. Inflammation, lipid infiltration, dystrophic calcification, ossification, platelet deposition and endothelial dysfunction have been observed in both diseases (Freeman & Otto 2005). In addition, several studies have suggested that AS and atherosclerosis share a number of risk factors, such as hypercholesterolemia, elevated lipoprotein (a), smoking, hypertension and diabetes (Helske *et al.* 2007a). These findings suggest that statin or angiotensin converting enzyme (ACE) inhibitor therapy, or both, could be beneficial in AS by their lipid-lowering and/or anti-inflammatory effects, as is the case in atherosclerosis (Freeman & Otto 2005, Otto 2007). Although this concept has been supported by experimental work, clinical trials have not been able to show benefits of lipid-lowering or ACE inhibitor use in AS (O'Brien *et al.* 2002, Rosenhek & Baumgartner 2008, Rossebo *et al.* 2008). Therefore, there is no effective pharmacologic treatment available for AS today and the only curative treatment today is still valve replacement surgery.

The aim of the present study was to analyze the role of endothelial factors (natriuretic peptide system, endothelin family, apelin pathway and renin-angiotensin system) in different stages of calcified aortic valve disease to reveal new possible targets for pharmacological interventions in AS.

2 Review of the literature

2.1 Aortic valve stenosis (AS)

2.1.1 Anatomy of normal aortic valve

A normal aortic valve consists of three leaflets located circumferentially attached to a crown-like structure, referred as the aortic annulus. The prevalence of bicuspid aortic valves in the general population is 0.5–2% (Schaefer *et al.* 2007). Monocuspid aortic valve, where the aortic valve is formed by single three layered membrane interrupted by commissure in one side of the aorta, is a rare curiosity, with prevalence of 1/10 when compared to prevalence of bicuspid aortic valves (Roberts & Ko 2005). Due to less effective stress-sharing properties, an aortic valve consisting of a less number of cusps predispose the person to earlier manifestation of AS; patients with bicuspid valves tend to present AS two decades younger than those with tricuspid valves (Beppu *et al.* 1993, Ward 2000, Friedman *et al.* 2008). The normal aortic valve is covered by endothelial cells and the valve histologically comprises of 3 layers. The ventricularis, on the ventricular side of the leaflet, is composed of elastin-rich fibers that are situated in a radial direction, perpendicular to the leaflet margin (Figure 1). The fibrosa, on the aortic side of the leaflet, comprises primarily fibroblasts and collagen fibers (providing strength and stiffness to the valve) aligned circumferentially, parallel to the leaflet margin. The spongiosa is a layer of loose connective tissue at the base of the leaflet, between the fibrosa and ventricularis, composed of fibroblasts, mesenchymal cells, and a mucopolysaccharide-rich matrix, providing flexibility and plasticity to leaflets (Figure 1). These layers work in concert to provide tensile strength and pliability for decades of repetitive motion depending on the systolic and diastolic phases of the heart.

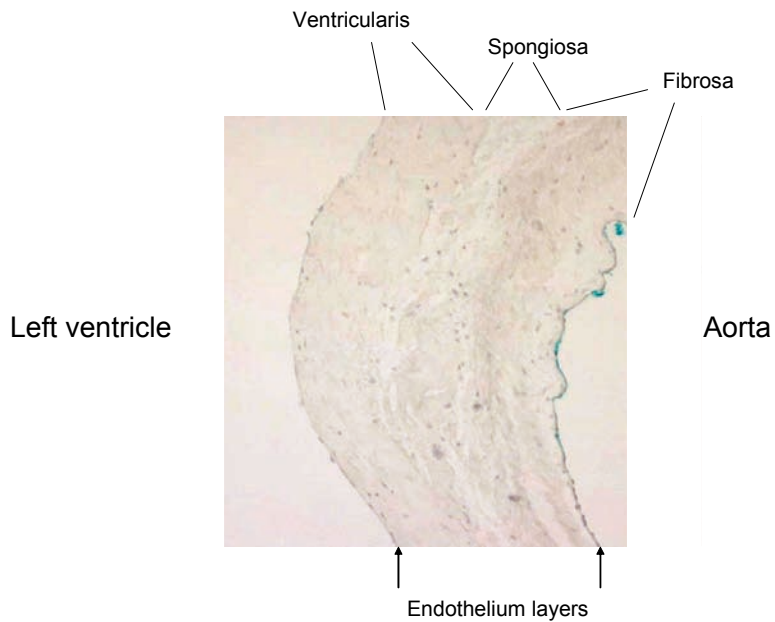


Fig. 1. Light microscopic cross section showing layers of a healthy human aortic valve. Blue ink shows the aortic surface of valve.

2.1.2 Definition of AS

In 2006, American College of Cardiology (ACC) together with American Heart Association (AHA), followed by the European Society of Cardiology (ESC) in 2007, published guidelines concerning the definition of AS and the categorization of the severity of the disease (American College of Cardiology/American Heart Association Task Force on Practice Guidelines 2006, Vahanian *et al.* 2007). The opening area of a normal aortic valve is approximately 3–4 cm² (aortic valve area, AVA) and hemodynamic complications do not usually occur until AVA is smaller than 1–1.2 cm². The degree of AS is graded as mild if AVA < 1½–2 cm² and the mean transaortic pressure gradient < 25 mmHg (aortic jet velocity 2½–3 m/s). In moderate AS, AVA is 1–1½ cm² (mean pressure gradient 25–40 mmHg, aortic jet velocity 3–4 m/s). Severe aortic stenosis is defined as AVA < 1 cm² (mean pressure gradient > 40 mmHg, aortic jet velocity > 4 m/s) (American College of Cardiology/American Heart Association Task Force on Practice Guidelines 2006) (Figure 2).

ESC guidelines, though, emphasizes potential inaccuracies of valve area measurements and points out that valve area measurements are less robust than gradient estimates in clinical practice. Thus, the aortic valve area alone with absolute cut-off points cannot be relied upon for clinical decision making and it should be considered in combination with flow rate, pressure gradient and ventricular function as well as functional status. AS with a valve area $< 1 \text{ cm}^2$ is considered severe; however, indexing to body surface area (BSA), with a cut-off value of $0.6 \text{ cm}^2 / \text{m}^2 \text{ BSA}$ is helpful, in particular in patients with either unusually small or large BSA. Severe AS is unlikely if cardiac output is normal, and there is a mean pressure gradient $< 50 \text{ mmHg}$. As soon as mean gradient is less than 40 mmHg , even a small valve area does not definitely confirm severe AS since mild-to-moderately diseased valves may not open fully, resulting in a “functionally small valve area” (pseudosevere AS) (deFilippi *et al.* 1995, Vahanian *et al.* 2007).

The development of symptoms identifies a critical point in the natural history of AS. Asymptomatic patients with AS have outcomes similar to age-matched normal adults. Patients with asymptomatic AS require frequent monitoring for development of symptoms and progressive disease.

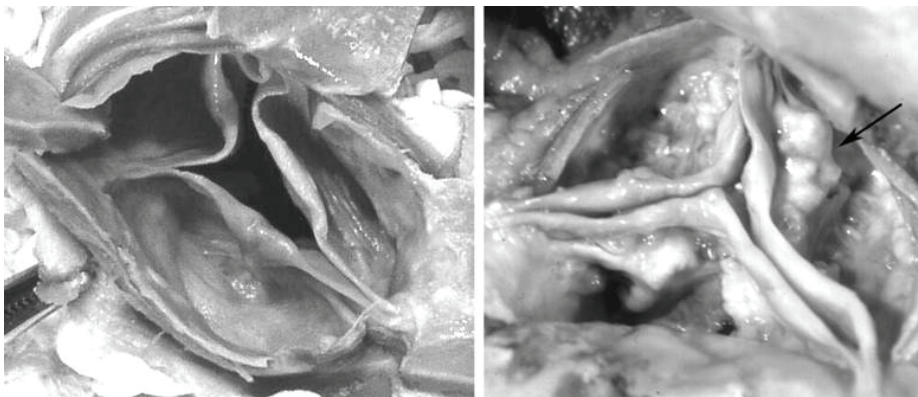


Fig. 2. Gross specimen of a minimally diseased aortic valve (left) and a severely stenotic aortic valve (right). In the severely stenotic valve, there are prominent lipocalcific changes on aortic side of valve cusps (arrow), with sparing of commissures. Freeman & Otto 2005. Reprint with permission from Lippincott, Williams & Wilkins and Professor Catherine M. Otto.

2.1.3 Diagnosis in AS

Careful exploration for the presence of symptoms (exertional shortness of breath, angina, dizziness or syncope) is critical for proper patient management. It has to be noted that the physician has to realize that patients may deny symptoms because they significantly reduce their activities. In addition, although uncommon, sudden death can occur without apparent symptoms (Frank *et al.* 1973, Pellikka *et al.* 1990)

The characteristic systolic murmur draws the attention and guides further diagnostic work into the right direction. However, occasionally the murmur may be faint and the primary presentation may be heart failure of unknown cause. The disappearance of the second aortic sound is specific to severe AS, although not a sensitive sign. The typical finding is a harsh systolic parasternal murmur, which increases in intensity toward the second right intercostals space.

The echocardiography has become the key diagnostic tool. A specificity of 96%, a sensitivity of 78%, and a predictive accuracy 93% have been reported (Ward 2000). Two different methods of echocardiography is in clinical use, transthoracic echocardiography (TTE) and transesophageal echocardiography (TEE), of which TEE has more predictive accuracy if patient is obese. First-class evaluation, in expert hands (Dal-Bianco *et al.* 2008), confirms the presence of AS revealing the aortic jet velocity, pressure gradients, left ventricular end-diastolic dimension (LVEDD), left ventricular ejection fraction (LVEF), grade of left ventricular hypertrophy (LVH) and aortic valve area (AVA). Furthermore, echocardiography assesses also the degree of valve calcification and finally provides prognostic information. Doppler echocardiography is the preferred technique in assessing the severity of the disease (Quinones *et al.* 2002). The clinical utility of measuring stenosis severity is two-fold: to reliably predict the optimal timing of valve replacement and to ensure that valve disease is the cause of the patient's symptoms.

There are biomarkers available to be used as secondary in diagnostics next to echocardiographic measurements. The role of C-reactive protein (CRP) is becoming more important (Imai *et al.* 2008), especially when combined with B-type natriuretic peptide (BNP) showing the systolic dysfunction of the heart (Moura *et al.* 2008). An interesting candidate among new biomarkers is an inactive form of matrix gla protein (ucMGP) as a biomarker for cardiovascular calcification (Cranenburg *et al.* 2008).

Computed tomography (CT) and magnetic resonance imaging (MRI) cojoin with echocardiography as diagnostic tools (John *et al.* 2003, Messika-Zeitoun *et al.* 2004). These tools present newer methods, with greater costs, feasible when diagnosing AS.

2.1.4 Genetic syndromes with increased prevalence of AS and calcific aortic valve disease

Human chromosomal regions 18q, 5q and 13q (between markers D18S68 and D18S1161, D5S644 and D5S2027, and D13S1265 and 13qter, respectively) have been associated with the inheritance of bicuspid aortic valves (Martin *et al.* 2007). The bicuspid valve is not the only variation which contains increased incidence of AS. Marfan syndrome and Ehlers-Danlos syndrome have higher prevalence of AR and higher prevalence of AS (Savunen 1987, American College of Cardiology/American Heart Association Task Force on Practice Guidelines 2006). Paget's syndrome (Strickberger *et al.* 1987), Ebstein anomaly (Waller *et al.* 1995b), Fabry's disease and Whipple's disease (Waller *et al.* 1995a) have individual genetic variations and disease specific characters, of which one clinical manifestation is higher prevalence of AS, as well as a small number of candidate genes, such as vitamin D receptor (VDR), apolipoprotein E (apoE4), apolipoprotein B (apoB), interleukin 10 (IL-10) and estrogen receptor α (ESR1) (Bosse *et al.* 2008). Interestingly, the knowledge of pathophysiology of AS in syndromes mentioned above is very weak. Some other genetic factors may contribute to the development of AS as well: early development of aortic valve calcification may be related to vitamin D receptor genotypes and mutations in the gene for the NOTCH1 transcriptional factor have been described in individuals with familial bicuspid aortic valves (Ortlepp *et al.* 2001, Garg *et al.* 2005). Stenotic bicuspid aortic valves show the same degree of T lymphocyte infiltration as tricuspid aortic valves irrespective of the primary valve anomaly (Wallby *et al.* 2002) and inflammation is considered to be involved in the pathogenesis of, thus, acquired aortic stenosis (See chapter 2.1.7 below).

2.1.5 Epidemiological risk factors in AS or calcific aortic valve disease

The prevalence of AS increases with age, being present in 2% to 4% of adults over the age of 65 years (Stewart *et al.* 1997, Otto *et al.* 1999). Epidemiological

risk factors for aortic stenosis are age hyperlipidemia, hypercholesterolemia (Deutscher *et al.* 1984), male sex, hypertension, smoking, diabetes mellitus (Deutscher *et al.* 1984), increased ionized calcium in serum (Lindroos *et al.* 1994), chronic dialysis (Baglin *et al.* 1997) and end-stage renal disease (Maher *et al.* 1987). Aortic valve calcification has also been associated with ageing (Wilson *et al.* 1998) and metabolic syndrome (Katz *et al.* 2006).

2.1.6 Prognosis of patient suffering from AS

The clinical history is one of the most useful and important predictor of death from aortic stenosis. Aortic stenosis generally has a long clinically latent period. Asymptomatic patients with AS have outcomes similar to age-matched normal adults and that is why patients with asymptomatic AS require frequent monitoring for development of symptoms and progressive disease (American College of Cardiology/American Heart Association Task Force on Practice Guidelines 2006). However, when symptoms, in particular angina, syncope, or heart failure develop, average survival dramatically worsens to 2–3 years (Turina *et al.* 1987, Horstkotte & Loogen 1988, Iivanainen *et al.* 1996). It can be noted the prognosis is as poor as 1.5 to 2 years after the onset of congestive heart failure (CHF) in patients with severe AS who do not undergo surgery (Aronow 2007). Therefore, the development of these symptoms represents an important clinical decision point mandating surgical treatment of aortic stenosis.

2.1.7 Pathophysiology of AS

Stress initiating AS

The early aortic lesions are likely initiated by endothelial disruption due to increased mechanical or decreased shear stress (Freeman & Otto 2005). Mechanical stress of the aortic valve is highest on the aortic side of the leaflet in the flexion area, near the attachment to the aortic root. Shear stress across the endothelium of the noncoronary cusp is lower than the left and right coronary cusps because of the absence of diastolic coronary flow, which likely explains why the noncoronary cusp is often the first cusp affected. Further supporting the effects of leaflet stress as an initiating event, is the discrepancy in average age at the time of presentation when tricuspid and bicuspid valves are compared, despite

the identical histological appearance of lesions. Patients with bicuspid valves, which are subjected to higher mechanical stress, tend to present two decades younger than those with tricuspid valves (Pachulski & Chan 1993, Freeman & Otto 2005). Nearly all patients with bicuspid valves develop significant outflow obstruction over time, whereas only a relatively small proportion of patients with a trileaflet valve progress to severe aortic stenosis.

