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RĪGA STRADIŅŠ UNIVERSITY

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Right Atrial Morphology in Coronary Heart Disease and Degenerative Aortic Valve Stenosis

Summary of the Doctoral Thesis
for obtaining a doctoral degree (*Ph.D.*)

Sector – Basic Sciences of Medicine
including Pharmacology
Sub-sector – Histology and Cytology

Riga, 2021

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Abbreviations

ANUP	Atrial natriuretic peptide
AoV	Aortic valve
BNP	Brain natriuretic peptide
CHD	Coronary heart disease
ChgA	Chromogranin A
CRP	C-reactive protein
DNS	Deoxyribonucleic acid
ET-1	Endothelin 1
HDL	High-density lipoprotein
IHC	Immunohistochemistry
Il-10	Interleukin 10
Il-1α	Interleukin 1α
LDL	Low-density lipoprotein
LVEF	Left ventricular ejection fraction
mRNS	Messenger ribonucleic acid
PGP 9.5	Protein gene product 9.5
RAA	Right atrial area
RVSP	Right ventricular systolic pressure
TG	Triglycerides
TNFα	Tumor necrosis factor α
VEGF	Vascular endothelial growth factor
βD1	Beta defensin 1
βD2	Beta defensin 2
βD3	Beta defensin 3
βD4	Beta defensin 4

Introduction

Diseases of the heart and circulatory system are the leading cause of mortality in Europe, responsible for over 3,9 million deaths a year (Wilkins et al., 2017). According to the World Health Organization, 17.9 million people died of cardiovascular disease worldwide in 2016, which is about a third of all deaths (World Health Organization, 2017). Diseases of the circulatory system are still the most common cause of death and the most common cause of premature death also in Latvia (Skrule, 2018). In addition, cardiovascular diseases are the cause of death, the share of which in the structure of causes of death of the Latvian population has increased the most significantly over a hundred years (Štāle, Skrule and Rožkalne, 2018).

One of the main forms of cardiovascular diseases is coronary heart disease (CHD), and it is the most common single cause of death in Europe, accounting for 19% of all deaths among men and 20% among women each year (Wilkins et al., 2017). The degenerative aortic valve (AoV) stenosis is the most frequent native valve disease (Iung et al., 2003), and it has become the most common indication for valve surgery as well as catheter intervention for structural heart disease (Baumgartner and Walther, 2018). Both CHD and degenerative AoV stenosis have common risk factors such as age, high blood cholesterol, diabetes, smoking, high blood pressure, inflammation, and metabolic syndrome (Stewart et al., 1997). CHD is present in 30% of patients with mild to moderate AoV stenosis and 50% with critical AoV stenosis. Not only risk factors, but also pathophysiological changes, especially in the early stages of degenerative aortic valve stenosis, are similar to atherosclerosis – endothelial damage, lipid deposition, focal sclerosis, inflammatory cell infiltration, cytokine release and calcification (Milin et al., 2014). However, these conditions are not always observed at the same time: CHD was reported in 60% of patients undergoing surgical aortic valve prosthesis and in 65% of patients undergoing transplant

catheter AoV prosthesis implantation (Sabbagh and Nishimura, 2017). This confirms the existence of risk and pathogenesis factors specific to each disease (Henein et al., 2015). As both of these diseases progress with age, their prevalence increases as the population ages.

Although these heart diseases have been known for a long time and are intensively studied, there is still a lack of reliable markers that could help predict disease progression, the need for further surgery and mortality, therefore the pathophysiological processes involved in disease pathogenesis should be re-evaluated. Tissue changes in these diseases are complex and include cell death, cardiac innervation, tissue ischemia, regulators of metabolism and homeostasis, markers of inflammation and anti-inflammation, and other changes that are still not fully understood. Understanding the pathomorphology and its clinical implications are important for the cardiovascular surgeon, because the therapeutic options may improve the patient's outcome. Therefore, an important task for scientists is to identify those biomarkers that would play a role in early diagnosis, treatment choice and that would predict the outcome of the disease.

Aim of the study

To determine the prevalence of markers of apoptosis, homeostasis regulating factors, innervation, ischemia and inflammation in right atrial tissue in cases of coronary heart disease and degenerative aortic valve stenosis.

Hypothesis of the study

In cases of coronary heart disease and degenerative aortic valve stenosis, the expression of homeostasis regulating factors, ischemia, innervation and inflammatory markers in the right atrial tissue is altered and different.

Objectives

To achieve the aim, following objectives were proposed:

1. To evaluate the structure of the right atrial tissue by routine microscopy after staining with hematoxylin and eosin in patients with coronary heart disease, degenerative aortic valve stenosis, and control tissues, as well as to statistically analyze the obtained data.
2. To determine the apoptosis index and the number of apoptotic cells in the right atrial tissue in cases of coronary heart disease, aortic valve stenosis and control tissues, as well as to statistically analyze the obtained data.
3. To determine the appearance and relative distribution of homeostasis regulating factor atrial natriuretic peptide in right atrial tissue in cases of coronary heart disease, aortic valve stenosis and control tissues, as well as to statistically analyze the obtained data.
4. To determine the appearance and relative distribution of ischemic marker vascular endothelial growth factor in right atrial tissue in cases of coronary heart disease, aortic valve stenosis and control tissues, as well as to statistically analyze the obtained data.
5. To determine the appearance and relative distribution of potent vasoconstrictor endothelin 1 in right atrial tissue in cases of coronary heart disease, aortic valve stenosis and control tissues, as well as to statistically analyze the obtained data.
6. To evaluate changes in innervation in right atrial tissue in patients with coronary heart disease, degenerative aortic valve stenosis, and control tissues, as well as to statistically analyze the obtained data.
7. To evaluate involvement of the neuroendocrine system (chromogranin A) in right atrial tissue in patients with coronary

heart disease, degenerative aortic valve stenosis, and control tissues, as well as to statistically analyze the obtained data.

8. To evaluate inflammatory and anti-inflammatory processes by determining interleukin 1α and interleukin 10 in right atrial tissue in cases of coronary heart disease, aortic valve stenosis and control tissues, as well as to statistically analyze the obtained data.
9. To determine the appearance and relative distribution of antibacterial peptides (beta defensins 2, 3 and 4) in right atrial tissue in cases of coronary heart disease, aortic valve stenosis and control tissues, as well as to statistically analyze the obtained data.

Novelty of the study

In this study, we identified various tissue, innervation, inflammatory, anti-inflammatory factors, and antimicrobial peptides in right atrial tissue in patients with CHD and degenerative AoV stenosis, using right atrial tissue samples from patients with congenital heart diseases operated at an early age as a control group. The number of positive structures was analyzed in 4 different groups of right atrial tissue - cardiomyocytes, connective tissue cells, vascular and endocardial endothelial cells. In total, we determined 11 different markers: apoptosis, ANUP, VEGF, PGP 9.5 innervation, ChgA, ET- 1, Il-1α, Il-10, βD2, βD3 and βD4. To date, they have not been studied in this combination, and as some of these markers have long been known and extensively studied in combination with lesser known ones, we gain a broader insight into the morphological processes in the right atrial tissue in patients with CHD and degenerative AoV stenosis, which is considered a novelty of the study.

Personal contribution

The author of this study has collected tissue samples of the study and analyzed each tissue sample under a light microscope, as well as performed statistical analysis of the obtained data. A total of 492 tissue samples from the right atrium were analyzed. The author has written all of this scientific work and is the author of all microphotographs included in the study.

Structure and volume of the Doctoral Thesis

The Doctoral Thesis is written in Latvian. It consists of 4 chapters: literature review, material and methods, results and discussion. The volume of the Doctoral Thesis is 136 pages; it contains 24 tables and 63 microphotographs. The bibliography contains 353 sources.

1 Materials and methods

The reported research activities are consistent with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving human subjects and the ethical requirements of Riga Stradins University (Ethics Committee meeting date 29.05.2014.). All tissue specimens have been obtained with informed consent.

The tissue material used in the study – fragments of the right atrial appendage from 36 unique patients, collected at the Pauls Stradins Clinical University Hospital during elective open-heart surgeries in the period from 2014 to 2017.

All patients underwent routine pre-operative examinations as usual prior to elective cardiac surgery, including echocardiography and coronary angiography, blood tests for C-reactive protein (CRO), cholesterol fractions including triglycerides, total cholesterol, high and low density cholesterol, and some patients also have brain natriuretic peptide (BNP). A history of diabetes mellitus, arrhythmias and statin use has been collected. All patients underwent preoperative echocardiography, where the right atrial area (RAA), left ventricular ejection fraction (LVEF), and right ventricular systolic pressure (RVSP) were determined.

1.1 Patients Baseline Characteristics

A total of 36 patients with acquired heart diseases were included in the study – 24 patients with coronary heart disease and 12 patients with degenerative aortic valve stenosis. Preoperative patient data are summarized in Table 1.1. In the CHD group, the mean patient age (mean ± SD) was 65 ± 8.6 years (range 52 to 80 years) and in the AoV stenosis group was 69 ± 10.1 years (range 52 to 83 years), and there was no statistically significant difference between the groups (p 0.653).

Table 1.1

Patients Baseline Characteristics

Diagnosis	Values	CHD	AoV stenosis
		n(%), n = 24	n(%), n = 12
Male		18 (75.0)	4 (33.3)
Diabetes mellitus		5 (20.8)	4 (33.3)
Use of statins		19 (79.2)	9 (75.0)
Atrial fibrillation		1 (4.2)	3 (25.0)
TG, mmol/L		n = 23	–
	< 1.7	15 (62.5)	8 (66.7)
	≥ 1.7	8 (33.3)	4 (33.3)
LDL, mmol/L		–	n = 22
	< 1.8	11 (45.8)	4 (33.3)
	1.81–2.5	5 (20.8)	4 (33.3)
	2.51–3.0	0 (0)	2 (16.7)
	> 3.0	6 (25.0)	2 (16.7)
HDL, mmol/L		–	n = 23
	≥ 1.2	4 (16.7)	10 (83.3)
	< 1.2	19 (79.2)	2 (16.7)
CRP, mg/l		–	–
	≤ 5.0	19 (79.2)	11 (91.7)
	> 5.0	5 (20.8)	1 (8.3)
LVEF, %		–	–
	> 52	16 (66.7)	12 (100)
	41–51	5 (20.8)	0 (0)
	30–41	3 (12.5)	0 (0)
	< 30	0 (0)	0 (0)
RAA, cm^2		–	–
	≤ 18	20 (83.3)	9 (75.0)
	> 1	4 (16.7)	3 (25.0)
RVSP, mmHg	8	–	–
	< 40	22 (91.7)	9 (75.0)
	41–55	1 (4.2)	2 (16.7)
	56–70	1 (4.2)	1 (8.3)
	> 70	0 (0)	0 (0)

* Abbreviations: AoV – aortic valve, CHD – coronary heart disease, CRP – C- reactive protein, HDL – High-density lipoprotein, LDL – Low-density lipoprotein, LVEF – left ventricular ejection fraction, RAA – right atrial area, RVSP – right ventricular systolic pressure, TG – Triglycerides.

1.2 Characteristics of control group patients

The reported research activities are consistent with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving human subjects and the ethical requirements of Riga Stradins University (Ethics Committee meeting date 30.04.2015.). Samples of right atrial tissue from 5 patients with congenital heart disease operated at an early age in the archives of the Institute of Anatomy and Anthropology of Rīga Stradiņš University were used as the study control group.

Control group patient data are summarized in Table 1.2. In this group, patients ranged in age from 16 days to 1 year and 2 months.

Table 1.2

Characteristics of control group patients

No.	Gender	Age on the day of surgery	Diagnosis
1.	Male	16 days	Pulmonary artery and tricuspid valve atresia. Hypoplastic right ventricle. Coronary artery fistula. Patent arterial duct.
2.	Female	6 months	Ventricular septal defect. Patent arterial duct.
3.	Female	6 months	Tetralogy of Fallot
4.	Male	1 year 2 months	Ventricular septal defect
5.	Male	4 months	Ventricular septal defect

1.3 Collection and preparation of tissue sections

Cardiac surgeries were performed under full intubation anesthesia with a longitudinal sternotomy approach. Tissue fragments (~2 mm^2) were taken from the venous cannulae insertion site before cardiopulmonary bypass had begun and cardioplegic solution was administered. Tissue fixation was carried out immediately in the operating room, and for this purpose, previously prepared Eppendorf tubes with saturated picric acid solution (2% formaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.2)) were used. Tissue fragments

were transported to a morphology laboratory at the Institute of Anatomy and Anthropology, Riga Stradins University. Tissue fragments were dehydrated, embedded in paraffin and cut into 3 μm thick slices. The sections were then prepared for staining - deparaffinized and rehydrated. The tissues were then stained with hematoxylin and eosin for routine light microscopy, treated with the biotin-streptovidine method for immunohistochemical detection of tissue markers and by the TUNEL method for the detection of apoptotic cells.

1.4 Routine histological staining method

Tissues for routine light-microscopical examination were stained with hematoxylin and eosin (Fischer et al., 2008). After rehydration, tissues were rinsed with distilled water and stained with hematoxylin (ab143166, *Abcam*, USA) to visualize cell nuclei. The tissues were then rinsed with running water and stained with eosin (05B1003, *Bio-Optica*, Italy) to visualize the cytoplasm. The tissues were then rinsed again with running water, dehydrated with increasing concentrations of alcohol, and covered with carboxylol and xylene. The preparation was then fixed with histological glue (00811, *HistoLab*, Sweden) and covered with a coverslip. All specimens were observed using a Leica VM 6000B microscope.

1.5 Immunohistochemical method

The following markers were detected in right atrial tissue by immunohistochemistry: atrial natriuretic peptide (ANUP), PGP 9.5 containing innervation, vascular endothelial growth factor (VEGF), chromogranin A (ChgA), endothelin 1 (ET-1), interleukin 1α (Il-1α), interleukin 10 (II-10), β defensins 2, 3 and 4 (βD2, βD3 and βD4, respectively). Tissue sections were stained by the biotin-streptavidin method (Hsu, Raine and Fanger, 1981). After rehydration, the tissues were washed with TRIS buffer solution (15-M106,

Bio-Optica, Italy) and boiled in EDTA buffer solution (T0103, *Diapath*, Italy). Tissue samples were then treated with 3% peroxide to block endogenous peroxidase and rinsed again with TRIS buffer. Further treated with the following primary antibodies:

- ANUP – 8515/6, working dilution 1:10, *Dako*, Denmark;
- PGP – 9.5 439273A, working dilution 1: 200, *Invitrogen*, USA;
- VEGF – SC7269, working dilution 1:50, *Santa Crus Biotechnology*, Inc., USA;
- ChgA – 910216A, working dilution 1: 100, *Invitrogen*, USA;
- ET-1 – ab2786, working dilution 1: 250, *Abcam*, UK;
- Il-1α – sc-9983, working dilution 1:50, *Santa Cruz Biotechnology*, Inc., USA;
- Il-10 – ab34843, working dilution 1: 400, *Abcam*, Great Britain;
- βD2 – AF2758, working dilution 1: 100, *R&D Systems*, Germany;
- βD3 – orb183268, working dilution 1: 100, *Biorbyt*, Great Britain;
- βD4 – sc-59496, working dilution 1:50, *Santa Cruz Biotechnology*, Inc., USA.

An antibody diluent (ab64211, *Abcam*, USA) was used to dilute the antibodies. After incubation with primary antibodies, samples were rinsed with TRIS buffer, HiDef Detection reaction amplifier (954D-31, *Sigma-Aldrich*, USA), rinsed again with TRIS buffer, and *HiDef Detection HRP* polymer label (954D-32, *Sigma-Aldrich*, USA) was applied. The samples were further rinsed with TRIS buffer and stained with hematoxylin (ab143166, *Abcam*, USA) to visualize the cell nuclei. The tissues were then rinsed again with running water, dehydrated with increasing concentrations of alcohol, and covered with carboxylol and xylene. The preparation was then fixed with histological glue (00811, *HistoLab*, Sweden) and covered with a coverslip.

For negative controls the primary antibody was replaced by a diluent. Positive controls (in tissues which always have positive reaction) were prepared for each preparation series as well.

All specimens were observed using a Leica VM 6000B microscope. Immunoreactive (positive) structures were evaluated in five randomly selected fields of view at 400 X magnification for each tissue section material. For the quantification of structures, a semiquantitative counting method was used (Pilmane et al., 1998). The criteria for evaluating the relative frequency of immunohistochemically positive structures are shown in Table 1.3.

Table 1.2

Criteria for evaluating the relative frequency of immunohistochemically positive structures

Grading	Explanation
0	Negative reaction
0/+	Occasional positive structures in the view field
+	Few positive structures in the view field
+/++	Few to moderate number of positive structures in the view field
++	Moderate number of positive structures in the view field
++/+++	Moderate to great number of positive structures in the view field
+++	Numerous positive structures in the view field;
+++/++++	Numerous to abundant positive structures in the view field
++++	Abundance of positive structures in the view field

1.6 TUNEL method

Programmed cell death or apoptosis was determined by deoxynucleotide transferase dUTP-labeled end labeling or TUNEL method (Negoescu et al., 1996). For TUNEL staining, tissues were prepared similarly to immunohistochemistry – after rehydration, tissues were washed in distilled water and TRIS buffer solution (15-M106, *Bio-Optica*, Italy). Tissue samples were then treated with 3% peroxide to block endogenous peroxidase and rinsed again with TRIS buffer. It was then boiled in EDTA buffer (T0103, *Diapath*, Italy),

cooled, rinsed again in TRIS buffer and left in 0.1% bovine serum albumin phosphate buffer. Tissue samples were then coated with TUNEL mix (11684817910, *Roche Diagnostics*, Germany) enzyme solution, then rinsed in TRIS buffer and incubated with horseradish peroxidase-containing reagent. After incubation, the samples were rinsed with TRIS buffer solution and covered with DAB solution for peroxidase detection. The samples were further rinsed with TRIS buffer and stained with hematoxylin (ab143166, *Abcam*, USA) to visualize the cell nuclei. The tissues were then rinsed again with running water, dehydrated with increasing concentrations of alcohol, and covered with carboxylol and xylene. The preparations were then fixed with histological glue (00811, *HistoLab*, Sweden) and covered with a coverslip.

All preparations were evaluated under a Leica VM 6000B light microscope. All TUNEL-positive cardiomyocytes were counted in three randomly selected non-overlapping view fields, and then the apoptotic index, which is the number of apoptotic cardiomyocytes as a percentage of all cardiomyocytes in one field of vision, was determined (Soini, Paakko and Lehto, 1998).

1.7 Statistical Analysis

All statistical analyses were performed with IBM SPSS Statistics 22. As most of the data were on a ranking scale, we used non-parametric statistical methods to process them. To determine the differences in the distribution between two different variables, we used the Mann–Whitney U-test, but to determine the differences between three or more variables, we used the Kruskal–Wallis H-test. A Spearman's rank-order correlation was used to determine the relationship between variables. Correlation effect size was set as following: r_s 0.00–0.3 “weak”; r_s 0.31–0.69 “moderate”; r_s 0.7–0.99 “strong”.

Statistical significance was considered at the level of $p < 0.05$. Data are presented as the mean ± standard deviation (SD).

2 Results

2.1 Review Pictures

Myocardial degeneration with diffuse cardiomyocyte vacuolization was observed in almost all preparations (Figure 2.1A). Severe diffuse vacuolization was more common in patients with CHD – six patients, while cardiomyocyte vacuolization was moderate in three or mild in nine patients with AoV stenosis. Cardiomyocytes and their nuclei varied in size in 28 specimens (18 specimens from patients with CHD and 10 specimens from patients with degenerative AoV stenosis), of which five patients with CHD (Figure 2.1B) and three patients with AoV stenosis had very large cardiomyocyte nuclei.

Significant connective tissue ingrowth into the right atrial myocardium was observed in three patients with CHD and one patient with degenerative AoV stenosis (Figure 2.1C). Significant vascular sclerosis was observed in three patients with degenerative AoV (Figure 2.1D) and two patients with CHD.

In most tissue fragments were detected regions with cube shaped endocardial endothelial or epicardial epithelial cells. Cube shaped epicardial epithelial cells were observed in 17 patients with CHD (Figure 2.1E) and five patients with degenerative AoV stenosis. Cube shaped endocardial endothelial cells were observed in 15 patients with CHD and seven patients with AoV stenosis.

Focal epicardial infiltration of inflammatory cells was observed in three patients with CHD and one patient with degenerative AoV stenosis (Figure 2.1F).

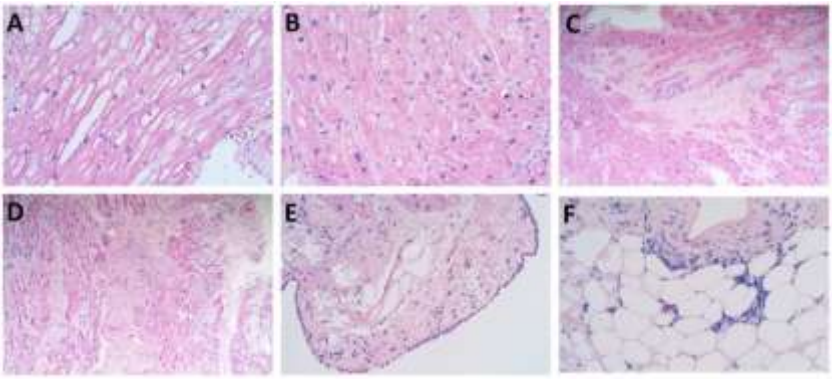

Figure 2.1. **A – Perinuclear vacuolization of cardiomyocytes in right atrial tissue in a 63-year-old patient with CHD (He / Eo, ×200). B – Nuclei of various sizes, including large, in atrial cardiomyocytes in a 78-year-old patient with CHD (He / Eo, ×200). C – Connective tissue ingrowth in the right atrial myocardium in a 63-year-old patient with CHD (He / Eo, ×100). D – Sclerotic blood vessels in the right atrial tissue in a 74-year-old patient with degenerative AoV stenosis (He / Eo, ×100). E – Cube shaped epicardial epithelial cells in right atrial tissue in a 55-year-old patient with CHD (He / Eo, ×200). F – Infiltration of inflammatory cells in the epicardial right atrial tissue in a 52-year-old patient with degenerative AoV stenosis (He / Eo, ×200)**

2.2 Apoptosis

Of the 24 right atrial tissue samples taken from patients with CHD, only two were free of apoptotic cardiomyocytes (Figure 2.2A); seven samples had moderate number (++) (Figure 2.2B), nine samples had moderate to great number (++ / +++) and six samples had abundance (+++) of apoptotic cardiomyocytes (Figure 2.2C). The apoptotic index in patients with CHD was 60.93 ± 21.68% and ranged from 0 to 83.82%.

More or less pronounced cardiomyocyte apoptosis was observed in absolutely all right atrial samples taken from patients with degenerative AoV stenosis. Few to a moderate number (+ / ++) of apoptotic cardiomyocytes were observed in one patient with degenerative AoV stenosis, but abundance (++++)

of apoptotic cardiomyocytes were observed in three patients. In five patients with AoV stenosis, moderate to great number (++ / +++) of apoptotic cardiomyocytes were detected in the right atrial tissue. The apoptotic index in patients with degenerative AoV stenosis was 70.55 ± 15.51% and ranged from 36.00 to 87.50%.

Comparing the relative number of apoptotic cardiomyocytes in the samples and the apoptotic index in patients with CHD and patients with degenerative AoV stenosis, no statistically significant difference was found between the two groups (p 0.115 and p 0.136).

Figure 2.2. **A – Lack of apoptotic cardiomyocytes in the right atrial myocardium in a 77-year-old patient with CHD (TUNEL, ×200). B – Moderate number of apoptotic cardiomyocytes in the right atrial myocardium in a 62 - year-old patient with CHD (TUNEL, ×200). C – Numerous apoptotic cardiomyocytes in the right atrial myocardium in a 66-year-old patient with CHD (TUNEL, ×200)**

2.3 Protein gene product 9.5 (PGP 9.5) containing innervation

In all study groups, the majority of patients in the right atrial tissue had at least numerous to abundant (+++ / ++++) PGP 9.5-containing nerve fibers (Figure 2.3A), respectively, in the CHD group, 83.3% of patients, in the AoV stenosis group 100% and in the control group or patients with congenital heart disease – 80% of patients. In the CHD group, two patients had few to moderate number (+ / ++) and one patient had moderate number (++) of PGP 9.5 – containing nerve fibers in the right atrial tissue (Table 2.1). In all

specimens, more PGP 9.5-containing nerve fibers were observed in the areas around the blood vessels (Figure 2.3B).

Comparing the relative number of PGP 9.5-containing nerve fibers in the right atrial tissue, no statistically significant difference was found between the three study groups (p 0.385). There was also no significant difference between patients with CHD and patients with degenerative AoV stenosis (p 0.558). In the CHD group, the relative number of nerve fibers containing PGP 9.5 showed a moderate negative correlation with right ventricular systolic pressure (r_s –0.477; p 0.018).

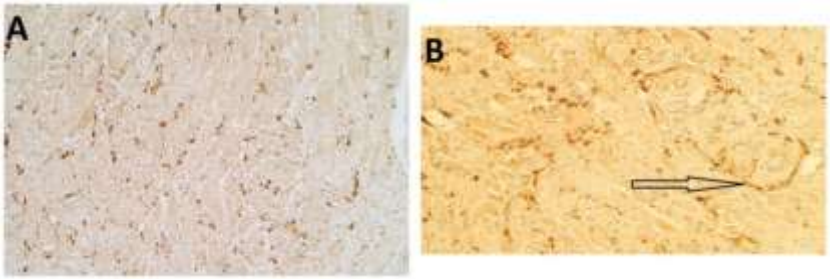

Figure 2.3. **A – Numerous PGP 9.5-containing nerve fibers in the right atrial tissue in a 66-year-old patient with CHD (PGP 9.5 IHC, ×200). B – Numerous PGP 9.5-containing nerve fibers around blood vessels in right atrial tissue in a 64-year-old patient with CHD (arrow, PGP 9.5 IHC, ×200)**

Table 2.1

Relative number of PGP 9.5-containing innervation in right atrial tissue and its statistical evaluation

	CHD n = 24	**AoV n = 12**	**Control group n = 5**
The median relative number of positive structures	+++/++++	+++/++++	++++
Kruskal–Wallis H-test	p 0.385		
Mann–Whitney U-test	p 0.558		

* Abbreviations: AoV – degenerative aortic valve stenosis group, CHD – coronary heart disease group, PGP 9.5 – protein gene product 9.5.
Grading: 0 Negative reaction; 0/+ occasional positive structures in the view field; + few positive structures in the view field; +/++ few to moderate number of positive structures in the view field; ++ moderate number of positive structures in the view field; ++/+++ moderate to great number of positive structures in the view field;+++ numerous positive structures in the view field; +++/++++ numerous to abundant positive structures in the view field; ++++ abundance of positive structures in the view field.