Inflammation in AS

Stenotic aortic valves show accumulation of macrophages and T lymphocytes (Olsson *et al.* 1994, Otto *et al.* 1994), when those are in practice absent in normal aortic valves. In stenotic valves, macrophages are present in form of foam cells as well. Biologically active T lymphocytes are mainly positioned in the very same surrounding area of calcific deposits and stenotic lesions, where accumulation of oxidized lipids can be seen (Olsson *et al.* 1994, Olsson *et al.* 1999). Mast cells have been identified in aortic valves with a possible role in valve pathology and injury (Mohler *et al.* 2001, Helske *et al.* 2004, Helske *et al.* 2006a, Veinot *et al.* 2006), in inflammatory heart diseases (Fairweather & Frisano-Kiss 2008) as well as in other atherosclerotic plaques containing pathologies (Kovanen 1995), with influence to progression (Lindstedt & Kovanen 2004, Kovanen 2007a, Lindstedt *et al.* 2007).

Mast cells are nearly linked to inflammation. They circulate in blood and lymphatic system as progenitor cells and differentiate, after migration, into mast cells in tissues under stimulus of specific cytokines. Mast cells are strategically located at sites that interface with our external environment, closely associated with blood vessels and nerves. They become also activated in different inflammatory states. That activation can be resulted from adjacent T lymphocytes or macrophages, different cytokines and chemokines as well as component of the complement system (Marshall 2004, Nakae *et al.* 2006). Mast cells are able to induce the formation of macrophage foam cells (Kovanen 2007b).

Inflammatory cells mentioned above, are able to contribute to the pathological changes in valve tissue in AS. Activated T lymphocytes within the subendothelial region release cytokines, such as tumour necrosis factor- α (TNF- α), which, in turn, promote the calcification of aortic valves (Kaden *et al.* 2005). Transforming growth factor- β 1 (TGF- β 1) and interleukin-1 β (IL-1 β) are released as well. IL-1 β increases local production of matrix metalloproteinases (MMPs), which contribute to extracellular matrix remodeling (Bosse *et al.* 2008).

Macrophages also express osteopontin, a bone-associated protein (Bosse *et al.* 2008). Additionally, there are changes in the expression profile of inflammation modulators, i.e. Toll-like receptors 2 and 4 (TLR2 and TLR4), expressed in stenotic valves but not in controls (Meng *et al.* 2008).

In addition, there is experimental animal data concerning AS. The problem is the fact that some species, such as swine, can develop spontaneous vascular and valvular atherosclerotic lesions, while others, such as rabbits and mice, have not been shown to develop lesions naturally and require an inciting factor, such as hypercholesterolemia (Guerraty & Mohler III 2007). Liberman *et al.* made an interesting study on reactive oxygen species (ROS) and the effect of antioxidants tempol and lipoic acid in rabbits with AS (induced by 0.5% cholesterol diet +10(4) IU/d Vitamin D2 for 12 weeks) (Liberman *et al.* 2008). Their data provide evidence that ROS, particularly hydrogen peroxide H₂O₂, promote progression of aortic valve calcification. van Wanrooij *et al.* constructed a DNA vaccine against CD99, capable of blocking CD99 cells by specific antibodies. CD99 is a leukocyte membrane protein that was initially described to function in T cell activation and lymphocyte aggregation. Results showed that vaccination decreases atherogenesis in aortic valve leaflets by the selective removal of CD99-expressing cells, which could reduce leukocyte recruitment into atherosclerotic lesions and attenuate atherogenesis (van Wanrooij *et al.* 2008).

Associations between respiratory infections and serious heart disease events have been demonstrated (Clayton *et al.* 2008). *Chlamydia pneumoniae* is associated with AS (Juvonen *et al.* 1997, Juvonen *et al.* 1998) as well as *Mycoplasma pneumoniae* (Higuchi-Dos-Santos *et al.* 2005). Proatherogenic pathogens *Chlamydiae pneumoniae* and *Aggregatibacter actinomycetemcomitans* have been shown to induce secretion of inflammatory molecules (IL-8, TNF- α and MCP-1) in cultured human peripheral blood-derived mast cells (Oksaharju *et al.* 2008). In addition, *Rickettsia helvetica* and *Bartonella spp.* has documented by RT-PCR to exist in diseased aortic valves (Nilsson *et al.* 2005). Nevertheless, CHD mortality rate does not decrease with pharmacological antimicrobe treatment (WIZARD–study, AZACS-study. Neumann 2002).

High sensitive C-reactive protein (Hs-CRP) have been reported to have a possible role in identifying patients in the early stages of calcific aortic valves (Jeevanantham *et al.* 2007) as well as in asymptomatic patients where CRP predicts severity, progression, and prognosis of the disease (Imai *et al.* 2008). CRP levels are reported to be higher in patients with degenerative AS who show rapid valve disease progression (Sanchez *et al.* 2006). On the other hand, very

weak (Agmon *et al.* 2004) or no correlation at all between CRP and AS / atherosclerotic diseases has been founded (Hunt *et al.* 2001, Gunduz *et al.* 2003, Reilly *et al.* 2003, Khera *et al.* 2006). The diagnostic role of CRP was decimated when the results of the Cardiovascular Health Study (n=5621) were published: C-reactive protein can be considered as poor indicator of AS when CRP was not associated with baseline AS, progression to aortic sclerosis or progression to AS (Novaro *et al.* 2007). The results concerning role of CRP in AS are controversial and for instance the guidelines of AHA / ACC or ESC do not mention CRP to have a diagnostic role in AS (American College of Cardiology/American Heart Association Task Force on Practice Guidelines 2006, Vahanian *et al.* 2007).

Lipid deposition, calcification, ossification and remodeling

Histopathologic studies show that lipid accumulation is present already in early aortic valve lesions. Focal subendothelial plaque like lesions are localized on the aortic side of the leaflet, deep enough to reach the adjacent fibrosa layer (Freeman & Otto 2005). Apolipoproteins B, (a), and E colocalize with extracellular valve lipids suggesting that lipids are from circulating plasma or locally produced atherogenic lipoproteins (O'Brien *et al.* 1996). Helske and colleagues have reported that valve lesions contain cholesterol precursors and plant sterols (Helske *et al.* 2008a). Similarities to atherosclerosis are present in form of LDL and evidence of LDL oxidation exists in these valvular plaques (Freeman & Otto 2005). T lymphocytes and macrophages colocalize with deposits of neutral and oxidized lipids, suggesting the close cooperation between tissue inflammation and lipid deposition in valve lesions (Olsson *et al.* 1999).

Calcification is the main phenomenon in the macroscopic inspection of a stenotic aortic valve. Calcification is an active process and this active calcification is prominent early in the disease process. The degree of valvular calcification is clinically important, as extensive calcification in a valve predicts rapid disease progression and poor outcome (Rosenhek *et al.* 2004).

In aortic sclerosis, microscopic areas of calcification colocalize in areas of lipoprotein accumulation and inflammatory cell infiltration. Oxidized LDL stimulates valvular fibroblasts to release matrix vesicles, a nidus for early calcification. In the end stage disease, calcification and ossification are the reason for the stiffness of the valve (Freeman & Otto 2005).

In the development of AS, a closely related phenomenon existing next to calcification is ossification (Pohjolainen *et al.* 2008). Interestingly, calcification

and cellularity in human aortic heart valve tissue determine the differentiation of bone-marrow-derived cells (Leskela *et al.* 2006) It has been shown that macrophages express osteopontin (Bosse *et al.* 2008), a protein needed in bone formation, with the degree of mRNA expression of osteopontin corresponding to the degree and location of valvular calcification (Mohler *et al.* 1997). Additional genes to osteopontin (O'Brien *et al.* 1995) reported to be expressed in calcified aortic valves are osteocalcin (Osman *et al.* 2007) osteonectin (OSN) (Charest *et al.* 2006), osteoprotegerin (Pohjolainen *et al.* 2008), tenascin-C (Satta *et al.* 2002), bone morphogenic protein (BMP), BMP II (Kaden *et al.* 2004), bone sialoprotein (BSP) (Kaden *et al.* 2004) and BSP II (Pohjolainen *et al.* 2008).

One set of these proteins is known to act as inhibitors of calcification (e.g., BSP and osteocalcin), whereas another set of proteins is known to act as activators of calcification (e.g., BMPs); and it has hence been hypothesized that the pathologic mineralization of cardiovascular matrixes is at least partly regulated by the balance between these procalcific and anticalcific regulatory proteins (Pohjolainen *et al.* 2008).

Of note, there is coexistence of extracellular matrix remodelling (ECM) and fibrosis / osteogenesis in stenotic aortic valves. It is not only increased collagen turnover and osteogenesis resulting loss of leaflet elasticity but disorganization of collagen and elastin fibers as well as degradation of them. Matrix metalloproteinases (MMPs) can be seen resulting this, together with suppression of tissue inhibitors of matrix metalloproteinases (TIMPs). Factors supporting ECM are elastolytic cathepsins G, S, K and V, tenascin-C (by increasing the activity of MMP-2), filamin-A (by maintaining cytoskeletal architecture) and fibulin-4 (by maintaining ECM integrity) (Helske *et al.* 2006b, Hakuno *et al.* 2008).

Angiogenesis in AS

One significant phenomenon in diseased stenotic aortic valve, correlating with the intensity of inflammation (Mazzone *et al.* 2004), is angiogenesis and neovascularisation, which does not exist in a normal aortic valve. This neovascularization increases the progression of stenosis in valves, when inflammatory cells as well as lipids accumulating to leaflets are able to effortlessly reach target tissue. Angiogenetic factors such as vascular endothelial growth factor (VEGF) (Soini *et al.* 2003, Salo *et al.* 2006), and Secreted Protein, Acidic and Rich in Cysteine/osteonectin (SPARC) (Charest *et al.* 2006a) are upregulated

in stenotic valves. Interestingly, cellular immunity causing endothelial growth factor receptor 2 (VEGFR2) -vaccine has been reported to lead to inhibition of angiogenesis in atherosclerosis-associated cells (Hauer *et al.* 2007).

2.2 Treatment of AS

2.2.1 Surgical management of AS patient

At present there is no approved pharmacologic treatment for AS and the unavoidable operation as curative therapy of the disease is valve replacement surgery (Figure 3). Symptomatic AS is an unequivocal indication for valve replacement surgery (Vahanian *et al.* 2007).

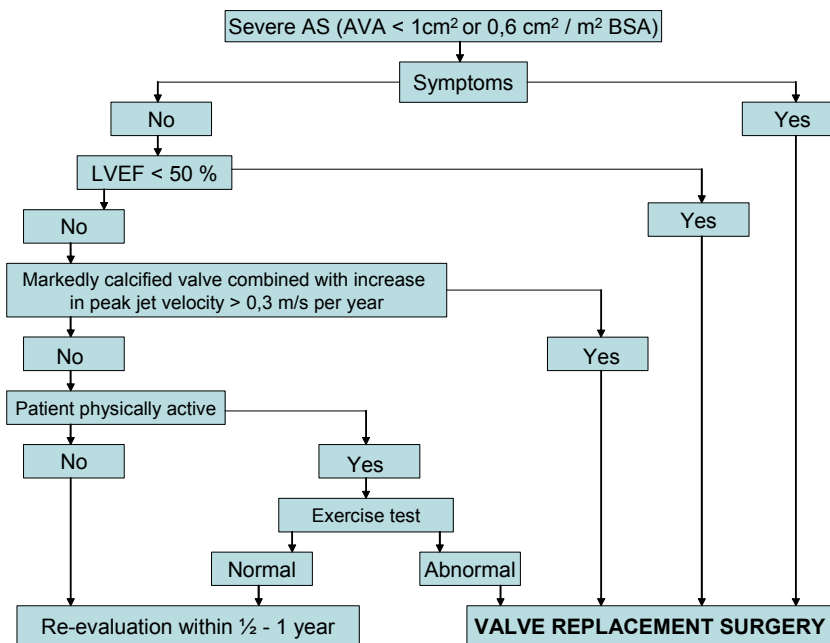


Fig. 3. Indications for aortic valve replacement in the treatment of severe aortic stenosis. AS indicates aortic stenosis. LVEF: left ventricular ejection fraction. BSA: body surface area. Modified from Vahanian *et al.* 2007 (Guidelines on the management of valvular heart disease: The Task Force on the Management of Valvular Heart Disease of the European Society of Cardiology 2007).

In contemporary series, mortality of isolated aortic valve replacement is ~3–5% in patients younger than 70 years and 5–15% in older adults (Nguyen 2000, STS National Cardiac Surgery Database; comprising of U.S. and Canada 2000, The UK Cardiac Surgical Register 2000, Iung *et al.* 2003). Comorbidities and high age increase both operative mortality and morbidity (Ambler *et al.* 2005). In symptomatic patients who undergo aortic valve replacement, age-adjusted postoperative survival is nearly normalized (Lindblom *et al.* 1990). Of note, advanced age is not considered a contraindication to surgery (American College of Cardiology/American Heart Association Task Force on Practice Guidelines 2006). Understanding operative risk is important in patient care and the selection of patients for aortic valve replacement (Table 1).

Table 1. Operative mortality after surgery for valvular heart disease.