2.4 Tissue factors

2.4.1 Atrial natriuretic peptide (ANUP)

In both specimens from patients with CHD and from patients with degenerative AoV stenosis, numerous (+++) ANUP-positive cardiomyocytes were observed in the majority of cases, in 14 patients (Figure 2.4A) and six patients, respectively (Table 2.2). Three patients in the CHD group (Figure 2.4B) and one patient in the AoV stenosis group had moderate number (++) of ANUP-positive myocardial cells, seven patients in the CHD group and four patients in the AoV stenosis group had moderate to great number (++ / +++) of positive cardiomyocytes. There was one patient in the AoV stenosis group who had numerous to abundant (+++ / ++++) ANUP-positive cardiomyocytes in the right atrial tissue sample.

In the control group, the following ANUP-positive cardiomyocyte relative numbers were observed in right atrial tissue samples: one patient had few (+), one patient had few to moderate number (+ / ++), two patients had moderate number (++), and one patient had numerous (+++) ANUP-positive cardiomyocytes.

There was a statistically significant difference in the relative number of ANUP-positive cardiomyocytes in all atrial tissues between all three groups (p 0.029), but no statistically significant difference was found between patients with CHD and patients with degenerative AoV stenosis (p 0.721).

The number of ANUP-positive structures in the CHD group showed a moderate positive correlation (Table 2.3) with patient age (r_s 0.428; p 0.037) and a strong positive correlation with blood BNP levels (r_s 0.867; p 0.002).

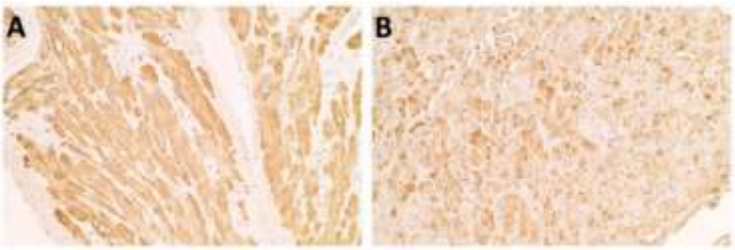

Figure 2.4. **A – Numerous ANUP-positive cardiomyocytes in right atrial tissue in a 56-year-old patient with CHD (ANUP IHC, ×200). B – Moderate number of ANUP-positive cardiomyocytes in right atrial tissue in a 52-year-old patient with CHD (ANUP IHC, ×200)**

Table 2.2

Relative number of ANUP-positive cardiomyocytes in right atrial tissue and its statistical evaluation

	CHD n = 24	**AoV n = 12**	**Control group n = 5**
The median relative number of positive structures	+++	+++	++
Kruskal–Wallis H-test	**p 0.029**		
Mann–Whitney U-test	p 0.721		

* Abbreviations: ANUP – atrial natriuretic peptide, AoV – degenerative aortic valve stenosis group, CHD – coronary heart disease group.
Grading: 0 Negative reaction; 0/+ occasional positive structures in the view field; + few positive structures in the view field; +/++ few to moderate number of positive structures in the view field; ++ moderate number of positive structures in the view field; ++/+++ moderate to great number of positive structures in the view field;+++ numerous positive structures in the view field; +++/++++ numerous to abundant positive structures in the view field; ++++ abundance of positive structures in the view field.

Table 2.3

Correlation of the relative number of ANUP-positive cardiomyocytes with pre-operative data

Factor	**Group**	**Factor2**	**r_s**	**p**
ANUP	CHD	Age	0.428	0.037
		BNP	0.867	0.002

* Abbreviations: ANUP – atrial natriuretic peptide, BNP – brain natriuretic peptide, CHD – coronary heart disease, r_s – Spearman's rank correlation coefficient.

2.4.2 Vascular endothelial growth factor (VEGF)

The number of VEGF-positive endothelial cells in the right atrial vessels (Table 2.4) in patients with CHD ranged from a lack of them (one patient) to abundance (++++) of positive cells in the view field (one patient), and in patients with AoV stenosis from no positive structures (one patient) to numerous to abundant positive structures in the view field (+++ / ++++) (one patient). Two

patients in the CHD group and one patient in the AoV stenosis group had occasional (0 / +) VEGF-positive vascular endothelial cells in the view field, six patients in the CHD group and two in the AoV stenosis group had few (+) and four patients in each group had moderate number (+ +) of VEGF-positive vascular endothelial cells. Two patients in the CHD group had moderate to great number (++ / +++), six patients in the CHD group, and three patients in the AoV stenosis group had numerous (+++) VEGF-positive vascular endothelial cells (Figure 2.5A).

In both patients with CHD and AoV stenosis, VEGF expression was observed not only in right atrial vessels but also in epicardial epithelial cells (Figure 2.5B) and endocardial endothelial cells (Figure 2.5C). Only two patients with CHD and one patient with AoV stenosis had no epicardial or endocardial VEGF-positive cells in the study samples. 62.5% of patients in the CHD group and 41.6% of patients in the AoV stenosis group had numerous to abundant (+++ / ++++) VEGF-positive cells in the epicardium and endocardium.

Although visually, the number of VEGF-positive endothelial cells in the right atrium and endocardium or epicardium was higher in the CHD group than in the AoV stenosis group, no statistically significant differences were found between these groups (p 0.745 and p 0.719).

In the congenital heart disease group, there were occasional (0 / +) VEGF-positive endothelial cells in one specimen, few (+) VEGF-positive endothelial cells in another one, one specimen had moderate to great number (++ / +++), and two specimens – abundance (++++) of VEGF-positive endotheliocytes (Table 2.4).

Table 2.4

Relative number of VEGF-positive structures in right atrial tissue and its statistical evaluation

	Vascular endothelium			Endocardial endothelium	
	CHD n = 24	**AoV n = 12**	**Control group n = 5**	**CHD n = 24**	**AoV n = 12**
The median relative number of positive structures	++	++	++/+++	+++	++ to ++/+++
Kruskal–Wallis H-test	p 0.779			–	
Mann–Whitney U-test	p 0.745			p 0.719	

* Abbreviations: AoV – degenerative aortic valve stenosis group, CHD – coronary heart disease group, VEGF – vascular endothelial growth factor.
Grading: 0 Negative reaction; 0/+ occasional positive structures in the view field; + few positive structures in the view field; +/++ few to moderate number of positive structures in the view field; ++ moderate number of positive structures in the view field; ++/+++ moderate to great number of positive structures in the view field;+++ numerous positive structures in the view field; +++/++++ numerous to abundant positive structures in the view field; ++++ abundance of positive structures in the view field.

Comparing the relative number of VEGF-positive cells in the right atrial blood vessels in all three groups, no statistically significant difference was found (p 0.779). In the CHD group, patients with cube shaped endocardial endothelial cells had significantly more VEGF-positive endocardial endothelial cells than patients with flat endocardial endothelial cells (p 0.007).

The relative number of VEGF-positive endocardial endothelial cells in the CHD group showed a statistically significant negative moderate correlation with the severity of pulmonary hypertension (r_s –0.429; p 0.036).

Figure 2.5. **A – Moderate number of VEGF-positive endothelial cells in the right atrial vessel in a 53-year-old patient with AoV stenosis (arrow, VEGF IHC, ×200). B – Numerous poorly stained VEGF-positive cube shaped epicardial epithelial cells in the right atrial tissue in a 63-year-old patient with CHD (VEGF IHC, ×200). C – Numerous VEGF-positive flat endocardial endothelial cells in right atrial tissue in a 78-year-old patient with AoV stenosis (VEGF IHC, ×100)**

2.4.3 Chromogranin A (ChgA)

In both groups of acquired heart diseases, the number of ChgA-positive endothelial cells in the right atrium (Table 2.5) ranged from no positive cell in the field of view (four patients in the CHD group and two patients in the AoV stenosis group) to numerous (+++) positive cells in the field of view (three patients in the CHD group (Figure 2.6A) and one patient in the AoV stenosis group). However, there was one patient in the AoV stenosis group with abundance (++++) of ChgA-positive endothelial cells in the right atrial blood vessels.

Table 2.5

Relative number of ChgA-positive structures in right atrial tissue and its statistical evaluation

	Vascular endothelium			Endocardial endothelium	
	CHD n = 24	**AoV n = 12**	**Control group n = 5**	**CHD n = 24**	**AoV n = 12**
The median relative number of positive structures	+/++	+/++	+++	++/+++	+++
Kruskal–Wallis H-test	**p 0.017**			–	
Mann–Whitney U-test	p 0.647			p 0.779	

* Abbreviations: AoV – degenerative aortic valve stenosis group, CHD – coronary heart disease group, ChgA – chromogranin A.
Grading: 0 Negative reaction; 0/+ occasional positive structures in the view field; + few positive structures in the view field; +/++ few to moderate number of positive structures in the view field; ++ moderate number of positive structures in the view field; ++/+++ moderate to great number of positive structures in the view field;+++ numerous positive structures in the view field; +++/++++ numerous to abundant positive structures in the view field; ++++ abundance of positive structures in the view field.

ChgA expression was also observed in right atrial endocardial endothelium (Figure 2.6B) and epicardial epithelial cells (Figure 2.6C) in a large proportion of acquired heart disease patients. In the CHD group, two patients had few (+), three patients had moderate (++), six patients had moderate to numerous (++ / +++), eight patients had numerous (+++), and two patients had numerous to abundant (+++ / ++++) positive endocardial or epicardial epithelial cells. In the AoV stenosis group, we found occasional (0 / +) positive cells in the visual field in one patient, one patient had few (+), one patient – moderate number (++), one patient moderate number to numerous (++ / +++), and seven patients numerous (+++) positive endocardial or epicardial cells in the field of view.

In contrast, in the control group or patients with congenital heart disease, the number of ChgA-positive endothelial cells in the right atrium (Table 8) varied from moderate to numerous (++ / +++) positive structures in the view field – one patient to numerous (+++) ChgA-positive structures in the view field – four patients. In addition, the relative number of ChgA-positive vascular endothelial cells was statistically significantly different between all three study groups (p 0.017).

Figure 2.3. **A – Moderate number of ChgA-positive endothelial cells in the right atrial blood vessels in a 63-year-old patient with CHD (arrows, ChgA IHC, ×200). B – Moderate number of ChgA-positive flat-shaped endocardial endothelial cells in right atrial tissue in a 63-year-old patient with CHD (ChgA IHC, ×200). C – Numerous ChgA-positive epicardial epithelial cells in right atrial tissue in a 63-year-old patient with CHD (ChgA IHC, ×200)**

2.4.4 Endothelin 1 (ET-1)

The majority of patients with acquired heart diseases (37.5% in the CHD group and 33.3% in the AoV stenosis group) had moderate number (++) of ET-1-positive endothelial cells in the right atrial vessels (Table 2.6). In the CHD group, the number of ET-1-positive endothelial cells in the right atrial blood vessels varied from lack of them - in two patients to numerous (+++) positive structures in the view field also in two patients (Figure 2.7A) but in the group of degenerative AoV stenosis from no positive cells in the field of view - one patient up to numerous to abundance (+++ / ++++) of ET-1 positive structures in the field of view – two patients.

In the congenital heart disease or control group, in most of the specimens (60%) were only occasional (0 / +) ET-1-positive endothelial cells in the blood vessels, in one specimen ET-1-positive endothelial cells in the blood vessels were not detected and in one specimen there was a moderate number (++) of ET-1-positive endothelial cells in blood vessels. There was no statistically significant difference in the relative number of ET-1-positive vascular endothelial cells between all three study groups (p. 0.109) or separately between patients with CHD and patients with degenerative AoV stenosis (p. 0.666). The relative number of ET-1-positive endothelial cells in the right ventricular blood vessels in patients with acquired heart diseases showed a weak negative correlation with right ventricular systolic pressure (r_s –0.345; p 0.039).

ET-1-positive endocardial endothelial cells were detected in almost all specimens with one exception - from a patient with degenerative AoV stenosis (Table 2.6). ET-1 expression was found in the endocardium both at sites with flat endothelial cells (Figure 2.7B) and at sites with cubic endothelium. Although the median relative number of ET-1-positive endocardial endothelial cells in the CHD group was moderate to numerous (++ / +++) and moderate (++) in the degenerative AoV stenosis group, no statistically significant difference was found between these groups (p 0.174). Patients with AoV stenosis who received statins had statistically significantly fewer ET-1-positive endothelial cells in the right atrial endocardium than patients who did not use them (p 0.047).

Table 2.6

Relative number of ET-1 positive structures in right atrial tissue and its statistical evaluation

	Vascular endothelium			**Endocardial endothelium**	
	CHD n = 24	**AoV n = 12**	**Control group n = 5**	**CHD n = 24**	**AoV n = 12**
The median relative number of positive structures	++	+/++ to ++	0/+	++/+++	++
Kruskal–Wallis H-test	p 0.109			–	
Mann–Whitney U-test	p 0.666			p 0.174	

* Abbreviations: AoV – degenerative aortic valve stenosis group, CHD – coronary heart disease group, ET-1 – endothelin 1.
Grading: 0 Negative reaction; 0/+ occasional positive structures in the view field; + few positive structures in the view field; +/++ few to moderate number of positive structures in the view field; ++ moderate number of positive structures in the view field; ++/+++ moderate to great number of positive structures in the view field;+++ numerous positive structures in the view field; +++/++++ numerous to abundant positive structures in the view field; ++++ abundance of positive structures in the view field.

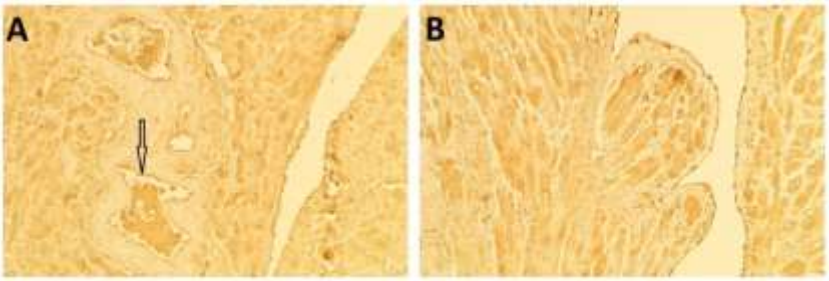

Figure 2.4. **A – Moderate number of ET-1-positive endothelial cells in the right atrial vessel in a 66-year-old patient with CHD (arrow, ET-1 IHC, ×200). B – Numerous ET-1 positive flat-shaped endocardial endothelial cells in right atrial tissue in a 78-year-old patient with degenerative AoV stenosis (ET-1 IHC, ×200)**

2.5 Inflammatory factors

2.5.1 Interleukin 1α (Il-1α)

In the tissue samples included in the study, Il-1α-positive were predominantly connective tissue and endocardial endothelial (Table 2.7) or epicardial epithelial cells (Figure 2.8A). Nine patients in the CHD group and four patients in the degenerative AoV stenosis group had no IL-1α-positive connective tissue cells in the right atrial tissue, nine patients in the CHD group and four patients in the AoV stenosis group had occasional (0/+) Il-1α-positive connective tissue cells, five patients in the CHD group and three patients in the AoV stenosis group had few (+) Il-1α-positive connective tissue cells, but one patient in the CHD group had few to moderate number (+/++) of Il-1α positive connective tissue cells (Figure 2.8B) and one patient in the AoV stenosis group had moderate to numerous (++/+++) Il-1α- positive connective tissue cells. There were also relatively few Il-1α-positive connective tissue cells in the control tissues: two patients had no Il-1α-positive connective tissue cells, two patients had occasional (0/+) Il-1α-positive connective tissue cells, and only one patient had moderate number (++) of Il-1α-positive connective tissue cells. The median

relative number of Il-1α-positive connective tissue cells in all three groups was occasional (0/+) positive structures in the field of view, and no statistically significant difference was found between all groups (p 0.883).

Table 2.7

Relative number of Il-1α-positive structures in right atrial tissue and its statistical evaluation

	Connective tissue			Endocardial endothelium	
	CHD n = 24	**AoV n = 12**	**Control group n = 5**	**CHD n = 24**	**AoV n = 12**
The median relative number of positive structures	0/+	0/+	0/+	+/++	+
Kruskal–Wallis H-test	p 0.883			–	
Mann–Whitney U-test	p 0.632			p 0.461	

* Abbreviations: AoV – degenerative aortic valve stenosis group, CHD – coronary heart disease group, Il-1α – interleukin 1α.
Grading: 0 Negative reaction; 0/+ occasional positive structures in the view field; + few positive structures in the view field; +/++ few to moderate number of positive structures in the view field; ++ moderate number of positive structures in the view field; ++/+++ moderate to great number of positive structures in the view field;+++ numerous positive structures in the view field; +++/++++ numerous to abundant positive structures in the view field; ++++ abundance of positive structures in the view field.

The number of Il-1α-positive endocardial endothelial cells in both acquired heart disease groups varied from their absence (29.2% in the CHD group, 33.3% in the AoV stenosis group to numerous (+++) positive structures in the view field (4.2% in the CHD group, 8.3% in the AoV stenosis group) and was not statistically different between the two groups (p 0.461). In the AoV stenosis group, the relative number of Il-1α-positive endocardial endothelial cells showed a moderate negative correlation with right ventricular systolic pressure (r_s –0.606; p 0.037).

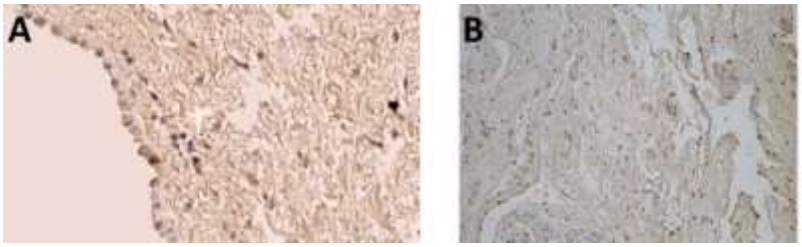

Figure 2.5. **A – Few Il-1α-positive cube shaped epicardial epithelial cells in right atrial tissue in a 69-year-old patient with degenerative AoV stenosis (Il-1α IHC, ×200). B – Few to moderate number of Il-1α-positive connective tissue cells in right atrial tissue in an 80-year-old patient with CHD (Il-1α IHC, ×200)**

2.5.2 Interleukin 10 (Il-10)

Expression of the anti-inflammatory cytokine IL-10 was observed in all tissue groups in the right atrial tissue samples (Tables 2.8 and 2.9) – cardiomyocytes, connective tissue, vascular and endocardial endothelium, and epicardial epithelium. Of the 24 right atrial tissue specimens taken from patients with CHD, 10 specimens contained moderate to numerous (++/+++) Il-10-positive cardiomyocytes, 12 specimens numerous (+++) Il-10-positive cardiomyocytes (Figure 2.9A), one specimen few to moderate (+/++) and one – moderate (++) IL-10 positive cardiomyocytes (Figure 2.9B). In addition, the relative number of IL-10-positive cardiomyocytes showed a moderate positive correlation with patient age (r_s 0.596; p 0.002) (Table 2.10). In the same group of patients, Il-10 expression in connective tissue cells was lower than in cardiomyocytes and ranged from occasional (0/+) Il-10-positive connective tissue cells in the view field in four patients to numerous (+++) positive connective tissue cells in the field of view in five patients. The median relative number of Il-10-positive connective tissue cells was moderate (++). Il-10 expression was also seen in right atrial vascular and endocardial endothelium. In the CHD group, only one patient had no IL-10 positive

endothelial cells in the right atrial blood vessels, but one patient had few (+), three patients had few to moderate (+/++), seven patients had moderate (++) (Figure 2.9C), four patients had moderate to numerous (++/+++), six patients had numerous (+++), one patient had numerous to abundant (+++/++++) and one patient had abundance (++++) of positive vascular endothelial cells. In addition, patients with CHD and CRO plasma levels greater than 5 mg /L had statistically significantly fewer IL-10-positive endothelial cells in the right atrial blood vessels (p 0.018). The majority of patients (66.7%) with CHD had numerous (+++) Il-10 positive endocardial endothelial cells in right atrial tissue (Figure 9D) and their relative numbers showed a moderate negative correlation with left ventricular ejection fraction (r_s –0.441; p 0.031) (Table 2.10).

In the AoV stenosis group, the majority of patients (58.3%) had moderate to numerous (++/+++) IL-10 positive cardiomyocytes in the right atrial tissue. The number of Il-10 positive connective tissue cells varied from few (0/+) positive cells in the view field in 16.7% of patients to numerous (+++) positive cells in the view field in 20.8% of patients. The median relative number of Il-10 positive connective tissue cells was moderate (++). In the same group of 12 patients, two had few to moderate (+/++), five had moderate (++), one had moderate to numerous (++/+++) and four had numerous (+++) Il-10 positive vascular endothelial cells. 91.7% of patients in the AoV stenosis group had at least moderate number (++) of IL-10-positive endocardial endothelial cells in the right atrial tissue (Figure 2.9E).

Table 2.8

Relative number of Il-10 positive cardiomyocytes and connective tissue cells in right atrial tissue and its statistical evaluation

	Cardiomyocytes			**Connective tissue cells**		
	CHD n = 24	**AoV n = 12**	**Control group n = 5**	**CHD n = 24**	**AoV n = 12**	**Control group n = 5**
The median relative number of positive structures	++/+++ līdz +++	++/+++	++/+++	++	+/++	0/+
Kruskal–Wallis H-test	**p 0.008**			**p 0.045**		
Mann–Whitney U-test	p 0.010			p 0.290		

* Abbreviations: AoV – degenerative aortic valve stenosis group, CHD – coronary heart disease group, Il-10 – interleukin 10.
Grading: 0 Negative reaction; 0/+ occasional positive structures in the view field; + few positive structures in the view field; +/++ few to moderate number of positive structures in the view field; ++ moderate number of positive structures in the view field; ++/+++ moderate to great number of positive structures in the view field; +++ numerous positive structures in the view field; +++/++++ numerous to abundant positive structures in the view field; ++++ abundance of positive structures in the view field.

Table 2.9

Relative number of Il-10 positive vascular and endocardial endothelial cells in right atrial tissue and its statistical evaluation

	Vascular endothelium			**Endocardial endothelium**		
	CHD n = 24	**AoV n = 12**	**Control group n = 5**	**CHD n = 24**	**AoV n = 12**	**Control group n = 5**
The median relative number of positive structures	++ līdz ++/+++	++	0/+	+++	++/+++	0/+
Kruskal–Wallis H-test	**p 0.003**			**p < 0.001**		
Mann–Whitney U-test	p 0.876			p 0.017		

* Abbreviations: AoV – degenerative aortic valve stenosis group, CHD – coronary heart disease group, Il-10 – interleukin 10.
Grading: 0 Negative reaction; 0/+ occasional positive structures in the view field; + few positive structures in the view field; +/++ few to moderate number of positive structures in the view field; ++ moderate number of positive structures in the view field; ++/+++ moderate to great number of positive structures in the view field; +++ numerous positive structures in the view field; +++/++++ numerous to abundant positive structures in the view field; ++++ abundance of positive structures in the view field.

In the congenital heart disease (control) group, two patients had moderate (++) and three patients had moderate to numerous (++/+++) IL-10-positive cardiomyocytes (Figure 2.9F), but there were only occasional (0/+) Il-10-positive connective tissue cells in 80% of samples. Control patients had relatively few IL-10 positive endothelial cells in the right atrial blood vessels and endocardium. 40% of patients had few (+) and 60% occasional (0/+) Il-10- positive vascular endothelial cells in the field of view. In two patients, IL-10-positive endocardial endothelial cells were not detected.

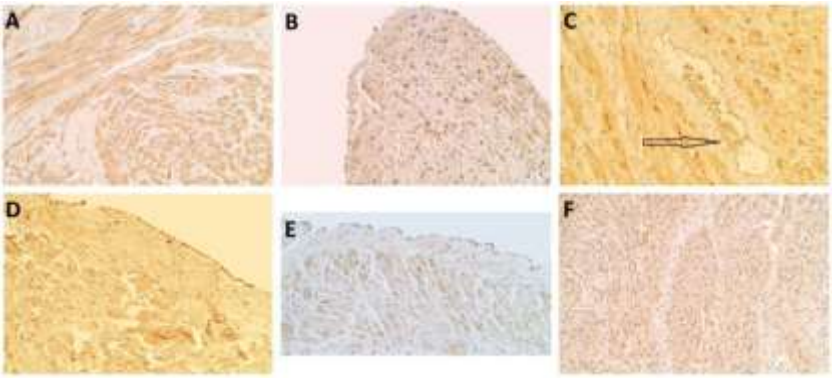

Figure 2.6. **A – Numerous Il-10-positive cardiomyocytes in right atrial tissue in a 63-year-old patient with CHD (Il-10 IHC, ×200). B – Moderate number of Il-10-positive cardiomyocytes in right atrial tissue in a 67-year-old patient with CHD (Il-10 IHC, ×200). C – Moderate number of Il-10-positive endothelial cells in the right atrial blood vessel in a patient with CHD (arrow, Il-10 IHC, ×200). D – Numerous Il-10 positive cube-shaped endocardial endothelial cells in right atrial tissue in a 57-year-old patient with CHD (IHC, ×200). E – Moderate number of Il-10 positive flat-shaped endocardial endothelial cells in right atrial tissue in a 74-year-old patient with AoV stenosis (Il-10 IHC, ×200). F – Moderate number to numerous Il-10 positive cardiomyocytes in right atrial tissue in a 4-month-old patient with ventricular septal defect (Il-10 IHC, ×200)**

There were statistically significantly more IL-10-positive cardiomyocytes and endocardial endothelial cells in the CHD group than in the AoV stenosis group (p 0.010 and p 0.017, respectively). Comparing all three groups, there were statistically significant differences in the relative numbers of Il-10-positive cardiomyocytes (p 0.008), the relative numbers of Il-10-positive connective tissue cells (p 0.045), and the relative numbers of Il-10-positive vascular and endocardial endothelial cells (p 0.003 and $p < 0.001$). In patients with acquired heart diseases, the number of IL-10 positive endothelial cells in the blood vessels of the right atrium showed a weak negative correlation with plasma CRO levels (r_s –0.354; p 0.034) (Table 2.10).

Patients with CHD and inflammatory cell infiltration in the epicardium had statistically significantly more IL-10-positive endothelial cells in the right atrial blood vessels (p 0.023) than patients with CHD without inflammatory cell infiltration. In patients with acquired heart diseases and inflammatory cell infiltration, the same trend was observed - more Il-10 positive endothelial cells in the right atrial blood vessels (p 0.006) than in patients with acquired heart diseases and without inflammatory cell infiltration in the right atrial epicardium.

Table 2.10

Correlations of the relative number of Il-10-positive cardiomyocytes with pre-operative data

Factor1	Study group	Factor2	r_s	p
Il-10-positive cardiomyocytes	CHD	Age	0.596	0.002
Il-10-positive vascular endothelial cells	CHD	CRP	–0.474	0.019
		RVSP	0.470	0.021
Il-10 endokarda endotēlijā	Acquired heart diseases	CRP	–0.354	0.034

* Abbreviations: CHD – coronary heart disease group, CRP – C reactive protein, Il-10 – interleukin 10, LVEF – left ventricular ejection fraction (groups: 52–72%, 41–51%, 30–40%, <30%), r_s – Spearman's rank correlation coefficient, RVSP – right ventricular systolic pressure.