Variable	STS (2001)	UKCSR (1999–2000)	EHS (2001)
Aortic valve replacement, no CABG (%)	3.7	3.1	2.7
Aortic valve replacement, with CABG (%)	6.3	7	4.3

STS = Society of Thoracic Surgeons (USA). (<http://www.sts.org/doc/3031>). UKCSR= United Kindom Cardiac Surgical Register. (<http://www.scts.org/file/NACSDreport2000ukcsr.pdf>). EHS= Euro Heart Survey (Iung *et al.* 2003). CABG= coronary artery bypass grafting. Modified from Vahanian *et al.* 2007

2.2.2 Pharmacological treatment available in AS

Histopathologic studies have now demonstrated that the development and progression of calcific AS is based on an active process, sharing a number of similarities with atherosclerosis. In addition, several studies have suggested that AS and atherosclerosis share a number of risk factors, such as hypercholesterolemia, elevated lipoprotein (a), smoking, hypertension and diabetes. These findings suggest that ACE-inhibitors or statins could be beneficial in AS, as is the case in atherosclerosis (Freeman & Otto 2005, Otto 2007). Although this concept has been supported by experimental work and by retrospective clinical studies, observing significantly slower rates of hemodynamic progression in statin-treated patients (Antonini-Canterin *et al.* 2008), three prospective randomized and well performed trials (SALTIRE, SEAS and TASS) yielded a negative result and RAAVE-study showed negative result among normocholesterolemic patients as well (Cowell *et al.* 2005, Dichtl *et al.*

2008, Rossebo *et al.* 2008, Rosenhek & Baumgartner 2008). SALTIRE trial (Scottish Aortic Stenosis and Lipid Lowering Trial, Impact on Regression) was double-blind, placebo-controlled trial, among patients (n=155) with calcific aortic stenosis, randomly assigned to receive either 80 mg of atorvastatin daily or a matched placebo (Cowell *et al.* 2005). SEAS –study (Simvastatin and Ezetimibe in Aortic Stenosis) was a double-blind multi center study with 1800 AS patients receiving 40 mg simvastatin combined with 10 mg ezetimibe or a matched placebo (Rossebo *et al.* 2008). RAAVE study (Rosuvastatin Affecting Aortic Valve Endothelium) was open-labelled prospective study evaluating 121 consecutive patients with asymptomatic moderate to severe AS. Rosuvastatin 80mg showed a significantly slower rate of progression of AS in hypercholesterolemic patients compared with patients with normal cholesterol levels but had no effect on progress of disease among patients with normal cholesterol levels (Rosenhek & Baumgartner 2008). TASS study (Tyrolean Aortic Stenosis Study) was a prospective placebo controlled study evaluating 47 patients with asymptomatic calcificated AS with atorvastatin treatment (20 mg/day vs placebo), showing that atorvastatin 20mg daily does not halt AS (Dichtl *et al.* 2008).

According to guidelines, the only recommended pharmacological treatment among AS patients is medication lowering blood pressure (American College of Cardiology/American Heart Association Task Force on Practice Guidelines 2006).

2.3 Autocrine / paracrine factors

2.3.1 Natriuretic peptide system

Natriuretic peptides

At present, the mammalian natriuretic peptide system including Atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) has been described (Figure 4). Natriuretic peptides are produced as proforms and they are converted into mature peptides by proteolytic processing of corin (ANP, BNP) and furin (CNP) (Figure 4). The three peptides share a common 17 amino acid ring structure in which most of the amino acid residues are conserved (Potter *et al.* 2006). As implied by the initial studies, the main biological function of ANP and BNP is the regulation of sodium and fluid

The atrial natriuretic peptide, ANP, was the first member of the family to be characterized. The major site of synthesis in normal hearts is the atrium, and secretion is rapidly stimulated by stretch (for review, see Ruskoaho 1992). ANP protein has been found 100 times higher concentrations in the atrial tissue than in ventricle, so that ANP mRNA can constitute up to 3% of all atrial mRNA. The induction of left ventricular ANP gene expression is seen in most of the clinical disorders as well as experimental models with pressure or volume overload, and the increase occurs within the first day of experimental overload (Gardner *et al.* 1986, Ruskoaho 1992).

BNP, originally termed brain natriuretic peptide, was discovered in 1988 in porcine brain (Sudoh *et al.* 1988), but it was soon discovered that the highest concentration of the peptide is found in cardiac ventricular myocytes. Its ventricular expression is dramatically increased during cardiac overload and hypertrophy, leading to plasma levels exceeding those of ANP in late phase heart failure (Gardner 2003). The hemodynamic effects of BNP are largely similar to ANP. BNP gene expression in atria and ventricles is induced within 1 hour in response to overload (Mantymaa *et al.* 1993, Magga *et al.* 1994). With chronic overload, BNP mRNA levels have been suggested to remain constantly increased (de Bold *et al.* 1996).

Two years after the discovery of BNP, the third member of the group, CNP, was found, again in the porcine brain (Sudoh *et al.* 1990). CNP was thought to act mainly as a local regulator in brain, the major site of synthesis, and in vessels, since it causes vigorous vasorelaxation of vascular smooth muscle, but only mild diuresis and natriuresis, and its plasma concentrations are very low (Sudoh *et al.* 1990, Ruskoaho 1992). In the human genome, CNP is located between 2q24 and the 2q terminus. CNP is found in the central nervous system, the endothelial cells and in low concentrations in the blood. CNP, produced mostly by the endothelial cells, is currently considered as an endothelium-derived hyperpolarizing factor with the local effect of dilation of vessels. CNP is produced from the precursor proCNP as the result of cleavage by endoprotease furin to the mature 53 amino acid peptide (Wu *et al.* 2003). In some tissues CNP precursor will be cleaved to CNP-22 by an unknown extracellular enzyme (Potter *et al.* 2006).

ANP and BNP has been reported to be increased in AS patients and, according to PEACE trial, BNP has prognostic value in stable coronary artery disease (Ikeda *et al.* 1997, Omland *et al.* 2007, Pedrazzini *et al.* 2008).

Natriuretic peptide receptors

Three single membrane-spanning natriuretic peptide receptors (NPRs) have been identified: NPR-A, NPR-B and NPR-C. The rank order of NPR-A activation by natriuretic peptides is ANP \geq BNP \gg CNP and the rank order of activation of NPR-B by natriuretic peptides is CNP \gg ANP \geq BNP (see reviews Potter 2006 and Rose 2008).

Two, NPR-A and NPR-B, are transmembrane guanylyl cyclases, enzymes that catalyze the synthesis of cGMP (Potter 2006, Rose 2008). One, NPR-C, lacks intrinsic enzymatic activity in terms of lacking the guanylyl cyclase domain and controls the local concentrations of natriuretic peptides through constitutive receptor-mediated internalization and degradation. The biological effects of NPs have been mainly attributed to changes in intracellular cGMP following their binding to NPR-A and NPR-B. NPR-C does not include a guanylyl cyclase domain and NPR-C has been denoted as a clearance receptor and is thought to bind and internalize NPs for ultimate degradation. However, a substantial body of biochemical work has demonstrated the ability of NPR-C to couple to inhibitory G proteins (Gi) and cause inhibition of adenylyl cyclase (Rose & Giles 2008).

NPR-A gene null rodents have high blood pressure, cardiac hypertrophy, and ventricular fibrosis (Lopez *et al.* 1995). In humans, a single allele mutation was identified in the promoter of the NPR-A gene that decreases receptor expression by about 70% (Nakayama *et al.* 2000). Interestingly, of the eight Japanese patients identified with this mutation, seven had hypertension and one had congestive heart failure. Hence, every time a loss of function mutation was identified in the NPR-A gene, it was associated with disease. In contrast, a separate study involving 498 New Zealand patients failed to observe this mutation, suggesting that it may be rare outside of Japan (Palmer *et al.* 2004).

Loss of function of NPR-B results in dwarfism and female sterility in rodents (Tamura *et al.* 2004). Homozygous loss of function mutations in human NPR-B have been identified in patients with a rare form of short-limbed dwarfism called acromesomelic dysplasia, type Maroteaux (Bartels *et al.* 2004)

Factors increasing synthesis and secretion of CNP

Opposite to ANP and BNP, CNP is not stored in granules. The secretion is increased by growth factors and cytokines, mainly by TNF- α (Suga *et al.* 1993), IL-1 (Suga *et al.* 1993), basic fibroblast growth factor, and transforming growth

factor β (Passino *et al.* 2008) as well as shear stress (Chun *et al.* 1997). Secretion is suppressed by insulin (Igaki *et al.* 1996). The production of CNP is also regulated by interaction between endothelial cells and macrophages (Suga *et al.* 1998).

Clinical and functional improvement after physical training in heart failure patients is associated with a decrease in adrenergic activation and in both CNP and BNP plasma concentration. Changes in CNP plasma concentration after physical training might reflect an improvement in endothelial function (Passino *et al.* 2008). Increased CNP secretion is observed as the result of tissue damage or hypoxia in different rat models (Klinger *et al.* 1998).

Degradation and elimination of CNP

CNP is degraded through two processes: NPR-C mediated internalization followed by lysosomal degradation and enzymatic degradation by neutral endopeptidase (NEP) (Marton *et al.* 2005). The human NPR-C -gene is located on chromosome 5p14-p13 (Rahmutula *et al.* 2002). The extracellular domain of NPR-C is about 30% identical to NPR-A and NPR-B (van den Akker 2001). However, unlike the cyclase-linked receptors, it contains only 37 intracellular amino acids and no guanylyl cyclase activity (Fuller *et al.* 1988). The affinity of NPR-C for natriuretic peptides is ANP \geq CNP>BNP in humans (Suga *et al.* 1992). The differential affinity of NPR-C for the cardiac family members may contribute to the longer serum half-life of BNP compared to ANP.

Neural endopeptidase (NEP; EC. 3.4.24.11) is a type 2 cell surface metalloprotease. NEP is a zinc-dependent enzyme expressed on the plasma membrane. It has broad substrate specificity and tissue distribution. NPR-C and NEP employ different degradation mechanisms (Fan *et al.* 2005). Targeted deletion of neutral endopeptidase (Lu *et al.* 1995) does not lead to skeletal overgrowth like targeted deletion on NPR-C (Matsukawa *et al.* 1999). NEP activity has been reported to be elevated in failing human hearts (Fielitz *et al.* 2002) and in stenotic aortic valves (Fielitz *et al.* 2002, Helske *et al.* 2007b).

Nevertheless, characterization of gene-expression natriuretic peptide system in human aortic valves has not been reported. Especially, gene-expression of endothelial derived factor CNP in aortic valves is not known.

2.3.2 Endothelin family

Endothelins

Since the discovery of an endothelium-derived constricting factor in 1985 and in 1988, four structurally different endothelin (ET) isoforms have been described (i.e. ET-1, ET-2, ET-3, ET-4) (Hickey *et al.* 1985, Yanagisawa *et al.* 1988). ET-1 shares structural homology with ET-2, ET-3 and ET-4 and snake venom sarafotoxin (Luscher & Barton 2000). In addition, 31-residue ETs have been identified (Kishi *et al.* 1998). Amongst the four ET isopeptides, the 21-amino acid peptide ET-1 is regarded as the most prominent isoform in the cardiovascular system (Kedzierski & Yanagisawa 2001).

Mature ET-1 is formed from pre-pro-ET-1 via a 39-amino acid intermediate, big ET-1 (Kedzierski *et al.* 2003). Big ET-1 is processed to ET-1 by a family of endothelin converting enzymes (ECEs) and other enzymes such as chymases, and endopeptidases (Barton *et al.* 2003, Kedzierski *et al.* 2003). Under physiological conditions, ET-1 is produced in small amounts mainly in endothelial cells, primarily acting as an autocrine/paracrine mediator. Low shear stress increases ET-1 mRNA, while high shear stress decreases it (Yoshizumi *et al.* 1989).

Under pathophysiological conditions the production of ET-1 is stimulated in a large number of different cell types, including endothelial cells, vascular smooth muscle cells, cardiac myocytes, and inflammatory cells such as macrophages and leukocytes as well as human neutrophils (Ehrenreich *et al.* 1990, Sessa *et al.* 1991, Ito *et al.* 1993, Cambiaggi *et al.* 2001).

Endothelin receptors

The biological effects of ET-1 are mediated by two G-protein coupled receptor (GPCR) subtypes, ET_A and ET_B receptors (Rubanyi & Polokoff 1994). In the vasculature, the ET_A receptor is mainly located on vascular smooth muscle cells. The vasoconstrictory effects of ET-1 are primarily mediated by ET_A (Luscher & Barton 2000) (Figure 5). ET-1 may also induce indirect vasoconstrictor effects due to the generation of endothelium-derived thromboxane A₂ (Taddei & Vanhoutte 1993). ET_A receptors bind preferentially ET-1 while ET_B receptors are non-isopeptide-selective (Sakurai *et al.* 1990, Hosoda *et al.* 1991). The rank order of potencies for the endothelins as ET-1 = ET-2 >> ET-3 for the ET_A receptor and ET-1 = ET-2 = ET-3 for the ET_B receptor (Davenport 2002). The ET_B receptor is

primarily located on endothelial cells, but may also be present on vascular smooth muscle cells. Stimulation of the endothelial ET_B receptor results in release of nitric oxide (NO) and prostacyclin which cause vasodilatation, whereas stimulation of the vascular smooth muscle cell ET_B receptor results in vasoconstriction (de Nucci *et al.* 1988). Under physiological conditions, the net effect is vasoconstriction mediated by the ET_A receptor, which is partly counteracted by ET_B receptor-mediated release of NO. In heart and vasculature NO is produced by three NOS isoforms (endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS)) which share over 50% homology (Kelly *et al.* 1996, Kone 2000, Kellogg *et al.* 2008). The net effect of ET-1 can be considered to be determined by receptor localisation and the balance between ET_A and ET_B receptors (Bohm & Pernow 2007).

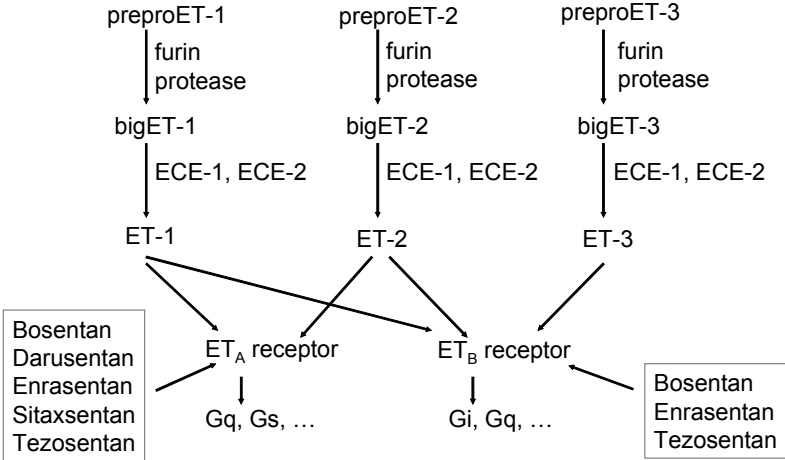


Fig. 5. Endothelins, endothelin receptors and endothelin receptor antagonists.

Pro-inflammatory and atherosclerotic effects

Apart from its direct vasomotor activity, ET-1 has been implicated in inflammatory processes within the vascular wall. Specifically, ET-1 in

subnanomolar concentrations has been demonstrated to activate macrophages, resulting in the release of pro-inflammatory and chemotactic mediators, including TNF- α , IL-1, IL-6 and IL-8 (Ruetten & Thiemermann 1997, Browatzki *et al.* 2000) which are of importance in the atherosclerotic process (Libby 2002). Cardiac overexpression of ET-1 in mice is associated with an inflammatory response involving increased activation of the pro-inflammatory transcription factor NF- κ B and expression of several pro-inflammatory cytokines including TNF- α , IL-1 and IL-6 (Yang *et al.* 2004). Interestingly, significant prolongation of survival was observed only with a dual ET_A/ET_B antagonist, but not with a selective ET_A antagonist (Yang *et al.* 2004). In turn, transcription factors and pro-inflammatory cytokines such as nuclear factor kappa-B (NF- κ B), TNF- α , and IL-6 stimulate ET-1 production (Viridis & Schiffrin 2003). ET-1 enhances the expression of adhesion molecules on TNF- α stimulated vascular endothelial cells and stimulates aggregation of polymorphonuclear neutrophils (Gomez-Garre *et al.* 1992, Ishizuka *et al.* 1999). Conversely, ET receptor blockade attenuates the accumulation of neutrophils and myeloperoxidase activity in the ischemic myocardium (Gonon *et al.* 2001). In circulation, increased plasma big-ET-1 levels have been reported in patients with AS, but big-ET-1 levels do not correlate with valve area or trans-valvular pressure gradient (Bergler-Klein *et al.* 2006). Characterization of endothelin family or nitric oxide synthases in stenotic aortic valves has not been reported.