2.6 Beta defensins

2.6.1 Beta defensin 2 (βD2)

βD2 expression was observed in all right atrial tissues examined in the study (Tables 2.11 and 2.12). Although there were more βD2-positive cells in the specimens taken from patients with acquired heart diseases, a statistically significant difference between all groups was only in the relative number of βD2-positive endocardial endothelial cells (p 0.005).

In the CHD group, all patients had at least moderate number (++) of βD2-positive cardiomyocytes in the right atrial tissue, of which moderate number (++) was observed in three patients, moderate number to numerous (++/+++) in six patients, and numerous (+++) in 15 patients (Figure 2.10A). In the AoV stenosis group, exactly 50% of patients had numerous (+++) βD2-positive cardiomyocytes, the remaining 41.7% had moderate number to numerous (++/+++) and 8.3% few to moderate number (+/++) βD2-positive cardiomyocytes (Figure 2.10B). As in patients with acquired heart diseases, four patients in the control group had moderate number to numerous (++/+++) and one patient had numerous (+++) βD2-positive cardiomyocytes (Figure 2.10C).

The relative number of βD2-positive connective tissue cells in the specimens in the CHD group ranged from their absence (0) in seven patients to numerous (+++) in 10 patients, in the AoV stenosis group from none (0) in two patients to numerous (+++) positive cells in five patients, and in the control group from occasional (0/+) positive structures in the view field in two patients to moderate number (++) of positive structures in the view field in one patient. The median relative number of βD2-positive connective tissue cells was moderate (++) in the CHD group, moderate to numerous (++/+++) in the AoV stenosis group, and few (+) in the control group.

At least occasional βD2-positive endothelial cells in the right atrial blood vessels were found in absolutely all samples included in the study. In the CHD group, one-third of the samples (33.3%) had moderate number of (++) βD2-positive vascular endothelial cells (Figure 2.10D). Two patients in the AoV stenosis group had an abundant number (++++), while the majority of patients in the control group (60%) had only few (+) βD2-positive endothelial cells in the right atrial blood vessels.

Table 2.11

Relative number of βD2-positive cardiomyocytes and connective tissue cells in right atrial tissue and its statistical evaluation

	Cardiomyocytes			Connective tissue cells		
	CHD n = 24	**AoV n = 12**	**Control group n = 5**	**CHD n = 24**	**AoV n = 12**	**Control group n = 5**
The median relative number of positive structures	+++	++/+++ to +++	++/+++	++	++/+++	+
Kruskal–Wallis H-test	p 0.425			p 0.328		
Mann–Whitney U-test	p 0.567			p 0.573		

* Abbreviations: AoV – degenerative aortic valve stenosis group, CHD – coronary heart disease group, βD2 – beta defensin 2.
Grading: 0 Negative reaction; 0/+ occasional positive structures in the view field; + few positive structures in the view field; +/++ few to moderate number of positive structures in the view field; ++ moderate number of positive structures in the view field; ++/+++ moderate to great number of positive structures in the view field; +++ numerous positive structures in the view field; +++/++++ numerous to abundant positive structures in the view field; ++++ abundance of positive structures in the view field.

One patient in the CHD group had few (+) βD2-positive endocardial endothelial cells in right atrial tissue, four patients had moderate (++), nine patients had numerous (+++), one patient had numerous to abundant number (+++/++++), six patients had abundant number (++++), but in three patients no βD2-positive endocardial endothelial cells were found in right atrial tissue. In the AoV stenosis group, one patient had occasional (0/+), two patients had few (+), one patient had moderate to numerous (++/+++) (Figure 10E), four patients had numerous (+++) and four patients had abundant number (++++) of βD2-positive

endocardial endothelial cells. In the congenital heart disease (control) group, only one patient had few (+) βD2-positive endocardial endothelial cells in the right atrial tissue sample (Figure 2.10F), but there were detected no βD2-positive endocardial endothelial cells in the remaining specimens.

Statistically significant correlations of the relative number of βD2-positive structures with pre-operative data are shown in Tables 2.13 and 2.14. Statistically significant strong positive correlations in the CHD group were found between the relative number of βD2-positive cardiomyocytes and vascular endothelial cells and BNP levels (r_s 0.779; p 0.013 and r_s 0.787; p 0.012). In the AoV stenosis group, the relative number of βD2-positive cardiomyocytes showed a moderate negative correlation with total cholesterol and LDL levels as well as with RVSP groups (r_s –0.685; p 0.014; r_s –0.619; p 0.032 and r_s –0.630; p 0.028). In the same group, similar strong and moderate negative correlations were also observed between the relative number of βD2-positive connective tissue cells and total cholesterol and HDL levels, as well as with RVSP groups (r_s –0.736; p 0.006; r_s –0.629; p 0.028 and r_s –0.630; p 0.028).

Table 2.12

Relative number of βD2-positive vascular and endocardial endothelial cells in right atrial tissue and its statistical evaluation

	Vascular endothelium			**Endocardial endothelium**		
	CHD n = 24	**AoV n = 12**	**Control group n = 5**	**CHD n = 24**	**AoV n = 12**	**Control group n = 5**
The median relative number of positive structures	++	++ to ++/+++	+	+++	+++	0
Kruskal–Wallis H-test	p 0.352			**p 0.005**		
Mann–Whitney U-test	p 0.583			p 0.781		

* Abbreviations: AoV – degenerative aortic valve stenosis group, CHD – coronary heart disease group, βD2 – beta defensin 2.
Grading: 0 Negative reaction; 0/+ occasional positive structures in the view field; + few positive structures in the view field; +/++ few to moderate number of positive structures in the view field; ++ moderate number of positive structures in the view field; ++/+++ moderate to great number of positive structures in the view field; +++ numerous positive structures in the view field; +++/++++ numerous to abundant positive structures in the view field; ++++ abundance of positive structures in the view field.

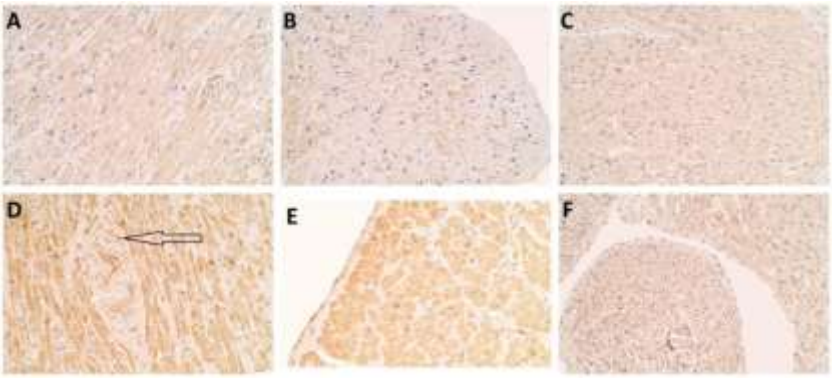

Figure 2.7. **A – Numerous βD2-positive cardiomyocytes in right atrial tissue in a 66-year-old patient with CHD (βD2 IHC, ×200). B – Few to moderate number of βD2-positive cardiomyocytes in right atrial tissue in a 53-year-old patient with AoV stenosis (βD2 IHC, ×200). C – Numerous βD2 poorly stained positive cardiomyocytes in right atrial tissue in a 16- day-old patient with combined congenital heart disease (βD2 IHC, ×200). D – Moderate number of βD2-positive endothelial cells in the right atrial blood vessel in a 77-year-old patient with CHD (arrows, βD2 IHC, ×200). E – Moderate number of βD2-positive flat-shaped endothelial cells in the right atrial endocardium in a 83-year-old patient with AoV stenosis (βD2 IHC, ×200). F – Few to moderate number βD2-positive flat-shaped endothelial cells in the right atrial endocardium in a 16-day-old patient with combined congenital heart disease (βD2 IHC, ×200)**

Table 2.13

Statistically significant correlations of the relative number of βD2-positive cardiomyocytes and connective tissue cells with pre-operative data

Factor1	Study group	Factor2	r_s	p
βD2- positive cardiomyocytes	CHD	Age	0.465	0.437
		LDL groups	–0.478	0.025
		BNP	0.779	0.013
	AoV	Age	0.579	0.049
		Total cholesterol	–0.685	0.014
		LDL	–0.619	0.032
		RVSP groups	–0.630	0.028

Table 2.13 continued

Factor1	Study group	Factor2	r_s	p
βD2- positive connective tissue cells	CHD	Age	0.437	0.033
		LDL groups	–0.548	0.008
	AoV	Total cholesterol	–0.736	0.006
		HDL	–0.629	0.028
		RVSP groups	–0.630	0.028

* Abbreviations: AoV – degenerative aortic valve stenosis group, BNP – brain natriuretic peptide, CHD – coronary heart disease group, HDL – high-density lipoprotein, LDL – low-density lipoprotein (groups: <1,8mmol/L; 1,81 – 2,5mmol/L; 2,5 – 3mmol/L and >3mmol/L), RVSP – right ventricular systolic pressure (groups: LKSS <40 mmHg, 41–55 mmHg, 55–70 mmHg, LKSS >70 mmHg), r_s – Spearman's rank correlation coefficient, βD2 – beta defensin 2.

Table 2.14

Statistically significant correlations of the relative number of βD2-positive vascular and endocardial endothelial cells with pre-operative data

Factor1	Study group	Factor2	r_s	p
βD2-positive vascular endothelium	CHD	Total cholesterol	–0.493	0.017
		LDL groups	–0.437	0.042
		BNP	0.787	0.012
		RVSP groups	0.426	0.038
	AoV	Age	0.590	0.043
βD2-positive endocardial endothelium	CHD	Total cholesterol	–0.474	0.022
		LDL groups	–0.459	0.031
	AoV	RVSP	–0.760	0.004

* Abbreviations: AoV – degenerative aortic valve stenosis group, BNP – brain natriuretic peptide, CHD – coronary heart disease group, HDL – high-density lipoprotein, LDL – low-density lipoprotein (groups: <1,8mmol/L; 1,81 – 2,5mmol/L; 2,5 – 3mmol/L and >3mmol/L), RVSP – right ventricular systolic pressure (groups: LKSS <40 mmHg, 41–55 mmHg, 55–70 mmHg, LKSS >70 mmHg), r_s – Spearman's rank correlation coefficient, βD2 – beta defensin 2.

2.6.2 Beta defensin 3 (βD3)

As with βD2, the relative number of βD3-positive cardiomyocytes in the right atrial tissue was at least moderate (++) or higher in all patients with CHD (Table 2.15), respectively moderate number (++) in three patients, moderate to

numerous (++/+++) – six patients, numerous (+++) – 14 patients and numerous to abundant number (+++/++++) – one patient (Figure 2.11A). In patients with AoV stenosis, the relative number of βD3-positive cardiomyocytes in the view field was as follows (Table 2.15): few (+) – one patient, moderate number (++) – five patients, moderate to numerous (++/+++) – three patients and one patient each had numerous (+++), numerous to abundance (+++/++++) and abundance (++++). All patients in the control group (Table 2.15) had moderate number to numerous (++/+++) βD3-positive cardiomyocytes in the right atrial tissue. There were no statistically significant differences in the relative number of βD3-positive cardiomyocytes between all three groups (p 0.061) or between patients with CHD and patients with degenerative AoV stenosis (p 0.052). Patients with acquired heart diseases and inflammatory cell infiltration in the epicardium had statistically significantly more βD3-positive cardiomyocytes in the right atrial tissue (p 0.034) than patients without inflammatory cell infiltration.

There were also no statistically significant differences in the relative number of βD3-positive connective tissue cells (Table 2.15) between all three groups (p 0.216) or separately between the acquired heart disease groups (p 0.178). In the CHD group, the relative number of βD3-positive connective tissue cells ranged from occasional positive cells (0/+) in five patients to numerous positive cells in the view field (+++) in two patients, but the median was moderate (++). In contrast, in the AoV stenosis group, the relative number of βD3-positive connective tissue cells ranged from none – two patients to numerous (+++) positive cells in the view field - in two patients, and the median was few (+) positive cells in the view field. In the control group, two patients had occasional (0/+) βD3-positive connective tissue cells, two patients had few to moderate number (+/++) and one patient had moderate number (++) of βD3-positive connective tissue cells.

Table 2.15

Relative number of βD3-positive cardiomyocytes and connective tissue cells in right atrial tissue and its statistical evaluation

	Cardiomyocytes			Connective tissue cells		
	CHD n = 24	**AoV n = 12**	**Control group n = 5**	**CHD n = 24**	**AoV n = 12**	**Control group n = 5**
The median relative number of positive structures	+++	++ to ++/+++	++/+++	++	+	+/++
Kruskal–Wallis H-test	p 0.061			p 0.216		
Mann–Whitney U-tests	p 0.052			p 0.178		

* Abbreviations: AoV – degenerative aortic valve stenosis group, CHD – coronary heart disease group, βD3 – beta defensin 3.
Grading: 0 Negative reaction; 0/+ occasional positive structures in the view field; + few positive structures in the view field; +/++ few to moderate number of positive structures in the view field; ++ moderate number of positive structures in the view field; ++/+++ moderate to great number of positive structures in the view field; +++ numerous positive structures in the view field; +++/++++ numerous to abundant positive structures in the view field; ++++ abundance of positive structures in the view field.

Figure 2.8. **A – Numerous to abundance of βD3-positive cardiomyocytes in the right atrial tissue in a 70-year-old patient with CHD (βD3 IHC, ×250). B – Moderate number of βD3-positive endothelial cells in the right atrial vessel in a 57-year-old patient with CHD (arrow, βD3 IHC, ×200). C – Moderate number of βD3-positive endocardial endothelial cells in right atrial tissue in a 78-year-old patient with CHD (βD3 IHC, ×200)**

In both the CHD group (Figure 2.11B) and the AoV stenosis group, the majority of patients had moderate (++) βD3-positive endothelial cells in the right atrial blood vessels, 45.8% and 33.3%, respectively (Table 2.16). In the

congenital heart disease group, one patient had few (+) and the other four had few to moderate number (+/++) of βD3-positive endothelial cells in the right atrial blood vessels.

βD3 expression was observed in both endocardial endothelial cells and epicardial epithelial cells. As with βD2 expression, the only statistically significant difference between all three groups was observed in the number of βD3-positive endocardial endothelial cells (p 0.017), where the median in both the CHD group and the AoV stenosis group was numerous (+++) positive cells in the view field but in the control group - occasional positive cells in the field of view (0/+) (Table 2.16). In the CHD group, only 16.6% of patients had less than moderate to numerous (++/+++) positive endocardial endothelial cells (Figure 2.11C). In the AoV stenosis group, one patient each had none (0), occasional (0/+) and few (+) βD3-positive endocardial endothelial cells, two patients each had moderate number (++), numerous (+++) and numerous to abundant number (+++ / ++++) and three patients had abundance of βD3-positive endocardial endothelial cells in the view field. In contrast, one patient in the control group did not have βD3-positive endothelial cells, two patients had occasional (0/+) βD3-positive endocardial endothelial cells, and two patients had few to moderate number (+/++) of βD3-positive endocardial endothelial cells.

Table 2.16

Relative number of βD3-positive vascular and endocardial endothelial cells in right atrial tissue and its statistical evaluation

	Vascular endothelium			**Endocardial endothelium**		
	CHD n = 24	**AoV n = 12**	**Control group n = 5**	**CHD n = 24**	**AoV n =12**	**Control group n = 5**
The median relative number of positive structures	++	+/++ to ++	+/++	+++	+++	0/+
Kruskal–Wallis H-test	p 0.064			**p 0.017**		
Mann–Whitney U-tests	p 0,147			p 0,785		

* Abbreviations: AoV – degenerative aortic valve stenosis group, CHD – coronary heart disease group, βD3 – beta defensin 3.
Grading: 0 Negative reaction; 0/+ occasional positive structures in the view field; + few positive structures in the view field; +/++ few to moderate number of positive structures in the view field; ++ moderate number of positive structures in the view field; ++/+++ moderate to great number of positive structures in the view field; +++ numerous positive structures in the view field; +++/++++ numerous to abundant positive structures in the view field; ++++ abundance of positive structures in the view field.

Correlations of the relative number of βD3 positive structures with pre-operative data are shown in Table 2.17. In the CHD group, the relative number of βD3-positive cardiomyocytes in the right atrial tissue showed a strong positive correlation with plasma levels of BNP (r_s 0.745; p 0.021), while the relative number of βD3-positive connective tissue and vascular endothelial cells showed a moderate strong positive correlation with echocardiographically measured RVSP and RVSP groups (r_s 0.431; p 0.036 and r_s 0.477; p 0.018). The relative number of βD3-positive connective tissue cells in the AoV stenosis group showed a moderate negative correlation with total cholesterol levels (r_s –0.627; p 0.029).

Table 2.17

Statistically significant correlations of the relative number of βD2-positive structures with pre-operative data

Factor1	Study group	Factor2	r_s	p
βD3-positive cardiomyocytes	CHD	BNP	0.745	0.021
βD3-positive connective tissue cells	CHD	RVSP groups	0.431	0.036
	AoV	Total cholesterol	–0.627	0.029
βD3-positive vascular endothelial cells	CHD	RVSP	0.477	0.018
βD3-positive endocardial endothelial cells	CHD	Total cholesterol	–0.553	0.006

* Abbreviations: AoV – degenerative aortic valve stenosis group, BNP – brain natriuretic peptide, CHD – coronary heart disease group, RVSP – right ventricular systolic pressure (groups: LKSS <40 mmHg, 41–55 mmHg, 55–70 mmHg, LKSS >70 mmHg), r_s – Spearman's rank correlation coefficient, βD2 – beta defensin 2.

2.6.3 Beta defensin 4 (βD4)

No βD4 positive structures were detected in any of the specimens (Figure 2.12).

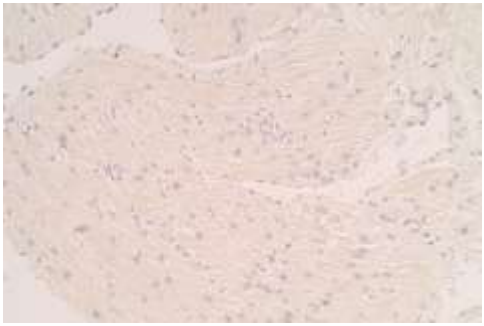

Figure 2.9. **Deficiency of βD4 in right atrial tissue in an 80-year-old patient with CHD (βD4 IHC, ×200)**

2.7 Statistical correlations of data

In the CHD group, a statistically significant strong positive correlation was found between the relative number of βD2-positive cardiomyocytes and the relative number of βD2-positive vascular endothelial cells in right atrial tissue (r_s 0.710, $p < 0.001$). The statistically significant moderate positive and negative correlations of the relative numbers of tissue markers in the study in the CHD group are shown in Tables 2.18 and 2.19.

Table 2.18

Statistically significant moderate positive correlations between tissue markers in coronary heart disease group

Factor 1	Factor 2	r_s	p
Apoptotic cardiomyocytes	Il-10-positive cardiomyocytes	0.438	0.032
ANUP-positive cardiomyocytes	ET-1-positive vascular endothelial cells	0.448	0.028
	βD2-positive vascular endothelial cells	0.407	0.048
ChgA-positive endocardial endothelial cells	VEGF-positive endocardial endothelial cells	0.645	0.001
	Il-1α-positive endocardial endothelial cells	0.490	0.015
	Il-10-positive endocardial endothelial cells	0.522	0.009
	βD2-positive endocardial endothelial cells	0.431	0.036
	βD2-positive vascular endothelial cells	0.405	0.049
	βD3-positive connective tissue cells	0.472	0.020
	βD3-positive endocardial endothelial cells	0.557	0.005
ET-1-positive vascular endothelial cells	ET-1-positive endocardial endothelial cells	0.486	0.016
VEGF-positive endocardial endothelial cells	Il-10-positive endocardial endothelial cells	0.468	0.021
	βD3-positive endocardial endothelial cells	0.431	0.036

Table 2.18 continued

Factor 1	Factor 2	r_s	p
Il-1α-positive endocardial endothelial cells	Il-10 -positive connective tissue cells	0.417	0.043
	βD3-positive cardiomyocytes	0.509	0.011
	βD3-positive connective tissue cells	0.528	0.008
Il-10-positive cardiomyocytes	βD2-positive cardiomyocytes	0.425	0.038
	βD2-positive connective tissue cells	0.571	0.004
Il-10-positive connective tissue cells	βD2-positive cardiomyocytes	0.512	0.011
	βD2-positive connective tissue cells	0.522	0.009
	βD2-positive vascular endothelial cells	0.464	0.022
	βD3-positive cardiomyocytes	0.521	0.009
	βD3-positive connective tissue cells	0.483	0.017
Il-10-positive vascular endothelial cells	βD3-positive vascular endothelial cells	0.435	0.034
Il-10-positive endocardial endothelial cells	βD2-positive cardiomyocytes	0.498	0.013
	βD2-positive vascular endothelial cells	0.452	0.026
βD2-positive cardiomyocytes	βD2-positive endocardial endothelial cells	0.603	0.002
	βD3-positive connective tissue cells	0.553	0.005
βD2-positive endocardial endothelial cells	βD2-positive connective tissue cells	0.644	0.001
	βD2-positive vascular endothelial cells	0.664	< 0.001
	βD3-positive connective tissue cells	0.408	0.048
	βD3-positive endocardial endothelial cells	0.688	< 0.001
βD2-positive connective tissue cells	βD2-positive vascular endothelial cells	0.622	0. 001
	βD3-positive cardiomyocytes	0.420	0.041
	βD3-positive connective tissue cells	0.554	0.005
	βD3-positive endocardial endothelial cells	0.495	0.014

Table 2.18 continued

Factor 1	Factor 2	r_s	p
βD2-positive vascular endothelial cells	βD3-positive connective tissue cells	0.498	0.013
βD3-positive cardiomyocytes	βD3-positive connective tissue cells	0.421	0.040
βD3-positive connective tissue cells	βD3-positive endocardial endothelial cells	0.410	0.047

* Abbreviations: ChgA – chromogranin A, ET-1 – endothelin 1, Il-10 – interleukin 10, Il-1α – interleukin 1α, PGP 9.5 – protein gene product 9.5, VEGF – vascular endothelial growth factor, βD2 – beta defensin 2, βD3 – beta defensin 3.

Table 2.19

Statistically significant moderate negative correlations between tissue markers in coronary heart disease group

Factor 1	Factor 2	r_s	p
ET-1-positive vascular endothelial cells	βD3-positive endocardial endothelial cells	–0.417	0.043
PGP 9.5-containing innervation	Il-10-positive cardiomyocytes	–0.456	0.025
	βD2-positive cardiomyocytes	–0.553	0.005
	βD2-positive endocardial endothelial cells	–0.438	0.032
	βD2-positive connective tissue cells	–0.610	0.002
	βD2-positive vascular endothelial cells	–0.525	0.008
	βD3-positive connective tissue cells	–0.501	0.013

* Abbreviations: ET-1 – endothelin 1, Il-10 – interleukin 10, PGP 9.5 – protein gene product 9.5, βD2 – beta defensin 2, βD3 – beta defensin 3.

In the group of degenerative AoV stenosis, a statistically significant moderate negative correlation was found between the relative number of apoptotic cardiomyocytes and the relative number of ChgA-positive endocardial endothelial cells in the right atrial tissue (r_s –0.633, p 0.027). The statistically significant moderate and strong positive correlations of the relative numbers of tissue markers in the AoV stenosis group are shown in Tables 2.20 and 2.21.

Table 2.20

Statistically significant strong positive correlations between tissue markers in the aortic valve stenosis group

Factor 1	Factor 2	r_s	p
ET-1-positive vascular endothelial cells	Il-10-positive vascular endothelial cells	0.777	0.003
ET-1-positive endocardial endothelial cells	VEGF-positive endocardial endothelial cells	0.732	0.010
Il-10-positive connective tissue cells	βD3-positive vascular endothelial cells	0.746	0.005
βD2-positive cardiomyocytes	βD2-positive connective tissue cells	0.901	<0.001
	βD2-positive vascular endothelial cells	0.711	0.009
	βD3-positive connective tissue cells	0.715	0.009
βD2-positive endocardial endothelial cells	βD2-positive connective tissue cells	0.713	0.009
βD2-positive connective tissue cells	βD2-positive vascular endothelial cells	0.707	0.010
	βD3-positive connective tissue cells	0.719	0.008
βD3-positive connective tissue cells	βD3-positive endocardial endothelial cells	0.769	0.003

* Abbreviations: ET-1 – endothelin 1, Il-10 – interleukin 10, VEGF – vascular endothelial growth factor, βD2 – beta defensin 2, βD3 – beta defensin 3.

Table 2.21

Statistically significant moderate positive correlations between tissue markers in the aortic valve stenosis group

Factor 1	Factor 2	r_s	p
Apoptotic cardiomyocytes	Il-1α-positive connective tissue cells	0.591	0.043
	βD2-positive cardiomyocytes	0.597	0.041
	βD2-positive connective tissue cells	0.613	0.034
	βD3-positive endocardial endothelial cells	0.628	0.029
Apoptotic index	VEGF-positive vascular endothelial cells	0.695	0.018
βD2-positive cardiomyocytes	βD2 endokardā	0.667	0.018
	βD3-positive endocardial endothelial cells	0.632	0.027
βD2-positive endocardial endothelial cells	βD2-positive vascular endothelial cells	0.640	0.025
βD2-positive vascular endothelial cells	βD3-positive connective tissue cells	0.654	0.021
	βD3-positive endocardial endothelial cells	0.646	0.023
	βD3-positive vascular endothelial cells	0.690	0.013

* Abbreviations: Il-1α – interleukin 1α, VEGF – vascular endothelial growth factor, βD2 – beta defensin 2, βD3 – beta defensin 3.

3 Discussion

In this study, we identified various tissue, innervation, inflammatory, anti-inflammatory factors, and antimicrobial peptides in right atrial tissue in patients with CHD and degenerative AoV stenosis, using right atrial tissue samples from patients with congenital heart diseases operated at an early age as a control group. Both CHD and degenerative AoV stenosis have similar risk factors and local pathogenesis processes, but are not always observed at the same time. In total, we determined 10 different markers: apoptosis, ANUP, VEGF, PGP 9.5 innervation, ChgA, ET-1, Il-1α, Il-10, βD2, βD3 and βD4. To date, they have not been studied in this combination, and as some of these markers have long been known and extensively studied in combination with lesser known ones, we gain a broader insight into the morphological processes in the right atrial tissue in patients with CHD and degenerative AoV stenosis, which is considered a novelty of the study.