2.3.3 The renin-angiotensin system (RAS)

Since the first identification of renin by physiologist Robert Tigerstedt in Stockholm in 1898, the renin-angiotensin system (RAS) has been extensively studied. The circulating renin-angiotensin system includes renin, which cleaves angiotensinogen to inactive angiotensin I, which in turn is further converted to the angiotensin II by the angiotensin converting enzyme (ACE) (Kim & Iwao 2000, Unger 2001). ACE2 produces Ang [1-7], effect of which is mediated via mas receptor, known as AT₄ as well (Fyhrquist & Saijonmaa 2008). Angiotensin II is considered to be the main effector peptide of the RAS (Fig. 6).

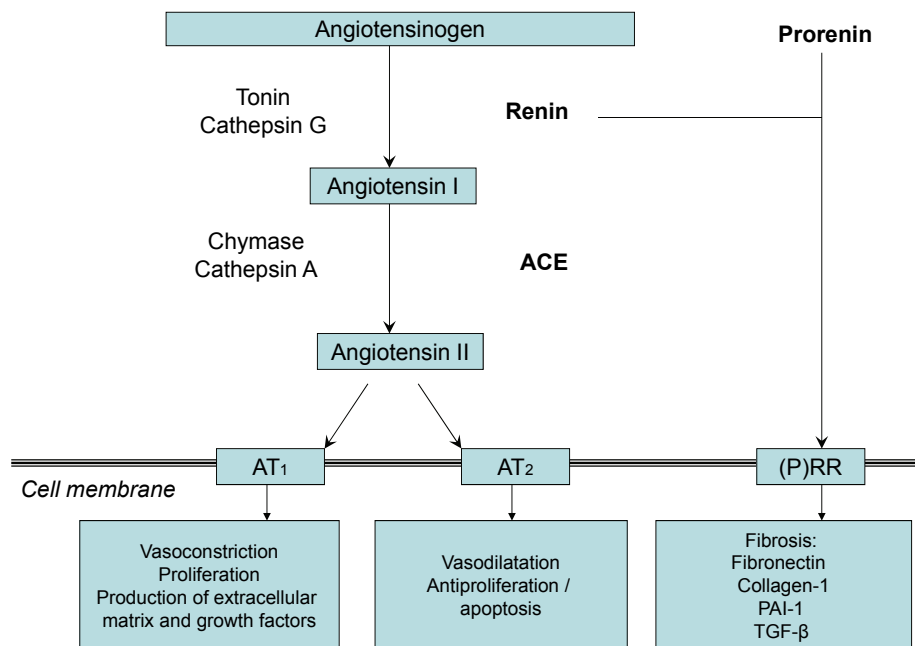


Fig. 6. Renin-angiotensin system. Angiotensinogen is secreted from the liver and renin (of kidney origin) modifies angiotensin I from angiotensinogen. Angiotensin I will in turn be modified angiotensin II by angiotensin converting enzyme (ACE). Angiotensin II receptors (AT₁ and AT₂) mediate the effect of angiotensin II. Prorenin is cleaved in kidneys to renin and both are ligands of (pro)renin receptor, known as (P)RR. Modified from Ruiz-Ortega M *et al.* 2007. PAI-1: plasminogen activator inhibitor-1. TGF- β : transforming growth factor β .

Both the low renal perfusion pressure and the increased beta-adrenergic stimulation, as well as low sodium concentration in plasma, activate the renin-angiotensin system, being the central regulator of blood pressure, vascular contractility and fluid balance (Schrier & Abraham 1999).

Although the renin-angiotensin system is considered to be endocrine in nature, evidence is accumulating that the heart and several other tissues contain and/or synthesize its components in an autocrine or paracrine manner, and the functional effects of renin and angiotensin in remodelling and heart failure have been at least partially attributed to local modulation of tissue function (Lindpaintner & Ganten 1991). This local autocrine / paracrine RAS can act independently of circulating RAS or maintain crosstalk with endocrine RAS (Paul *et al.* 2006).

Renin, prorenin and (pro)renin receptor

Renin is an aspartyl protease synthesized as prorenin, a proenzyme that contains an additional 43-amino acid N-terminal fragment. The physiological maturation of prorenin into active renin takes place exclusively in the juxtaglomerular cells of the kidney (Sealey *et al.* 1977). Renin has a high substrate specificity, and its only known substrate is angiotensinogen. Renin cleaves the N terminus of circulating angiotensinogen to angiotensin I (Nguyen *et al.* 2002).

The existence and relevance of cardiac renin and prorenin expression has been a matter of controversial debate, since renin and prorenin mRNA expression in heart is low (Paul *et al.* 1993). Less controversial is the evidence for the presence of renin protein in the heart attributed to uptake from the circulation (Danser *et al.* 1997) either due to nonspecific uptake (diffusion) into the cellular interstitium (de Lannoy *et al.* 1997, Jan Danser & Saris 2002) or through the actions of specific functional binding sites or receptor for prorenin and renin (Nguyen *et al.* 2002, Nguyen *et al.* 2004).

The binding of prorenin and renin to the cell surface in tissues is of essential importance in terms of physiology of local RAS, since it provides a mechanism to generate angiotensin II locally, amounts equal with angiotensin II in plasma. It has been suggested that binding of prorenin and/or renin from circulation is responsible for uptake into tissues (Admiraal *et al.* 1999). Furthermore, it has been postulated that this mechanism (which is known also for other enzymes) rather than de novo renin biosynthesis at peripheral local sites is responsible for local actions of Ang II. The understanding of the potential role of prorenin and renin binding has been significantly expanded by the characterization of several proteins capable of binding prorenin and/or renin (Nguyen *et al.* 2002, Nguyen *et al.* 2004, Catanzaro 2005)

A specific human renin receptor has been recently identified by expression cloning. The complementary DNA of this renin receptor encodes a 350-amino acid protein with a single transmembrane domain and no homology to any known membrane protein (Nguyen *et al.* 2002). High expression levels are detected in the heart, brain, and placenta at the mRNA level, while lower levels are observed in the kidney and liver (Nguyen *et al.* 2002). This 45-kDa membrane protein binds both prorenin and renin and shows a dual function (Nguyen *et al.* 2004). First, binding of prorenin activates cellular effects that are independent from angiotensin II generation by activating the mitogen-activated protein (MAP) kinases p(42)/p(44) and extracellular signal-regulated kinases (ERK) 1/2 (Nguyen

et al. 2002, Nguyen *et al.* 2004). Second, this receptor acts as a cofactor by increasing the efficiency of angiotensin I generation on the cell surface by receptor-bound prorenin and renin (Nguyen *et al.* 2002, Nguyen *et al.* 2004). Additional studies showed that the receptor is localized in the kidney in the mesangium and in the subendothelium of renal, uterine, and cardiac blood vessels (Nguyen *et al.* 2002, Nguyen *et al.* 2004).

ACE

Angiotensin converting enzyme, ACE, originally called “kininase II”, was isolated from snake poison of *Bothrops Jararaca* in 1965. Peptides isolated from poison seemed to inhibit enzyme degrading bradykinin. Later, it became obvious that ACE and kininase II are the very same peptide.

ACE, a membrane bound metalloproteinase, cleaves blood pressure neutral decapeptide angiotensin I, by removing a histidyl-leusin-dipeptide, into octapeptide angiotensin II. In systemic RAS, action of ACE occurs in lungs. In terms of tissue RAS, increase of activity of ACE has been documented in stenotic aortic valves (Helske *et al.* 2004). Furthermore, there are two other enzymes, chymase and cathepsin G, capable of increasing angiotensin II amounts locally in stenotic aortic valves (Helske *et al.* 2004, Helske *et al.* 2006).

ACE 2, the first known human homologue of ACE, was discovered in 2000 (Tipnis *et al.* 2000). ACE2 is thought to counteract with Ang II in terms of hydrolysing Ang II to angiotensin 1–7 [Ang (1–7)], having opposite effects to those of Ang II. The role of the ACE2-Ang (1–7) axis in cardiac function is largely obscure (Keidar *et al.* 2007).

AT₁ and AT₂ receptors

The functional effects of Angiotensin II are mediated via guanine nucleotide binding protein coupled receptors (GPCRs). Angiotensin II is bound by two distinct receptor isoforms, angiotensin II receptors type 1 and 2 (AT₁ and AT₂). Both AT₁ and AT₂ are expressed in the heart (Kim & Iwao 2000) and AT₁ has been reported to be upregulated in stenotic aortic valves (Helske *et al.* 2004). Although AT₁ shares structural homology with AT₂, they are functionally distinct and differentially distributed (Unger *et al.* 1996, Kim & Iwao 2000). The classical systemic effects of angiotensin II include vasoconstriction via increased contraction of the vascular smooth muscle, aldosterone biosynthesis, increased

sodium and water reabsorption and increased catecholamine synthesis, which are mediated via AT₁. Furthermore, angiotensin II increases collagen synthesis and fibrosis locally and is able to directly induce hypertrophic growth of the heart. Angiotensin II also has a direct positive inotropic effect (Kim & Iwao 2000). According to theory, the beneficial effects in cardiovascular system (antifibrotic effect, antihypertrophic effect, vasodilating effect and opposing the antinatriuretic effect mediated by AT₁) of angiotensin II are mediated via AT₂ receptor (Steckelings *et al.* 2005). Renin angiotensin system has been reported to be upregulated in stenotic aortic valves, but the gene-expression of AT₂ receptor in calcified aortic valve disease is not known (O'Brien *et al.* 2002, Helske *et al.* 2004, Helske *et al.* 2007a).

2.3.4 Apelin–APJ axis

In 1992, a gene for a receptor with marked similarities to the AT₁ receptor was identified. At that time there was no known ligand and it was named the APJ receptor (also known as the Angiotensin-like 1 receptor) (O'Dowd *et al.* 1993). Angiotensin II is unable to activate the APJ receptor despite its similarities to the AT₁ receptor. It was considered as an “orphan” receptor until 1998 apelin was isolated (Tatemoto *et al.* 1998). There is no evidence to date that the apelin-APJ pathway involves more than one receptor.

Apelin and APJ mRNA is expressed extensively in body tissue. There are particularly high concentrations in the cerebellum, vascular endothelium, heart, lung and kidney (Hosoya *et al.* 2000, De Falco *et al.* 2002). There are high concentrations of APJ receptors in the heart and at a similar density to AT₁ receptors. Apelin immunoreactivity is found in endothelial cells of human cardiac and vascular tissue but not in other tissues suggesting that the physiological effects of apelin may be mediated by its actions on the endothelium (Kleinz *et al.* 2005).

The role of the apelin-APJ system in cardiovascular physiology and its interaction with other neuroendocrine pathways has not been fully elucidated. However, the small number of reported studies, which have been performed predominantly in animals, indicate that apelin signalling may be involved in the regulation of vascular tone, cardiac contractile function and fluid balance (De Mota *et al.* 2000, Tatemoto *et al.* 2001, Szokodi *et al.* 2002, Charles *et al.* 2006).

The proposed cardiovascular effects of the apelin-APJ system are opposite to the effects of the renin-angiotensin system (RAS), which is known to play a vital

role of heart failure (Kim & Iwao 2000). ACE II, breaking down Ang II to Ang [1–7], acts on apelin as well, suggesting a dynamic interaction between apelin and Ang II pathways (Vickers *et al.* 2002). In acute ischaemic injury, apelin may have a protective role since apelin and APJ gene expression is upregulated in response to hypoxia in cardiac tissue (Ronkainen *et al.* 2007). Animal models of heart failure show marked down-regulation of cultured myocytes subjected to mechanical stretch as well as the rat heart model of chronic pressure overload (Szokodi *et al.* 2002). In humans, apelin immunoreactivity was reduced in patients with left ventricular dysfunction (Foldes *et al.* 2003). In patients with chronic heart failure, secondary to idiopathic dilated cardiomyopathy, plasma apelin was no different to controls (Miettinen *et al.* 2007). In atherosclerosis, there was no increase in the expression of APJ receptors in atherosclerotic vessels when comparing to controls (Katugampola *et al.* 2002). Gene-expression or role of apelin-APJ-axis in aortic stenosis has not been reported.

3 Aims of the research

The aim of this research was to determine the role of different endothelial derived factors in calcified aortic valve disease

More specifically, the objectives were:

- To identify expression of CNP-system in human aortic valves and to compare expression profiles in CNP, its processing enzyme furin and target receptor NPR-B in four patient groups presenting different stages of calcified aortic valve disease. (Study I)
- To identify expression of endothelin family in human aortic valves and compare expression profiles on ET-1, its processing enzyme ECE-1 and target receptor ET_A in calcified aortic valve disease. (Study II)
- To identify expression of apelin-APJ axis in human aortic valves and compare expression profiles on apelin and target receptor APJ in patient groups presenting different stages of calcified aortic valve disease. (Study III)
- To identify expression of angiotensin II receptors AT₁ and AT₂ in human aortic valves presenting different stages of calcified aortic valve disease. (Study III)

4 Materials and methods

The different methods utilized in this doctoral thesis and their use in the three original publications is shown in Table 2.

Table 2. Methods used in the original publications I–III.

Method	Publication	Reference
RNA isolation	I,II,III	Magga 1994
RT-PCR	I,II,III	Majalahti-Palviainen 2000
Extraction of cytoplasmic and nuclear protein	II	Tenhunen 2006
Radioimmunoassay	II	
Gel shift assay	II	Hautala 2001
Histological analysis	I,II,III	Soini 2003
Statistical analysis	I,II,III	

4.1 Study population

Aortic valves were obtained from 75 patients (56 male and 19 female) undergoing valve replacement surgery between 2006 and May 2008 at the Department of Cardiovascular Surgery, University Hospital of Oulu, Oulu, Finland. The study protocol was approved by the Research Ethics Committee of Oulu University Hospital and it conforms the principles outlined in the Declaration of Helsinki. All operations were made following normal surgical procedures. Aortic valve cusps were placed immediately after removal on liquid nitrogen and stored at -70°C until analysed. Part of the samples were placed in 10% buffered formalin solution for histological analysis. All patients underwent Doppler echocardiography and some (in the AS group) additional cardiac catheterization.