No morphologically healthy right atrial tissue was detected in any of the specimens, neither in patients with CHD, nor in patients with degenerative AoV stenosis, or in patients with congenital heart disease. Myocardial degeneration with more or less pronounced vacuolization of cardiomyocytes was observed in almost all specimens. Vacuolization may be reversible, occur during exposure to the harmful agent, and the cell structure recovers when this exposure is eliminated (Cohen et al., 1979). However, vacuolization may also be irreversible, in which case it is a non-specific feature that indicates pathological conditions that lead to cell death (Shubin et al., 2016). These changes coincide with the large apoptotic index found in the study samples, although we did not find a statistically significant correlation between these features. It should be noted that the changes in the shape of the cardiomyocyte nuclei observed in our study also indirectly confirm the onset of apoptosis based on DNA fragmentation. One of the possible causes of irreversible cell vacuolization and apoptosis is the

pathogenic effect of bacteria or viruses on the cell, for example, hepatitis B virus surface protein causes acute vacuolization of liver cells followed by apoptosis (Foo et al., 2002).

3.1 Endocardial endothelium

Another characteristic feature found in a large proportion of the study tissues taken from patients with acquired heart diseases was changes in the shape of endocardial endothelial cells. Healthy endocardial endothelium is a single layer of squamous endothelial cells that line the interior surface of the heart, forming a barrier between circulating blood and the myocardium, while interacting directly with adjacent cardiomyocytes. Like vascular endothelial cells, they produce and secrete a variety of substances, such as nitric oxide, ET- 1, prostaglandin I2 and angiotensin II, with auto- and paracrine function, thereby regulating myocardial metabolism, growth and contractility (Brutsaert et al., 1988; Brutsaert, 2003). Unlike blood vessels, where the endothelium interacts with the smooth muscle of the blood vessel itself, regulating the blood supply, the endocardial endothelium is located close to adjacent cardiomyocytes, providing direct signal transmission and communication. Endocardial fragments with cube-shaped endotheliocytes were observed in a large number of tissue samples from both patients with CHD and patients with degenerative AoV stenosis, which in most cases showed a positive response to all tissue factors tested. It should be noted that such changes were not observed in the control group - tissue samples taken from patients with congenital heart disease. Studies show that, for example, in congenital hypertrophic cardiomyopathy, endocardial endothelial hypertrophy develops before myocardial hypertrophy, and increased endothelial cell volume persist throughout the development of hypertrophic cardiomyopathy (Jacques and Bkaily, 2019). In our study, patients with CHD and cube-shaped endocardium had significantly more VEGF-positive

endocardial endothelial cells than patients with CHD and flat endocardial endothelial cells, which could indicate a possible effect of ischemia on endocardial endothelial cell changes similar to ocular retinal capillaries (Hofman et al., 2001). Both genetically programmed and hypoxia-induced VEGF production are also critical for embryonic endocardial endothelial differentiation and proliferation (Dor et al., 2003).

In both the CHD and AoV stenosis groups, endocardial endothelial cells also showed a positive response to ChgA, ET-1, Il-1α, Il-10, βD2, and βD3 in most samples. In the congenital heart disease or control group, only anti-inflammatory cytokine Il-10 and antimicrobial peptides βD2 and βD3 were detected in the right atrial endocardial endothelium.

Studies on vascular endothelium have shown that ChgA inhibits the cytoskeletal reorganization of endothelial cells induced by the proinflammatory cytokine tumor necrosis factor alpha (TNFα) preventing increased vascular permeability (Ferrero et al., 2004). TNFα is an important factor in the pathogenesis of both patients with CHD and patients with degenerative AoV stenosis (Galeone et al., 2013; Carlsson et al., 2018), it causes an excessive inflammatory response, disrupting homeostasis and potentiating heart failure (Schumacher and Naga Prasad, 2018). One of the main mechanisms of action of TNFα is an increase in vascular permeability (Ferrero et al., 2001); therefore, ChgA in the endocardial endothelium could protect the endocardial endothelium from it.

In the heart, ET-1 is secreted mainly by intramyocardial capillary endothelial cells, but ET expression is also found in endocardial endothelial cells, cardiomyocytes, vascular smooth muscle cells, macrophages, etc. (Miyauchi and Sakai, 2019). In our study, both CHD and AoV stenosis patients had more ET-1-positive endocardial endothelial cells. Similar to studies of ET-1 expression in heart failure patients (Fukuchi and Giaid, 1998), in our study, the presence of ET-1 was detected in the right atrial endocardial endothelium in

absolutely all specimens from patients with acquired heart diseases except one specimen from a patient with degenerative AoV stenosis. In contrast to studies on plasma ET-1 (Cody et al., 1993), our study found no correlation between the relative number of ET-1 positive right atrial endocardial endothelial cells and pulmonary hypertension. It should be noted that patients with AoV stenosis who received statins had statistically significantly fewer ET-1-positive endothelial cells in the right atrial endocardium than patients who did not use statins. This finding is supported by the fact that statins inhibit the transcription of the preproendothelin-1 gene in endothelial cells, thereby reducing ET-1 synthesis (Hernández-Perera et al., 2000). In addition, statins not only reduce ET-1 synthesis in endothelial cells, but also inhibit ET-1-induced vasoconstriction (Mraiche et al., 2005).

The above indicates that in case of CHD and degenerative AoV stenosis, activation of right atrial endocardial endothelial cells occurs, thus endocardial endothelium is also a target tissue in the complex treatment of these diseases.

3.2 Innervation

A healthy heart is richly innervated by sympathetic and parasympathetic nerve fibers, which provides electrophysiological stability both at rest and under increased load (Mitchell, 1953). In most of the right atrial tissue specimens of our study, we observed rich innervation – numerous to abundance of PGP 9.5-containing nerve fibers. Although their relative numbers did not differ statistically between groups, some CHD patients had only few to moderate number of PGP 9.5-containing nerve fibers in the right atrial tissue. Based on the literature, this could be related to chronic ischemia, but no significant difference in the relative number of PGP 9.5-containing nerve fibers was observed when comparing patients with and without right coronary artery disease (Dae et al., 1995). It should be noted that in this group we also found a moderate negative correlation between the RVSP and PGP 9.5-containing nerve fibers in the right

atrial tissue, but there was no significant difference in the relative number of PGP 9.5-containing nerve fibers between patients with increased and normal right atrial size.

Cardiac innervation changes increase the risk of malignant arrhythmias and sudden cardiac death, so it is now very important to find visualization methods of cardiac innervation that can be used in clinical practice to predict the risk of arrhythmias and choose the appropriate treatment method (Huang, Boyle and Vaseghi, 2017; Travin, 2017). To evaluate the cardiac innervation visualization methods available in clinical practice, it is important to understand the changes in various heart diseases.

One of the most common arrhythmias with a significant risk of morbidity and mortality is atrial fibrillation (Hindricks et al., 2020). Cardiac innervation changes have been reported in the literature as one of the factors causing and influencing atrial fibrillation, for example, patients with atrial fibrillation have a higher density of sympathetic nerve fibers than patients with sinus rhythm (Nguyen et al., 2009; Scridon, Şerban and Chevalier, 2018). In our study, we did not find differences in PGP 9.5-containing innervation in right atrial tissue between patients with and without atrial fibrillation.

In our study patients with CHD, we observed a statistically significant moderate negative correlation between the relative number of PGP 9.5-containing nerve fibers and the relative number of Il-10, βD2, and βD3-positive cells in the right atrial tissue. Il-10 is a potent anti-inflammatory cytokine, but βD2 and βD3 are antimicrobial peptides, so it is thought that the density of innervation containing PGP 9.5 is influenced by the inflammatory process in the right atrial tissue. In animal studies, local sympathetic denervation has been shown to reduce the infiltration of inflammatory cells (macrophages, neutrophils and T cells) after myocardial infarction, preventing myocardial hypertrophy and maintaining cardiac muscle function (Ziegler et al., 2018).

3.3 Atrial natriuretic peptide (ANUP)

Atrial natriuretic peptide (ANUP) is a well-known marker of heart failure, the blood levels of which correlate with the severity of symptomatic heart failure and are elevated in asymptomatic left ventricular dysfunction (Volpe, Carnovali and Mastromarino, 2016; Cannone et al., 2019). All patients with acquired heart diseases in our study showed signs of congestion - at least a moderate number of ANUP-positive cardiomyocytes in the right atrial tissue. Besides, since in our study, patients with acquired heart diseases had statistically significantly more ANUP-positive cardiomyocytes in the right atrial tissue than the control group or patients with congenital heart disease, we can conclude that patients with congenital heart diseases underwent surgery in a better compensated state.

Since ANUP and brain or type B natriuretic peptide (BNP) in cardiomyocytes are synthesized and secreted under similar conditions – in response to myocardial stretching, (Nakagawa, Nishikimi and Kuwahara, 2019), naturally, we observed a close positive correlation between the relative number of ANUP-positive cardiomyocytes in right atrial tissue and patients' blood BNP levels.

Another important factor that potentiates the release of ANUP is hypoxia (Baertschi et al., 1986). Both hypoxia and mechanical stretching of ANUP are secreted and enhanced by locally produced endothelin. ANUP secretion caused by both hypoxia and mechanical stretching is enhanced by locally produced endothelin (ET-1) (Skvorak, Nazian and Dietz, 1995; Skvorak et al., 1996). In the CHD group, we observed a moderate positive correlation between the relative number of ET-1-positive endothelial cells in the right atrium and the number of ANUP-positive cardiomyocytes. Because ANUP and ET-1 have opposite effects – ANUP causes vasodilation and ET-1 is a potent vasoconstrictor, ANUP provides homeostasis, causing the blood vessels in the heart to dilate and improve the supply of oxygen (Barton and Yanagisawa, 2008; Cannone et al., 2019).

In patients with CHD but not in patients with AoV stenosis, we observed a moderate positive correlation between age and the number of ANUP-positive cardiomyocytes in the right atrium. A similar tendency was observed in healthy subjects and patients with heart failure, but as the changes are not large enough, age-indexing of reference intervals is not used in clinical practice (Clerico et al., 2002; Hogenhuis et al., 2005).

3.4 Vascular endothelial growth factor (VEGF)

VEGF is required in the body for normal physiological processes, where it participates in the maintenance of endothelial function, but altered VEGF secretion is observed in the pathogenesis of various diseases (Laakkonen et al., 2019). The main factor that enhances VEGF secretion in endothelial cells is hypoxia (Namiki et al., 1995). In our study, the relative numbers of VEGF-positive endothelial cells in all study groups were highly variable, ranging from a lack of them to a large number of VEGF-positive endothelial cells, with no statistically significant differences between groups. Thus, VEGF secretion in right atrial endothelial cells is not specific to any of the diseases studied in our study, but varies from patient to patient.

In the CHD group, we found moderate positive correlations between the relative number of VEGF-positive endocardial endothelial cells and the number of IL-10 and βD3-positive endocardial endothelial cells. It is well known that hypoxia causes an inflammatory reaction, but the inflammatory process can also lead to local hypoxia if oxygen availability is exceeded as metabolic activity increases (Ramakrishnan, Anand and Roy, 2014; Takahashi, 2015). In addition, for example, in tumors, VEGF causes Il-10 production, thereby inhibiting the inflammatory process (Shin et al., 2009).

Interestingly, in patients with AoV stenosis and a higher relative number of VEGF-positive endothelial cells in the right atrial blood vessels, we observed a higher right atrial cardiomyocyte apoptosis index. The opposite was observed in a similar animal model - in myocardial hypertrophy, VEGF protects against cardiomyocyte apoptosis maintaining cardiac contractile function (Friehs et al., 2006). Other studies have shown that VEGF protects endothelial cells from apoptosis and promotes angiogenesis, as well as protects against ischemia/reperfusion injury by inhibiting cardiomyocyte apoptosis (Mabeta, 2013; Chen et al., 2016).

3.5 Chromogranin A (ChgA)

ChgA is a marker of the neuroendocrine system that is elevated in neuroendocrine tumors and other diseases, including heart failure and hypertension (Mahata and Corti, 2019). In heart failure, activation of the neuroendocrine system is a protective mechanism that causes vasoconstriction by maintaining blood pressure and helps maintain cardiac output by increasing myocardial contractile force and rate (Kjær and Hesse, 2001). Initially, such changes help to maintain the body's vital functions, but in the long run cause decompensation. In our study, there were statistically significantly more ChgA-positive cells in the control group - in patients with early-operated congenital heart disease. ChgA-positive cells in right atrial tissue were also found in a large number of patients with acquired heart diseases, but their relative number was very variable and no significant difference was found between the CHD and AoV stenosis groups.

In the heart, ChgA is stored with natriuretic peptides and, like elevated levels of natriuretic peptides in the blood, elevated levels of ChgA are associated with increased mortality and re-hospitalizations due to decompensation of heart failure and myocardial infarction (Goetze et al., 2013). It should be noted that in

our study, no statistically significant correlations were observed between natriuretic peptides, left ventricular ejection fraction, and relative number of ChgA-positive cells in right atrial tissue.

Elevated blood ChgA levels are also associated with high mortality in critically ill intensive care and septic patients (Zhang et al., 2009; Hsu et al., 2015). Although one of the end products of ChgA cleavage has anti-inflammatory activity, the other biologically active peptides obtained by cleavage of ChgA have inflammation-promoting effects (Muntjewerff et al., 2018). In the CHD group, we observed a moderate positive correlation between the relative number of ChgA-positive cells and the relative number of inflammatory cytokine IL-1α positive cells, and between the relative number of ChgA-positive cells and the relative number of anti-inflammatory cytokine IL- 10 positive cells. In addition, there was a moderate positive correlation in this group between the relative number of ChgA and beta defensin-positive cells. Thus, we can conclude that patients with CHD and a more pronounced anti-inflammatory process have a more pronounced activation of the neuroendocrine system.