The control group consisted of 23 patients (21 male and 2 female) who were operated on due to aortic root pathology or aortic insufficiency with normal, non-calcified, smooth, and pliable valve cusps (Table 3). A subgroup, belonging to the control group, was an aortic regurgitation group (AR, 14 patients, all male); these individuals were operated on due to chronic aortic regurgitation with non-calcified, smooth, and pliable valve cusps. The aortic fibrosis group consisted of 15 (11 male and 4 female) patients, who were operated on due to chronic aortic regurgitation, but who were identified to have macroscopic thickenings of aortic valve cusps, which were microscopically identified mainly as fibrotic lesions. Patients in the aortic regurgitation group or aortic regurgitation combined with fibrosis had no significant transvalvular gradient (as patients in the aortic stenosis

group) and the mean aortic valve area was normal. Stenotic aortic valves (AS group) were obtained from 37 patients (24 male and 13 female) with clinically significant isolated, non-rheumatic AS. Patients in this group had significant transvalvular gradient. Distribution of bicuspid aortic valves was similar in all groups. There were no significant differences in the left ventricular ejection fraction or RAS affecting medications between the groups. Patients in the AS group were significantly older than the patients in the control group.

Table 3. Patient characteristics (n=75) of study population. Values are mean and standard deviation (SD) unless other stated.

	Control	Fibrosis / mild sclerosis	Aortic stenosis
Patients, n	23	15	37
Male, n (%)	21 (91%)	11 (73%)	24 (65%)
Bicuspid valve, n (%)	9 (39%)	5 (33%)	9 (24%)
Age, yrs (range)	45.2 ± 14.6 (21–70)	57.9 ± 13.2 (37–77)	66.6 ± 11.7 (47–82)
Hypertension, n (%)	0	4 (27%)	8 (22%)
Left ventricular EF (%)	57.0 ± 10.0	58.5 ± 10.0	61.6 ± 11.0
> 50%, n	16	9	27
30–50%, n	7	6	10
< 30%, n	0	0	0
LVEDD, (mm)	66.2 ± 10.4	60.8 ± 11.5	50.4 ± 7.2
DM, n (%)	0	1 (7%)	5 (14%)
Coronary disease, n (%)	3 (13%)	4 (27%)	18 (49%)
ASO, n (%)	0	1 (7%)	1 (3%)
COPD, n (%)	0	0	3 (8%)
Statins, n (%)	1 (4%)	3 (20%)	18 (49%)
Paget's disease, n	0	0	0
ESRD, n	0	0	0

EF, ejection fraction; LVEDD, left ventricular end diastolic diameter; DM, diabetes mellitus; ASO, peripheral atherosclerosis; COPD, chronic obstructive pulmonary disease; ESRD, end-stage renal disease

Study population in study I contained aortic valves from 42 patients (33 male and 9 female, mean age 57.7±17.0 years) as follows: aortic valves of patients with normal valves (n=4), aortic regurgitation (n=11), regurgitation and fibrosis (n=6) and aortic valve stenosis (n=21).

Study population in study II contained aortic valves from 36 patients (26 male and 10 female, mean age 58.6±16.9 years) as follows: control valves (n=12), regurgitation and fibrosis group (n=6) and aortic valve stenosis (n=18). Some of the patients of this study were enclosed in study I.

Study population in study III contained aortic valves from 55 patients (40 male and 15 female, mean age $58,3 \pm 16.0$ years) as follows: aortic valves of patients with normal valves (n=6), aortic regurgitation (n=9), regurgitation and fibrosis (n=14) and aortic valve stenosis (n=26). Approximately 50% of the patients of this study were enclosed in studies I and II.

4.2 Anaesthesia and cardiopulmonary bypass

The anaesthesia and cardiopulmonary bypass techniques were primarily based on the normal standards and practices of the Oulu University Hospital during the years 2006–2008. The patients were premedicated with benzodiazepine derivatives the night before the operation and with oral diazepam and intramuscular morphine one hour before the induction of anaesthesia. Antianginal medication and β -blockers at a reduced dose were continued, but calcium channel blockers and ACE inhibitors were discontinued before the operation. Before aortic cannulation, heparin (3mg/kg) was given and additional heparin was administered if necessary to maintain an activated clotting time of more than 400 seconds during the bypass. The effect of heparin was inhibited by protamine sulphate after bypass.

4.3 Echocardiographic measurements

Doppler echocardiography was conducted in accordance to the routines of Oulu University Hospital by a cardiologist. Measurements used for research purposes were left ventricular end diastolic diameter (LVEDD), ejection fraction (EF), aortic valve area (AVA), jet velocity and aortic peak gradient. “Peak to peak”-gradient was measured (invasively by catheter) additionally from some of the patients but aortic peak gradient, assessed in echocardiography, was used to document valvular function.

4.4 Isolation of RNA (I–III)

The aortic valve samples were immersed immediately to liquid nitrogen. Samples were stored next to operation room in -70 °C in the Department of Cardiovascular Surgery until immersed to liquid nitrogen for transportation to the laboratory at the Department of Pharmacology and Toxicology. Cytoplasmic RNA was extracted from human aortic valve cusps by the guanidine isothiocyanate-CsCl method (Magga *et al.* 1994). Attention was given to the proper destruction

of deep frozen extensively calcified valve tissue samples to enable high-class RNA catch.

4.5 Real-time RT-PCR (I–III)

The mRNA expression levels of angiotensin II, ANP, apelin, APJ, AT₁, AT₂, BNP, CNP, corin, ECE-1, eNOS, ET-1, ET_A, ET_B, furin, iNOS, NPR-A, NPR-B, NPR-C and 18S genes were analyzed with a real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) method. The first cDNA strand was synthesized from 0,5 µg of purified total RNA (First Prime Kit, Amersham) and the reactions were performed using TaqMan chemistry on an ABI 7700 Sequence Detection System (Applied Biosystems). The sequences of the forward and reverse primers and probes for RNA detection are shown in Tables 4 and 5. Primers and probes for the detection of respective mRNA sequences were designed with the Primer Express software (Applied Biosystems). The results were normalized to 18S RNA quantified from the same samples.

Table 4. Forward and reverse primers used for real time quantitative RT-PCR.

Probe	Sense primer (forward), Anti-sense primer (reverse)	Fluorogenic Probe
ANP	GATCTGCCCTCCTAAAAAGCAA, ATCTCCGCAGGCTCCGA	CTGAGGGCGCTGCTCACTGCC
Apelin	GATGCCGCTTCCCGATG, ATTCTTGACCCTCTGGGCT	ATGGGCTGGAAGACGGCAATGTCC
APJ	AGCATCATCGTGGTGCTGGT, TGTACAGCGTCTTCACCAGGTG	TGACCTTTGCCCTGTGCTGGATGC
AT ₁	CAGCATTGATCGATACTGGC, GCTACAAGCATTGTGCGTGC	TTGTTACCCAATGAAGTCCCGCC
AT ₂	CCCGTGACCAAGTCCTGAAG, TGAAGGGAAGCCAGCAA	CAGCTGCTGTTGTTCTGGCCTTCATCA
BNP	CAGGAGCAGCGCAACCAT, CAGGGATGTCTGCTCCACCT	TGCAGGGCAAACCTGTCGGAGCTG
CNP	GCTCGCCTTCTGCAAGAGC, CGAAGCAGCCCTTGGACA	CCCCAACGCGCGCAAATACAAA
corin	GAGCACTGTGGTGTCTACTGCAC, TGGGAGAGAAGCATCCGTAGTC	CCCGACCAACACGTTCCAGCCT
ECE-1	TGGATCAAGGCCAACCCA, CAGAGTTGCTGAAGGTCCC	TCCCTGATGGCCACTCACGCTG
eNOS	GAAAGACAAGGCAGCAGTGGA, TGGTGACTTTGGCTAGCTGGT	CAACGTGGCCGTGCTGCACA
ET-1	CCCTCCAGAGAGCGTTATGTG, TCAGACAGGCCCCGAAGTC	CCCACAACCGAGCACATTGGTGAC
ET _A	GCTCAGGATCATTTACCAGAACAA, CAAGGGCAAGACTGGCTATCA	TGAGGAATGGCCCCAACGCG

Table 5. Forward and reverse primers used for real time quantitative RT-PCR.

Probe	Sense primer (forward), Anti-sense primer (reverse)	Fluorogenic Probe
ETB	TTATGGCCCAAGGGTTCCA, TTTAGGCACCTCCGCAGG	AGTCTGGCGCGGTGCGTTGGC
furin	CCAGCCTCGGTACACACAGA, GCCACTCCCCCGCAC	ATGACAACAGGCACGGCACACGG
iNOS	TGGATGCAACCCATTGTC, CCCCTGCCCCAGTTT	TCCCCACGGCATGTGAGGATCA
NPR-A	GCCAAGCGCTCATGCTCTAC, ATGAACAGCCCCTCCACG	CCGGCCGGGTGACGAAGAGC
NPR-B	CCTCAGGGTCTATTTCGCC, TCCTGTGCCCAGAGCCAC	TCAGCTCCACTTTCAACCCTGGCAA
NPR-C	ACAGTTTCGACGAGACCAAAGAC, CACTCTCTCACTGGCCTGGATA	TGGATCTGGAAGACATCGTGCGCA
18S	TGGTTGCAAAGCTGAACTTAAAG, AGTCAAATTAAGCCGCAGGC	CCTGGTGGTGCCCTTCCGTCA

4.6 Extraction of cytoplasmic and nuclear protein (II)

For the total protein extracts, the aortic valve samples were broken and pulverized in liquid nitrogen, and homogenized for 10 minutes in a lysis buffer consisting of 20 mM Tris (pH 7.5), 10 mM NaCl, 0.1 mM ethylene diaminetetraacetic acid (EDTA), 0.1 mM ethylene glycol tetraacetic acid (EGTA), 1 mmol/L β -glycerophosphate, 1 mM Na_3VO_4 , 2 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM NaF, 1 mM dithiothreitol (DTT) and 10 $\mu\text{g}/\text{mL}$ each of leupeptin, pepstatin and aprotinin. The valve tissue homogenates were centrifuged at 2000 rpm for 1 minute at +4 °C. The total protein fraction was separated by the addition of 5x nuclear extraction buffer (NEB) (100 mM Tris-HCl [pH 7.5], 750 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5% Triton X 100, 12 mM sodium pyrophosphate, 5 mM β -glycerophosphate, 5 mM Na_3VO_4) to the tissue homogenate, followed by centrifugation at 12500 rpm for 20 minutes. The supernatant was frozen in liquid nitrogen and stored at -70 °C until assayed.

To extract the nuclear fraction, the supernatant from the first centrifugation was incubated in an ice solution for 15 minutes, NP-40 was added, and the nuclei were collected by centrifugation at 12 500 rpm for 30 seconds. The pellet was resuspended in a solution containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM

EDTA, 1 mM EGTA, 1 mM Na₃VO₄, 2 mM benzamidine, 1 mM PMSF, 50 mM NaF, 1 mM DTT, 3 µg/mL 1-chloro-3-tosylamido-7-phenyl-2-butanone (TPCK), 3 µg/mL L 1-tosylamido-2-phenylethyl chloromethyl ketone (TLCK), and 10 µg/mL of each leupeptin, pepstatin and aprotinin. The samples were incubated at +4 °C for 30 minutes, centrifuged at 12 500 rpm for 5 minutes and the resulting supernatants frozen in liquid nitrogen and stored -70 °C until assayed. The protein concentrations of each sample were determined colorimetrically by using Protein Assay (Bio-Rad Laboratories).

4.7 Radioimmunoassays (RIA) (II)

Parts of calcific aortic valve samples were broken and pulverized. The powder was homogenized in 9 volumes (vol/wet weight) of ice cold 1 mol/l acetic acid, 0.02 mol/l HCl using a Ultra-Turrax blender. The homogenates were centrifuged 10000g for 30 min at +4 °C. The supernatants were lyophilized in aliquots corresponding to 100 mg original tissue wet weight. The dry residues were dissolved in 1 ml of a radioimmunoassay buffer, centrifuged and duplicate aliquots of 100 µl, 40 µl and 16 µl of the clear supernatants were subjected to ET-1 radioimmunoassay.

ET-1 was measured using the rabbit-human ET-1 antiserum E1645 purchased from Sigma. According to the supplier's datasheet, the antiserum cross-reacts 100% with human ET-1, ET-2 and ET-3, but does not recognize big endothelin, ANP, or angiotensin I (cross-reaction ≤ 0.1%). Synthetic human ET-1 (Bachem) was used for calibration. Human ET-1 was radioiodinated using chloramine-T and purified by gel filtration followed by a reverse-phase HPLC as described previously (Ala-Kopsala *et al.* 2004).

4.8 Gel mobility shift assay (II)

Gel mobility shift assay was performed as described previously (Hautala *et al.* 2001) with double-stranded synthetic oligonucleotides containing activator protein-1 (AP-1) (5'-GGAAGTGTGTTTTGATGAGTCACCCCA-3') motif of the BNP, labelled with [α -³²P]dCTP using the Klenow enzyme. The binding reaction consisted of 30 µg of nuclear protein extract, the labelled probe and 2 µg of poly-(dI-dC)(dI-dC) in a buffer containing 10 mM HEPES pH 7.9, 1 mM MgCl₂, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 10% glycerol, 0.025% NP-40, 0.25 mM PMSF and 1mM each of aprotinin, leupeptin and pepstatin. The binding reaction

were carried out at room temperature for 20 minutes, and the protein-DNA complexes were subsequently separated out by electrophoresis on a 5% polyacrylamide gel in 0.5 x Tris-borate-EDTA buffer at +4 °C. Nuclear extracts used in assay were from human aortic valves. Nonlabeled double-stranded oligonucleotides corresponding to AP-1 binding site of the BNP promoter were used as specific competitor DNAs. After electrophoresis the gels were dried and exposed to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA,USA), which were scanned using Biorad Molecular Imager FX Pro Plus (Bio-Rad Laboratories, Hercules,CA,USA). Results were quantified using the Quantity One Software.

4.9 Histological analysis (I–III)

For histological analyses the aortic valves were fixed in 10% buffered formalin solution and embedded in paraffin. 5- μ m-thick sections were cut and stained with hematoxylin and eosin for calculation of the aortic valve cusp area and quantification of the calcified area. Blue ink was used in control valves to show the orientation of aortic side of these valves. FVIII-staining (Dako, Glostrup, Denmark) was used to visualize density of vessels in aortic valves, alpha smooth muscle actin (α SMA) -staining (Dako, Glostrup, Denmark) for visualizing myofibroblasts and CD 68 -staining (Dako, Glostrup, Denmark) for visualizing macrophages. Localization of apelin, AT₂, CNP, ECE-1, ET-1 and ET_A in aortic valve cusps was analyzed by using immunohistochemical staining. Specific antibodies (antibodies for apelin and ET-1, see below; ab 19134 (Abcam, Cambridge, UK) for AT₂ receptor, T-4223 (Bachem, USA) for CNP, HPA001490 (Atlas Antibodies, Stockholm, Sweden) for ECE-1 and 16201 (IBL Co., Gunma, Japan) for ET_A receptor) were used to stain positive cells in aortic valves.

ET-1 and apelin antibodies were produced as follows: before immunization, 0.4 mg of synthetic endothelin-1 or 3 mg of synthetic apelin-12, respectively, was coupled by water-soluble carbodiimide to 5 mg or 14 mg, respectively, horseshoe crab hemocyanin. A goat received injections at multiple sites in the back (1.0 mg of immunogen emulsified in an equal volume of Freund's complete adjuvant). Two boosters of 0.5 mg in Freund's incomplete adjuvant were given at 2-week intervals, and the goat was bled 14 days after the last injections. The specificity of ET-1 and apelin staining was assessed by parallel reactions in the presence of 40 μ g/ml or 100 μ g/ml, respectively, of synthetic endothelin-1 or apelin-12.

The immunostainings with the antibodies were performed as follows: Before application of the primary antibodies, the sections were heated in a microwave oven in 10 mmol Tris-EDTA, pH 9.0 for 10 minutes. The dilutions for the primary antibody were 1:100 for apelin, 1:250 for AT₂, 1:2000 for CNP, 1:25 for ECE-1, 1:150 for ET-1 and 1:20 for ET_A. Immunostaining was performed using Dako Real EnVision HRP Rabbit/mouse -Kit. With primary goat antibodies (endothelin-1 and apelin) the secondary antibody used was Zymed antigoat (1:300) and avidin-biotin complex was used (DAKO ABCComplex). Amino-9-ethylcarbazole (Zymed) was used as the chromogen. Negative control stainings were carried out by substituting nonimmune rabbit or goat serum for the primary antibodies. All histological analyses were made blinded by experienced pathologist with a Leica DM 3000 microscope and Leica DFC420 camera. Area calculations were performed on a standard PC, connected to the microscope and camera, with a public domain Java image processing program ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997–2008.). Analysis of immunohistochemical staining was semi quantitative. Intensity of staining in different cell types was measured subjectively.

4.10 Statistical analysis (I–III)

Results are expressed as mean and standard deviation (SD). Demographic data between the groups was compared by analysis of variance (continuous variables) or by Fisher's exact test (categorical variables). Because groups differed by age and sex (study I), these variables were set as covariates into analysis of covariance (ANCOVA) to control their influence on measurements. If analysis of covariance showed a significant group effect the adjusted means between groups were compared pair wise. Sidak's adjustment with four replications was used to control the multiple comparison problem. P values for group effect (p_g) and adjusted p-values (p) according to Sidak's were reported. If the dependent variable was heavily right-skewed then logarithmic or square-root transformation was used. Analyses were performed by SPSS for windows (version 14.0, SPSS inc., Chicago, IL). Two-tailed P-values were reported. Differences were considered statistically significant when P-values < 0.05.

5 Results

5.1 CNP-system in AS (I)

To study the expression level of CNP, its processing enzyme furin and its target receptors NPR-B, i.e. CNP-system, mRNA levels of CNP, furin and NPR-B were measured and control valves were compared to stenotic valves.

5.1.1 Down-regulation of furin in AS

The endothelium derived factor CNP is produced from the precursor proCNP as the result of cleavage by endoprotease furin to the mature 53 amino acid peptide (Wu *et al.* 2003a). The expression of furin, measured by RT-PCR, was markedly lower (54%, $p_g=0.045$, $p=0.04$) in stenotic aortic valves when compared to AR group, as seen in Figure 7. The mRNA levels of corin, identified as a proANP- and proBNP-converting enzyme, did not differ between the groups.

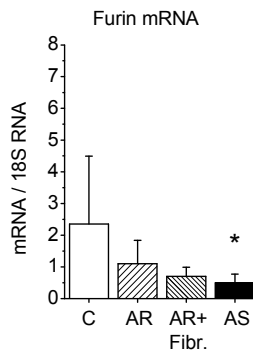


Fig. 7. Furin mRNA levels in aortic valves. Results are expressed as ratio of furin mRNA to 18S as determined by RT-PCR analysis. Results are mean±SD. * $P<0.05$ vs AR group. C indicates control group (n=4); AR, AR group (n=11); AR+Fibr, AR+fibrosis group (n=6); and AS, AS group (n=21).

5.1.2 Down-regulation of CNP in AS

The measurement of CNP mRNA levels in human aortic valve cusps was performed by RT-PCR. CNP mRNA levels were 92% lower ($p_g < 0.001$, $p < 0.001$) in stenotic valves when compared to AR group and 83 % lower ($p = 0.002$) when compared to AR + fibrosis group (Figure 8A). The localization of CNP was determined by immunistochemistry. CNP positive staining was found in valvular endothelial cells and myofibroblasts and stromal cells (Figures 8 D and E). There was a tendency for mRNA levels of ANP to decrease in AS group. There were no statistically significant changes in the expression of BNP gene between groups.

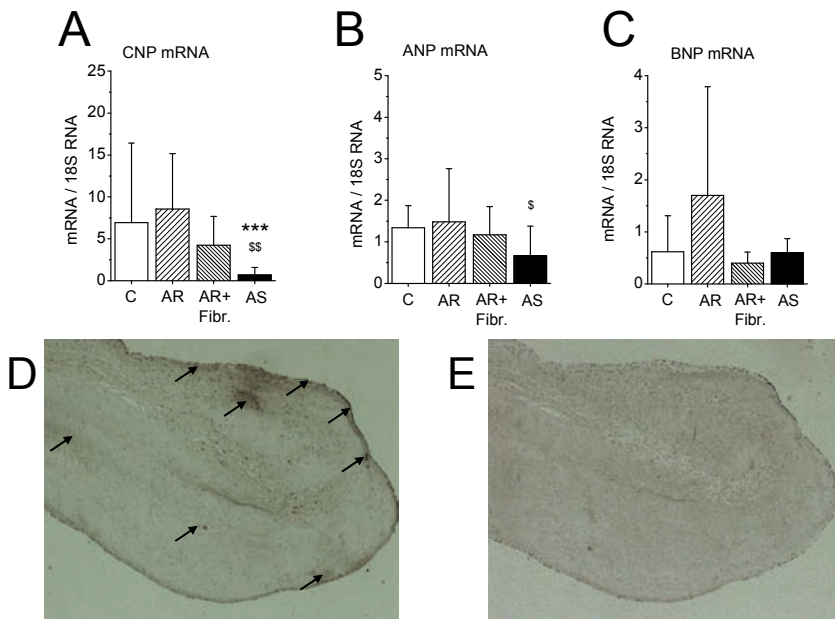


Fig. 8. A. CNP B. ANP and C. BNP mRNA levels in aortic valves. Results are expressed as ratio of CNP, ANP and BNP mRNA to 18S as determined by RT-PCR analysis. Results are mean \pm SD. * $P < 0.001$ vs AR group. \$\$ $P = 0.002$ vs AR+fibrosis group. § $P = 0.048$ vs AR+fibrosis group. C indicates control group (n=4); AR, AR group (n=11); AR+Fibr, AR+fibrosis group (n=6); and AS, AS group (n=21). Representative light photomicrographs (D and E) showing CNP immunostaining in aortic valve. D. Cross-section image of CNP stained aortic valve (arrows denote CNP positive cells). E. PBS-stained cross-section of aortic valve as negative control.**

5.1.3 Down-regulation of NPR-A and NPR-B in AS

Three natriuretic peptide receptors (NPRs) have been identified (NPR-A, NPR-B and NPR-C), of which two, NPR-A and NPR-B, has CNP as a functional ligand. NPR-A and NPR-B mRNA levels were 78% ($p_g=0.004$, $p=0.005$) and 76% ($p_g=0.001$, $p=0.002$) lower, respectively, in patients with aortic valve stenosis when compared to AR group (Figure 9).

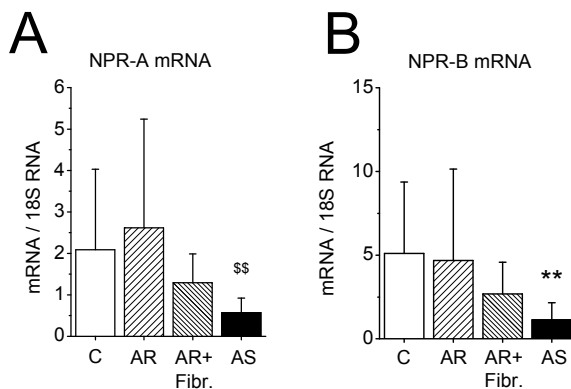


Fig. 9. A. NPR-A and B. NPR-B mRNA levels in aortic valves. Results are expressed as ratio of NPR-A and NPR-B mRNA to 18S as determined by RT-PCR analysis. Results are mean \pm SD. ** P=0.002 and ss P=0.005 vs. AR group. C, control (n=4); AR, aortic regurgitation (n=11); AR + fibr., aortic regurgitation and fibrosis (n=6); AS, aortic stenosis (n=21). NPR-A: natriuretic peptide receptor A. NPR-B: natriuretic peptide receptor B.

5.2 Endothelin family in AS (II)

Aortic stenosis, showing some hallmarks of atherosclerosis, can be considered to reveal dysfunction of endothelium, similar to the atherosclerosis. Interestingly, endothelin-1 has growth promoting and pro-inflammatory effects (Yang *et al.* 2005, Lalich *et al.* 2007). In subnanomolar concentrations, ET-1 is able to activate macrophages resulting in release of pro-inflammatory and chemotactic mediators (Libby 2002). To study the expression level of endothelin 1, its processing enzyme ECE-1 and its target receptors ET_A and ET_B, we measured mRNA levels of ET-1, ECE-1, ET_A and ET_B and compared control valves to stenotic valves.

5.2.1 Endothelin 1 (ET-1) in AS

ET-1 is the main endothelin peptide generated by endothelium in humans and is recognized as the major isoform of relevance in cardiovascular physiology and pathophysiology (Schneider *et al.* 2007). The mRNA levels of ET-1, by RT-PCR, did not differ significantly between patient groups ($P_g=0.45$, Figure 10A). The number of ET-1 positive cells in aortic valves was higher in stenotic than in control valves (Figures 1 B-C, study II). ET-1-positive staining was found in endothelial cells and myofibroblasts, adjacent to vessels, in stenotic aortic valves (Figure 10 B and C). Very few macrophages, if none, showed ET-1-positive staining (Figures 10 B and D).

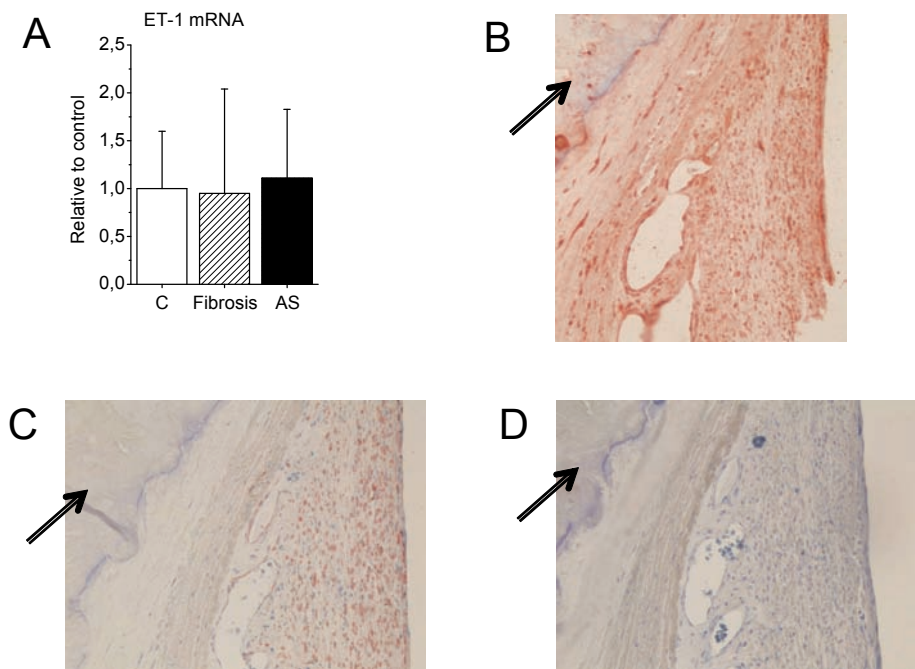


Fig. 10. A. ET-1 mRNA levels in human aortic valves. Results (mean±SD) are expressed as ratio of ET-1 mRNA to 18S as determined by RT-PCR analysis and are relative to control group. P-values are reported vs. control group. C, control (n=12); Fibrosis, fibrosis (n=6); AS, aortic stenosis (n=18). B–D. Representative light photomicrographs showing ET-1 immunostaining in aortic valves. Adjacent sections of ET-1 staining (B), alpha-smooth muscle actin staining (myofibroblasts, C) and CD 68 staining (macrophages, D) showing sorting of cell types in stenotic valve. A stenotic plaque typical to aortic stenosis can be seen in figures (arrows).

5.2.2 Endothelin converting enzyme 1 (ECE-1) in AS

The mRNA levels of ECE-1, cleaving big-ET-1 to mature biologically active ET-1, were 42% lower ($P_g=0.012$, $P=0.007$) in stenotic valves when compared to control valves (Figure 11A). A potential mechanism related to this down-regulation is significantly decreased binding activity of AP-1, which has a binding site in the promoter region of ECE-1 (Naomi *et al.* 1998). The binding activity of AP-1 was significantly lowered by 66% in AS group when compared to control group ($P_g=0.003$, $P=0.003$, Figures 11 B and C.)

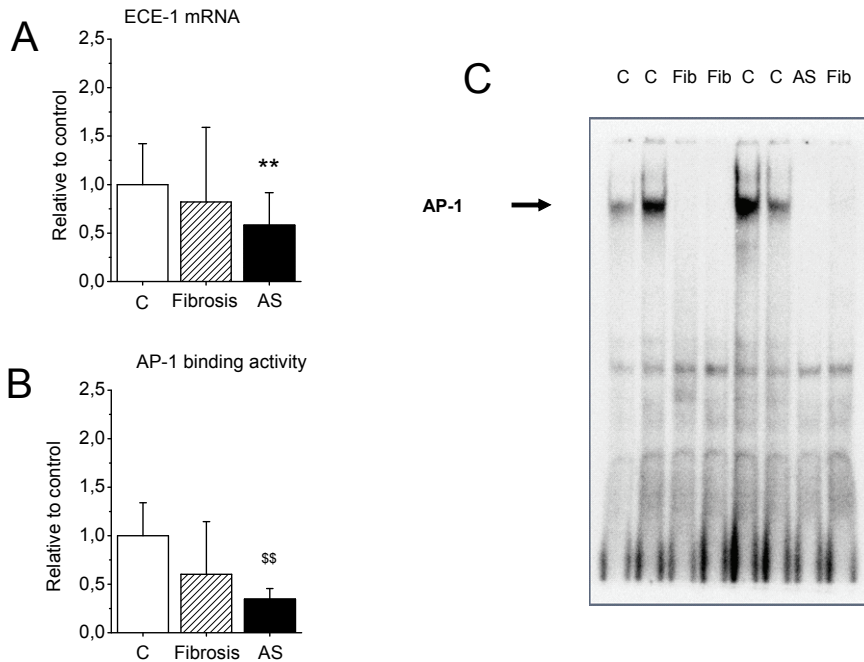


Fig. 11. A. ECE-1 mRNA levels in human aortic valves. Results (mean \pm SD) are expressed as ratio of ECE-1 mRNA to 18S and are relative to control group. C, control (n=12); Fibrosis (n=6); AS, aortic stenosis (n=18). **B.** AP-1 binding activity in human aortic valves. Results are mean \pm SD and are relative to control group. ** $P=0.007$ vs. control group. \$\$ $P=0.003$ vs. control group. C (n=10); Fibrosis, (n=5); AS (n=12). **C.** Gel mobility shift assay of nuclear extracts from human aortic valves.

5.2.3 Endothelin receptors ET_A and ET_B in AS

The levels of ET_A , mediating contractility and growth-promoting effects of ET-1 (Ihling *et al.* 2004), were significantly upregulated in stenotic aortic valves (4.3-fold, $P_g=0.016$, $P=0.032$ control vs. AS groups and 2.9-fold $P_g=0.016$, $P=0.044$ fibrosis vs. AS groups) (Figure 12A). The results of the RT-PCR analysis were supported by immunostaining, which showed a remarkable increase in the number of ET_A immunopositive cells in stenotic compared with control valves (Figures 12C and D). ET_A positive cells were located mainly to endothelial layer of neovessels. The mRNA levels of ET_B receptor did not differ between groups ($P_g=0.88$, Figure 12B).

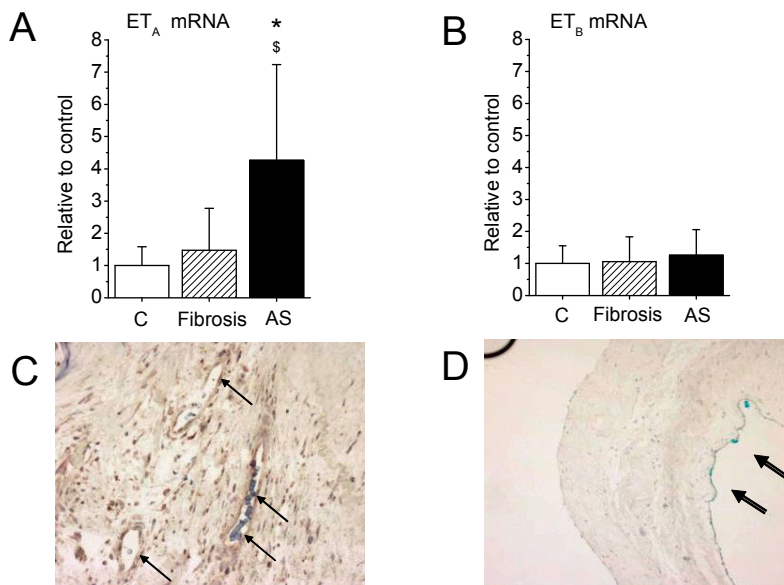


Fig. 12. A. ET_A and B. ET_B mRNA levels in human aortic valves. Results (mean \pm SD) are expressed as ratio of ET_A or ET_B mRNA to 18S as relative to control group. * $P=0.032$ vs control group. § $P=0.016$ vs fibrosis group. C, control (n=12); Fibrosis (n=6); AS, aortic stenosis (n=18). C. Graph of a stenotic valve showing ET_A receptor positivity in endothelial cells in neovessels (arrows). D. A control valve stained with ET_A receptor antibody. No positive staining was detected in the valve. Blue ink (double arrows) indicates the aortic side of the valve.

5.2.4 Nitric oxide synthases eNOS and iNOS in AS

The mRNA levels of eNOS and iNOS were measurable in all stages of the calcificated aortic valve disease. The expression of eNOS gene was 75 % ($P < 0.001$) (Figure 13A) lower than in control group. Furthermore, in stenotic valves the expression level of eNOS were 61% lower ($P = 0.032$) when AS was compared with fibrosis group (Figure 13A). Statistically significant changes in iNOS levels between groups was not seen ($P = 0.27$) (Figure 13B).

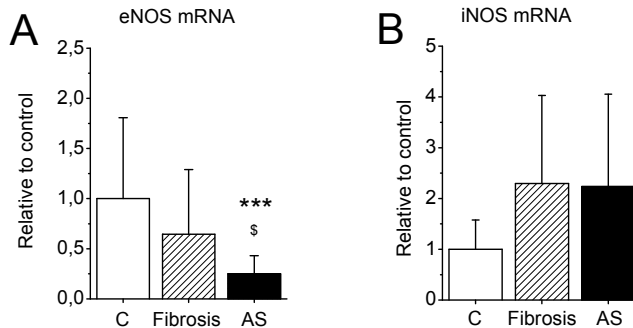


Fig. 13. A. eNOS and B. iNOS mRNA levels in human aortic valves. Results are expressed as ratio of eNOS or iNOS mRNA to 18S as determined by RT-PCR analysis and are relative to control group. Results are mean + SD. * $P < 0.001$ vs control group. [§] $P = 0.032$ vs fibrosis group C: control group (n=18). Fibrosis: fibrosis group (n=6), AS: aortic stenosis group (n=12). eNOS: endothelial nitric oxide synthase. iNOS: inducible nitric oxide synthase.**

5.3 Apelin-APJ pathway in AS (III)

It has been proposed that apelin-APJ axis acts as a compensatory mechanism of harmful effects of angiotensin II-AT₁ axis (Chandrasekaran *et al.* 2008). To study the expression level of apelin, its (angiotensin II receptor like) receptor APJ and angiotensin II receptors in AS, mRNA levels of apelin, APJ, AT₁ and AT₂ were measured and control valves were compared to stenotic valves.

5.3.1 Apelin in AS

Apelin, the ligand of orphan receptor APJ, was significantly up-regulated when control group (C) or aortic regurgitation group (AR) was compared to aortic stenosis group (AS) (3.63 -fold, $P=0.001$ and 3.60 -fold $p=0.008$, respectively). (Figure 14A). There was no statistical difference in apelin levels between AS patients with or without statin medication. The localization of apelin in aortic valves was determined by immunohistochemistry. Apelin positive staining was found in vascular endothelial cells in neovessels and fibroblast and macrophages adjacent to them (Figure 14B). Valvular endothelial cells in the surface of valve did not contain apelin positivity, unlike the stromal area next to them.

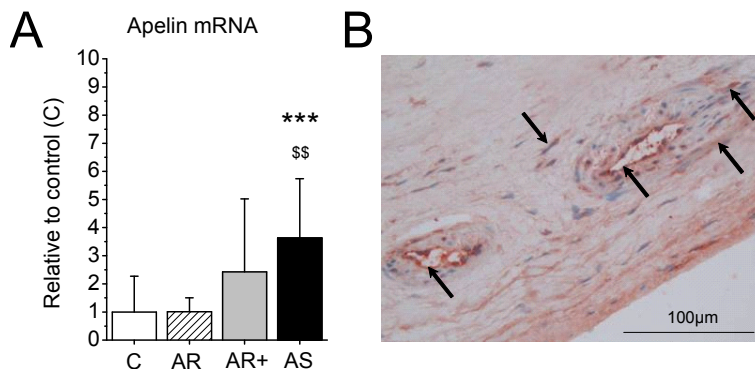


Fig. 14. A. Apelin mRNA levels in aortic valves. Results are as relative to control group. Results are mean \pm SD, * $p=0.001$ vs C. \$\$ $p=0.008$ vs AR. C indicates control group (n=6); AR, aortic regurgitation group (n=9); Fibr., aortic regurgitation and fibrosis group (n=14); and AS, aortic stenosis (n=26). B. Graph of a stenotic valve showing apelin positivity. Arrows denote apelin positive cells.**

5.3.2 APJ in AS

Similarities between the structure and anatomical distribution of apelin and its receptor APJ and that of angiotensin II and the angiotensin AT_1 receptor, respectively, provide clues about the physiological functions of this novel signal-transduction system. The apelin receptor, APJ, also known as angiotensin II receptor-like 1 (Agtr11), was initially classed as an orphan G-protein-coupled receptor, and little was known about its physiological functions until apelin, the endogenous ligand, was identified.

The receptor of apelin, APJ, was upregulated in the progression of aortic valve stenosis by 2.90 -fold, ($P=0.010$) and by 2.70 -fold ($p=0.011$) when control group was compared with a sclerotic or stenotic group, respectively (Figure 15). There was no statistical difference in APJ levels between AS patients with or without statin medication.

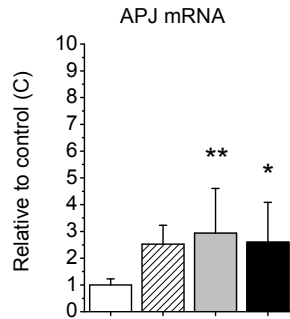


Fig. 15. APJ mRNA levels in human aortic valves. Results are expressed as relative to control group. Results are mean \pm SD. * $P=0.011$ vs C., ** $p=0.01$ vs C. C indicates control group (n=6); AR, aortic regurgitation group (n=9); Fibr., aortic regurgitation and fibrosis group (n=14); and AS, aortic stenosis group (n=26).

5.4 AT_1 and AT_2 receptors in AS (III)

There was not statistically significant increase in AT_1 mRNA levels during progression of calcific aortic valve disease (1.75 -fold, $P=0.324$, Figure 16A). AT_2 mRNA levels were 90% lower ($P<0.001$) in stenotic valves when compared to control valves and 72% lower ($P=0.005$) when compared to AR group (Figure 16B). To evaluate the localization of AT_2 in aortic valves, we used immunohistochemistry. AT_2 positivity could not be detected in stenotic aortic valves

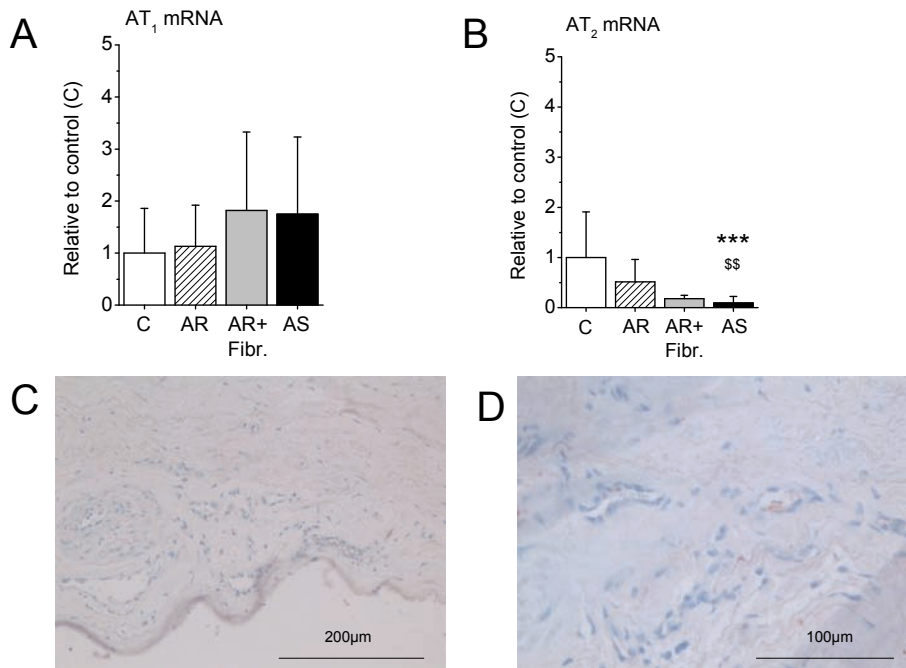


Fig. 16. A. AT₁ and B. AT₂ receptor mRNA levels in human aortic valves. Results are relative to control group. Results are mean±SD. * p≤0.001 vs C. \$\$ p =0.005 vs AR. C indicates control group (n=6 (n=5 for AT₂)); AR, aortic regurgitation group (n=9 (7 for AT₂)); Fibr., aortic regurgitation and fibrosis group (n=14 (5 for AT₂)); and AS, aortic stenosis group (n=26 (14 for AT₂₂ immunostaining in stenotic aortic valves. Section C is stained with a specific AT₂ antibody. Very little, if none, AT₂ -positivity can be seen in stenotic aortic valve (C), when compared with negative control staining (goat isotype) (D).**

6 Discussion

6.1 CNP system in AS

The results of the present study show for the first time that all components of natriuretic peptide system are expressed in human aortic valves. Remarkably, aortic valve stenosis was characterized by a distinct down-regulation of gene expression of CNP, its processing enzyme furin and target receptor NPR-B suggesting that CNP may be an important regulator of calcification process in aortic valves. Since CNP has been shown to suppress expression of vascular adhesion molecules as well as to inhibit leukocyte recruitment and platelet-leukocyte interactions, CNP has been considered as an anti-atherogenic factor (Qian *et al.* 2002, Scotland *et al.* 2005). Since CNP mRNA levels were markedly lower in stenotic valves, these findings support the hypothesis that the calcification process may be enhanced by decreased capability of valve endothelial cells to produce anti-atherogenic CNP.

CNP has highest receptor affinity to NPR-B among natriuretic peptide receptors. The pathophysiological consequences of reduced CNP gene expression in aortic valve stenosis may be further enhanced by the concomitant down-regulation of NPR-B receptors, mediating the biological effects of CNP. In addition, there was down-regulation of NPR-A as well, functioning as a target receptor for CNP with lower receptor affinity (Potter *et al.* 2006)

Another major finding was that the gene expression of proprotein convertase furin, which modulates formation of biologically active CNP, was down-regulated similarly to the CNP gene expression during calcification process. Therefore, impairment of CNP production in aortic valve stenosis occurs both at the level of CNP gene expression as well as at the post-translational proteolytic cleavage process converting precursor molecule proCNP to a mature, biologically active peptide. However, in addition to CNP, several substrates activated by furin-like proprotein convertases have been described, e.g. transforming growth factor β (TGF- β) precursor (Stawowy & Fleck 2005). Previous immunohistochemical studies have demonstrated the presence of higher levels of TGF- β 1 in calcified human aortic valve cusps in comparison to noncalcified cusps (Jian *et al.* 2003). Therefore, furin may have opposing effects in the calcification process. Increased production of biologically active CNP with anti-atherogenic properties would be a beneficial process, whereas increased production of TGF- β serves as pro-

atherogenic mechanisms (Freeman & Otto 2005). Interestingly, TGF- β 1 is generally thought to be an antiatherosclerotic molecule (Back *et al.* 2007).

A major question to be studied is whether the 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 the development of atherosclerotic lesions. Furthermore, it will be of interest to investigate whether CNP has therapeutic potential in aortic valve calcification.

6.2 Endothelin family

Another major finding of the study is that aortic valve stenosis was characterized by an increased amount of ET-1 peptide in stenotic aortic valves. Thus, many features which are typical to human calcified aortic valve disease such as uptake of oxidized LDL (Morawietz *et al.* 2002), atherosclerosis lesion formation (Schiffrin 1999), calcification (Wu *et al.* 2003), inflammation via actions and secretion of inflammatory cytokines and chemotactic mediators (Libby 2002, Yang *et al.* 2004) and local renin-angiotensin system activation may be consequences of increased paracrine/endocrine production of ET-1 in aortic valve (Muller *et al.* 2003).

The pathophysiological significance of increased ET-1 peptide levels in aortic valve stenosis may be further enhanced by the concomitant up-regulation of ET_A receptors. The molecular mechanisms that mediate up-regulation of ET-1 in aortic valve stenosis remain to be studied, but one possibility would be the changes in the degradation of ET-1. This seems, however, unlikely because an increase in neutral endopeptidase activity in stenotic aortic valves has been described recently (Helske *et al.* 2004). Increased amount of ET-1 in stenotic valves may also be related to uptake of circulating ET-1 via ET_B receptors (Dupuis *et al.* 1996). Previously, plasma levels of ET-1 have been shown to be increased in patients with AS and ET_B receptors are involved in elimination and uptake of ET-1 (Jensen *et al.* 1996, Luscher & Barton 2000). Yet, the levels of ET_B receptors were not different between control and stenotic valves in the present study.

Since continued stimulation of cells with agonists generally result in a down-regulation of receptors, one would expect either ET_A or ET_B or both receptors to be down-regulated due to higher ET-1 peptide levels in stenotic valves. However, in human calcified aortic valves several recent studies have reported higher mediator activity in parallel with increased receptor levels or decreased ligand levels in combination with down-regulation of target receptors. For example,

angiotensin II-forming enzymes and AT₁ receptors are all upregulated, and NEP activity increased in parallel with increased expression of profibrotic BK₁ receptors in stenotic valves (Helske *et al.* 2004, Freeman & Otto 2005, Helske *et al.* 2007b). Moreover, a significant down-regulation of both CNP levels and its target receptors has been reported in calcific stenotic valves, as mentioned earlier. Thus, although unanticipated, results showing increased amount of ET-1 and its target receptor ET_A agree with these observations.

Although aortic valve stenosis was characterized by distinct up-regulation of ET-1 peptide and its target receptor ET_A, promoting growth, inflammation and fibrosis, it remains to be determined whether ET receptor antagonists might have beneficial effects in slowing down the progression of disease or which effect ET receptor antagonists in general or selective ET_A receptor antagonists might have in patients with AS. Experimentally, ET_A receptor blockade prevents endothelial dysfunction and structural vascular changes in atherosclerosis as well as inhibits hypercholesterolemia-induced atherosclerosis (Barton *et al.* 1998, Tepe *et al.* 2002). On the other hand, the vasodilating and blood pressure lowering properties of ET receptor antagonists might present a clinical problem in AS. In patients with heart failure, ET_A receptor antagonists have controversial effects (e.g., darusentan), although neurohormones and natriuretic peptides would decrease favourably by treatment (Anand *et al.* 2004, Bergler-Klein *et al.* 2004).

Taken together, these findings raise the possibility that the process of AS, showing hallmarks of atherosclerosis, might be attenuated by therapeutic interventions aiming at blocking ET receptors before the late-stage pathology of the disease. In view of present results, it would be of interest to investigate whether selective ET_A receptor antagonists, particularly, have therapeutic potential in aortic valve stenosis.

6.3 Apelin-APJ axis

The results of the present study show that apelin pathway, i.e., apelin and its AT₁ receptor like receptor APJ, is expressed in human aortic valve cusps representing different stages of valve calcification disease. Interestingly, calcified aortic valve disease was characterized by significant up-regulation of apelin pathway.

The present finding of the existence of apelin-APJ pathway in human aortic valve cusps represents a new possible regulatory system involved in the valve pathophysiology. APJ receptors exhibit high levels of mRNA expression in the heart and APJ is expressed on a number of cell types including endothelium,

smooth muscle and the myocyte (Chandrasekaran *et al.* 2008). Apelin immunoreactivity is found in endothelial cells of human cardiac and vascular tissue but not in other tissues suggesting that the physiological effects of apelin may be mediated by its actions on the endothelium (Lee *et al.* 2006, Quertermous 2007, Chandrasekaran *et al.* 2008). In endothelial cells, apelin is expressed primarily in cells at sites of active vascular growth (Saint-Geniez *et al.* 2002). *In vitro*, apelin promotes chemotaxis in human endothelial cells (Cox *et al.* 2006), a well-established phenomenon in the plaque formation in stenotic aortic valves (Helske *et al.* 2008b). There is also growing evidence that apelin and APJ receptor may play an important role in angiogenesis, suggested to influence on the pathogenesis of aortic valve stenosis (Otto *et al.* 1997, Freeman & Otto 2005, Helske *et al.* 2007). Loss of function experiments in frog embryos have shown vascular developmental abnormalities and decreased numbers of endothelial cells (Cox *et al.* 2006). In zebrafish, APJ receptor knockdown was found to decrease the hypoxia-induced vessel regeneration in the *Fli-1* transgenic zebrafish model (Eyries *et al.* 2008). Moreover, blood vessel growth-promoting functions of apelin have been demonstrated in the Matrigel plug assay in the mouse (Kasai *et al.* 2004) and chick chorioallantoic membrane assay (Cox *et al.* 2006). In addition, apelin has been reported to stimulate proliferation and to suppress apoptosis of mouse osteoblastic cell line (Tang *et al.* 2007, Eyries *et al.* 2008).

Because apelin and APJ receptor levels were higher in stenotic valves, APJ receptor antagonists might be beneficial in the treatment of aortic valve stenosis by suppressing chemotaxis, angiogenesis and osteoblast activity. In the light of these results it would be interesting to investigate, whether apelin-APJ antagonists have therapeutic potential in terms of decreasing the angiogenesis and neovascularization and inhibiting the function of osteoblasts in stenotic aortic valves.

6.4 AT₁ and AT₂ receptors

Angiotensin II acts via both AT₁ and AT₂ receptors. AT₁ receptor stimulation mediates all of the classical actions of angiotensin II including vasoconstriction, cardiovascular hypertrophy and fibrosis (de Gasparo *et al.* 2000, Kim & Iwao 2000). By contrast, AT₂ receptor stimulation is thought to exert a counter-regulatory effect on AT₁ and mediate direct vasodilatation as well as antiproliferative and anti-inflammatory effects (Stoll *et al.* 1995, Tsutsumi *et al.* 1998, Harada *et al.* 1999). Since angiotensin II-forming enzymes are upregulated

and AT₁ receptor levels either increased (O'Brien *et al.* 2002, Helske *et al.* 2004) or unchanged (present study) in stenotic valves, the pathophysiological significance of activated apelin-APJ signaling, for example, may be further enhanced by the concomitant downregulation AT₂ receptor expression via unopposed AT₁ receptor activation.

On the other hand, the effects of the apelin-APJ pathway appear to be opposite to the effects of the renin–angiotensin system (Lee *et al.* 2006, Quertermous 2007, Chandrasekaran *et al.* 2008). Therefore, the upregulated apelin-APJ axis may also act as a compensatory mechanism ameliorating the harmful effects of AT₁ receptor activation in the pathogenesis of aortic valve stenosis. Importantly, recent data have shown that apelin can block a number of angiotensin II-related pathological processes associated with atherosclerosis (Chun *et al.* 2008). Thus, further studies will be required to determine the pathophysiological role of the apelin-APJ signaling pathway in stenotic valves and how the interactions of the apelin-APJ pathway with angiotensin II affect the pathobiological process in the calcified aortic valve diseases.

6.5 Nitric oxide synthases (NOS)

The disruption of the balance between ET-1 and vasodilating and anti-proliferative endothelium-derived factors, such as NO is associated with various vascular pathologies (Jensen *et al.* 1996, Bohm & Pernow 2007). However, very little information exists concerning changes of NOS enzymes in human aortic valves. It has been documented that eNOS and iNOS positivity exists in nonrheumatic aortic valve stenosis cusps when measured immunohistochemically, while gene expression levels as well as comparison to normal cusps has not been reported (Soini *et al.* 2003). This study shows that eNOS and iNOS are expressed in human aortic valves in all phases of calcified aortic valve disease. Interestingly, there was no significant compensatory activation of NOS in diseased valves, and in fact, eNOS gene was significantly downregulated in calcific aortic valve disease. Thus, the progressive, active process of aortic valve sclerosis and calcification involves alterations in both the ET-1 and NO signaling pathways in that ET-1 signaling appears to be potentiated by reduced bioavailability of protective NO, similar to endothelial dysfunction in atherosclerosis (Bousette & Giaid 2003, Bohm & Pernow 2007). Oxidative stress, resulting reduced bioavailability of NO, has been reported to exist in AS as well (Miller *et al.* 2008). The downregulated NOS activity may be one of the central factors common to

cardiovascular disease, although it is unclear whether this is a cause of, or result of, e.g. human calcific aortic stenosis.

6.6 Pharmacological treatment of AS

At present there is no approved pharmacologic treatment for normocholesterolemic AS patients (Rosenhek & Baumgartner 2008). Symptomatic AS is an unequivocal indication for valve replacement surgery (Vahanian *et al.* 2007). It will be of interest to investigate whether CNP, ET_A-antagonists or apelin-APJ-antagonists have therapeutic potential in aortic valve calcification. The hypothetical role of CNP, ET-1 and apelin in the pathophysiology of calcified stenotic aortic valve disease is presented in Fig. 17.

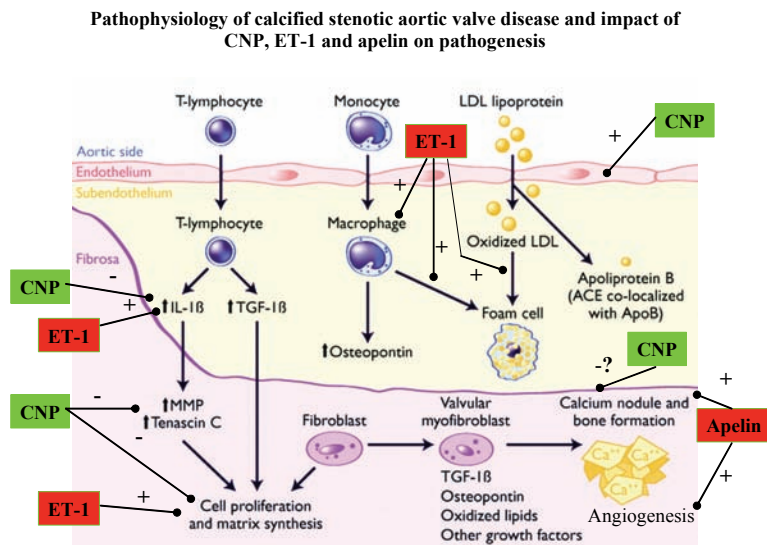


Fig. 17. Potential pathways depicting calcific aortic valve disease and possible role of CNP, ET-1 and apelin in the phenomena. T lymphocytes and macrophages infiltrate endothelium and release cytokines that act on valvular fibroblasts to promote cellular proliferation and extracellular matrix remodeling. CNP decreases cell proliferation and secretion of interleukins. ET-1 stimulates macrophages, increases secretion of interleukin-1 β and cell proliferation. Apelin increases neoangiogenesis and osteoblast activity. Modified from Freeman & Otto 2005.

6.7 Limitations of the study

The results should be interpreted with caution, however, because the control group, although unique, was small. Moreover, the groups differed by age; the patients in AS group being older than patients in control and AR groups. However, the only significant correlation in AS group between age and measured parameters was with NPR-B mRNA levels. In addition, there was no follow up or experimental treatment of the patients with CNP, ET receptor or APJ receptor antagonists, therefore the possible treatment impact in aortic valve stenosis is unknown. Moreover, there is no data on the plasma levels of apelin, ET-1 or bigET-1 in the study population.

7 Summary and conclusions

The aim of this work was to characterize endothelial factors expressed in different stages of calcified aortic valve disease, with a specific focus on potential target molecules for pharmacologic treatment of AS.

1. Aortic valve stenosis was characterized by distinct down-regulation of gene expression of CNP, its processing enzyme furin and target receptor NPR-B suggesting that CNP may be an important regulator of calcification process in aortic valves. A major question to be studied is whether the 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.
2. Aortic valve stenosis was characterized by increased amount of tissue ET-1 and its target receptor ET_A, promoting growth, inflammation and fibrosis. Furthermore, ET-1 signaling via ET_A receptors in calcific aortic valve disease appeared to be potentiated by an imbalance between ET_A and ET_B receptor gene expressions. Therefore, these findings raise the possibility that the process of AS might be attenuated by therapeutic interventions aiming at blocking ET_A receptors before the late-stage pathology of the disease.
3. Aortic valve stenosis was characterized by significant up-regulation of apelin pathway, i.e., apelin and its target receptor APJ. Aortic valve stenosis was also characterized by a highly significant down-regulation of AT₂, proposed to mediate the suppression of fibroblast proliferation and other beneficial effects of angiotensin II. These findings raise the possibility that the process of AS may be attenuated by therapeutic interventions blocking apelin-APJ system and AT₁ receptors in aortic valves.

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Original papers

- I Peltonen TO, Taskinen P, Soini Y, Rysä J, Ronkainen J, Ohtonen P, Satta J, Juvonen T, Ruskoaho H & Leskinen H (2007) Distinct down-regulation of C-type natriuretic peptide system in human aortic valve stenosis. *Circulation* 116: 1283–1289.
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- III Peltonen TO, Näpänkangas J, Vuolteenaho O, Ohtonen P, Soini Y, Juvonen T, Satta J, Ruskoaho H & Taskinen P (2008) Apelin and its receptor APJ in human aortic valve stenosis. Manuscript.

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Publications Editor Kirsti Nurkkala

ISBN 978-951-42-8987-3 (Paperback)

ISBN 978-951-42-8988-0 (PDF)

ISSN 0355-3221 (Print)

ISSN 1796-2234 (Online)