Another tendency observed in patients with CHD was a moderate positive correlation between the relative number of VEGF-positive and ChgA-positive endocardial endothelial cells. The ChgA fragment vasostatin may inhibit TNF α- and VEGF-induced changes in endothelial cell shape and barrier function, preventing an increase in vascular permeability, which is important in tumor pathogenesis and therapeutic efficacy (Ferrero et al., 2004). Elevated levels of ChgA in the blood cause uneven delivery and penetration of drugs in tumors, thereby limiting the response to chemotherapy (Loh et al., 2012).

In the AoV stenosis group, we found a moderate negative correlation between the relative number of cardiomyocytes affected by apoptosis and the relative number of ChgA-positive cells in the right atrial tissue. Although ChgA may stimulate various cell apoptosis, such as prostate carcinoma cell apoptosis

or microglia and neuronal apoptosis in Alzheimer's disease (Kingham and Pocock, 2000; Yu, Hsieh and Chang, 2003), other studies have shown that ChgA protects cardiomyocytes from the chemotherapy drug doxorubicin-induced cardiomyocyte apoptosis (Rocca et al., 2019), but the ChgA fragment catestatin protects the hypertrophied myocardium from ischemia/reperfusion-induced cardiomyocyte apoptosis (Penna et al., 2014). Based on the results of our study and the data available in the literature on the effect of ChgA on cardiomyocyte apoptosis, we can conclude that ChgA and its derivatives have a cardioprotective effect.

3.6 Endothelin 1 (ET-1)

Increased production of the potent vasoconstrictor ET-1 is a characteristic feature of endothelial dysfunction, whereas endothelial dysfunction is a key mechanism in the pathogenesis of atherosclerosis as well as degenerative AoV stenosis (Akahori et al., 2018; Persic et al., 2018; Miyauchi and Sakai, 2019). In our study, we observed a variable relative number of ET-1-positive endothelial cells in the CHD and AoV stenosis groups, but although the difference was not statistically significant, there were fewer ET-1-positive endothelial cells in the control group. ET-1 acts paracrine on cardiomyocytes by immediately increasing vascular smooth muscle and myocardial contractility, in the long run leading to cardiomyocyte damage and the progression of heart failure (Meyer et al., 1996; Miyauchi and Sakai, 2019). Interestingly, in the right atrium, ET-1 first causes a transient negative inotropic effect, followed by a prolonged positive inotropic reaction (Dhein et al., 2000). Another significant effect of ET-1 on the myocardium is excessive cardiomyocyte hypertrophy (Ito et al., 1994). Thus, increased production of ET-1 initially helps to maintain important vital functions, but in the long run causes decompensation.

In patients with AoV stenosis, we observed a strong positive correlation between the relative number of ET-1-positive and VEGF-positive right ventricular endocardial endothelial cells. VEGF and ET-1 stimulate each other's secretion, but inhibition of VEGF in the treatment of tumors leads to an increase in ET-1 levels and arterial hypertension (Matsuura et al., 1998; Lankhorst, Danser and Meiracker, 2016). In addition, pronounced vasoconstriction induced by ET-1 in myocardial blood vessels may reduce oxygen supply, followed by increased VEGF production. Hypoxia may also lead to increased production of ET-1 through the hipoxia-inducible factor (Heyman, Khamaisi and Abassi, 2018).

Today, no one has any doubt about the role of ET-1 in the pathogenesis of pulmonary hypertension (Shao, Park and Wort, 2011), so it is interesting that in this study we observed the opposite tendency – a statistically significant moderate negative correlation between the relative number of ET-1 positive endothelial cells in right atrial tissue in patients with increased right ventricular systolic pressure. A similar situation has been observed previously – patients with primary pulmonary hypertension had many ET-1-positive cells in the pulmonary vessels, but little or no in myocardium (Giaid et al., 1993). In addition, in the same study, patients with primary pulmonary hypertension had more ET-1-positive cells in their pulmonary vessels than patients with secondary pulmonary hypertension, and their number correlated with the severity of the disease. Thus, ET-1 production in right atrial tissue is not conclusively associated with pulmonary hypertension but with the underlying disease.

Several experimental studies have also linked ET-1 to inflammatory processes, such as more severe encephalomyelitis and higher levels of inflammatory cytokines interleukins 6 and 17, interferon γ and TNFα in mice with higher ET-1 levels (Guo et al., 2014). In contrast, in patients with multiple sclerosis, no correlation was observed between ET-1 levels and plasma cytokine levels (interleukins 1β, 2, 4, 5, 6, 10, 12, 13 and TNFα) (Rocha et al., 2019).

Therefore, there is currently no convincing evidence for the association of ET-1 with inflammatory processes. In our study, patients with CHD showed a moderate positive correlation between the number of ET-1 and βD3-positive cells, but in the AoV stenosis group there was a strong positive correlation between the relative number of ET-1 and Il-10-positive cells in right atrial tissue. It should be noted that Il-10 protects blood vessels from ET-1-induced vasoconstriction (Giachini et al., 2009).

In the case of CHD and AoV stenosis, ET-1 production is more characteristic of right atrial endocardial endothelial cells and is not specific for any of the diseases studied.

3.7 Inflammation

Inflammation is a protective response to tissue damage but an incorrect, excessive or persistent inflammatory response damages healthy tissues and is involved in the pathogenesis of cardiovascular diseases, so preventing inflammation is one of the key strategies both to reduce the impact of risk factors and to treat an existing disease. To make it possible, all factors involved in the pathogenesis must be fully understood. In this study, we identified inflammatory cytokine interleukin 1α, anti-inflammatory cytokine interleukin 10, and antimicrobial peptides beta defensin 2, 3, and 4 in right atrial tissue in patients with CHD, degenerative AoV stenosis, and congenital heart disease (control group).

3.7.1 Infiltration of inflammatory cells

Focal inflammatory cell infiltration was observed in the right atrial epicardium in three patients with CHD and one patient with degenerative AoV stenosis. In 2016, Andersen et al. published a study in which 60% of patients with CHD and no other known inflammatory diseases had focal infiltration of

inflammatory cells in right atrial tissue taken during coronary artery bypass graft surgery (Andersen et al., 2016). Similar to this study in our study, although the difference was not statistically significant, patients with inflammatory cell infiltration in the right atrial epicardium were on average nine years younger than patients without inflammatory cell infiltration. Due to the lack of data in the literature on epicardial infiltration of inflammatory cells, it is difficult to judge its clinical significance and association with the pathogenesis of CHD or degenerative AoV stenosis. Infiltration of inflammatory cells in younger patients could indicate a more aggressive form of the disease, as coronary artery bypass graft surgery was required at an earlier age.

Other studies have shown that inflammatory cell infiltration into the ventricular myocardium is common in patients with dilated and hypertrophic cardiomyopathy, aortic valve stenosis, after myocardial infarction, but not in patients with physiological left ventricular hypertrophy (Hofmann et al., 2012; Laroumanie et al., 2014; Patel et al., 2018). A recent animal study identified inflammatory cells infiltrating cardiac tissue after partial aortic ligation (model corresponds to AoV stenosis) and found that myocardium was infiltrated by macrophages, CD8 + and CD4 + T cells, B cells, natural killers, mast cells, neutrophils and regulatory T cells, but only mast cell infiltration was observed in the epicardium (Martini et al., 2019). In addition, in a similar animal model of abnormal left ventricular hypertrophy, suppression of T cell activation both early and at a later stage when the disease had already progressed resulted in an increase in anti-inflammatory cytokine IL-10 levels and significantly reduced the progression of heart failure (Kallikourdis et al., 2017). Although no focal inflammatory cell infiltration in the myocardium were observed in the tissue samples of our study, it should be noted that samples with inflammatory cell infiltration in the epicardium had statistically significantly more Il-10 positive endothelial cells in the right atrial blood vessels than in samples without inflammatory cell infiltration.

3.7.2 Interleukin1α

The cytokine Il-1α is an important element in inflammatory processes, but relatively little is known about its expression in human heart tissue. In cases of cardiovascular disease has been studied the therapeutic effect of its blockade (Szekely and Arbel, 2018). Il-1α is known to be found in atherosclerotic plaques, but we do not find conclusive data in the literature on the presence of Il-1α in degenerative AoV leaflets (Jiang et al., 2019). In our study, in all patient groups, approximately one-third of patients in the right atrial tissue samples did not detect Il-1α-positive cells at all, while the rest showed occasional Il-1α-positive connective tissue cells and few to moderate number of Il-1α-positive endocardial endothelial cells. The amount of Il-1α in steady-state cells varies depending on its type, but in cells that form barriers in the body, such as epithelial and endothelial cells, the amount of Il-1α is significantly higher (di Paolo and Shayakhmetov, 2016). We also observed more Il-1α-positive endocardial endothelial cells than Il-1α-positive connective tissue cells in the right atrial tissue samples of our study.

In several studies, Il-1α mRNA was found neither in healthy nor in dilated cardiomyopathy and viral inflammation affected adult heart muscle cells, but in animal studies, Il-1α was also found in cardiomyocytes (Han et al., 1991; Westphal et al., 2007). After myocardial infarction, an increased amount of Il-1α is observed in the necrosis zone of cardiomyocytes, where it initiates a sterile inflammatory process, thus its blockage reduces infarct size as well as protects left ventricular function (Timmers et al., 2008; Lugrin et al., 2015; Mauro et al., 2017).

In our study in the AoV stenosis group, we found a moderate negative correlation between the relative number of IL-1α positive right atrial endocardial endothelial cells and right ventricular systolic pressure (RVSP). RVSP reflects the degree of pulmonary hypertension and increases with decompensation of

AoV stenosis, therefore patients with increased RVSP have a higher risk of surgical treatment of AoV stenosis (Lancellotti et al., 2012; Cavender and Kolarczyk, 2019). Increased levels of inflammatory cytokines in the blood have been observed in patients with pulmonary arterial hypertension, and higher levels of Il-1α are associated with increased mortality (Cracowski et al., 2014; Rabinovitch et al., 2014). As no such association was observed in the CHD group and there were few patients with increased RVSP in both groups of acquired heart diseases, additional studies with a larger number of patients may be required to investigate the effect of increased RVSP on IL-1α expression in right atrial tissue in patients with decompensated AoV stenosis.

3.7.3 Interleukin 10

In our study, there were significantly more Il-10-positive cardiomyocytes and endocardial endothelial cells in the right atrial tissue in the CHD group, but comparing the two groups of acquired heart diseases with the control group, the relative numbers of all examined IL-10 positive cell groups were different. This indicates that increased IL-10 production in right atrial tissue is more common in patients with CHD.

Il-10 is the main anti-inflammatory cytokine that protects the body against an excessive inflammatory response, mainly by inhibiting the production of inflammatory cytokines (Saraiva et al., 2020). In animal models, deletion of the Il-10 gene increases inflammatory cytokine levels in the myocardium, as well as increases infarct size and mortality (Yang, Zingarelli and Szabó, 2000; Meador et al., 2008). However, Il-1α-induced production of other inflammatory cytokines, such as interleukin 1β, TNF α and interleukin 6, in cardiac myofibroblasts is not affected by Il-10 (Turner et al., 2009). In the right atrial tissue samples of our study, there were significantly more Il-10 positive cells

than Il-1α positive cells, indicating the superiority of anti-inflammatory processes.

However, the effect of Il-10 is ambiguous, so the negative effect of Il-10 on the myocardium should be mentioned - Il-10 promotes the production of osteopontin and growth factor beta in cardiac macrophages, which activates collagen synthesis in fibroblasts, leading to fibrosis and diastolic failure (Hulsmans et al., 2018). In this study, we did not evaluate the parameters of diastolic dysfunction, but it should be noted that we found a moderate positive correlation between the relative amount of IL-10 positive endothelial cells and right ventricular systolic pressure, and a moderate negative correlation between the relative number of IL-10 positive endocardial endothelial cells and left ventricular ejection fraction. This means more Il-10 positive endothelial cells in patients with a worse preoperative condition: lower left ventricular ejection fraction and higher pulmonary pressure. Although in a study of left ventricular remodelling in patients after myocardial infarction, blood IL-10 levels did not correlate with changes in ejection fraction, a significant negative correlation was found between IL-10 levels and left ventricular end-diastolic diameter 30 days after myocardial infarction (Zarrouk-Mahjoub et al., 2016). This suggests a positive association of IL-10 with left ventricular regeneration after myocardial infarction. In another study, patients with systolic heart failure and elevated blood levels of IL-10 had higher mortality (Amir et al., 2010).

Age is an independent risk factor for the pathogenesis of both CHD and degenerative AoV stenosis (Baumgartner et al., 2017; Knuuti et al., 2019). In the CHD group of our study, we observed a moderate positive correlation between the relative number of IL-10 positive cardiomyocytes and the age of the patients. Studies on the effect of age on Il-10 production are contradictory (Kelly et al., 2007; Zhang et al., 2015), but Il-10 is known to protect blood vessels from age-induced endothelial dysfunction, which is one of the main elements of

pathogenesis in the development of CHD (Kinzenbaw et al., 2013; Rajendran et al., 2013).

Another important risk factor for cardiovascular disease is altered cholesterol metabolism. Although elevated triglycerides are less associated with the risk of heart disease than hypercholesterolemia, they are an important independent risk factor for cardiovascular disease(Piepoli et al., 2016). In this study, we found a moderate negative correlation between the relative number of Il-10 positive cardiomyocytes in the right atrial tissue and triglyceride levels. No other significant association was found between the relative number of Il-10 positive cells and other cholesterol fractions. Although Il-10 has no significant effect on circulating cholesterol levels, it does affect the metabolism of cholesterol in macrophages by stimulating the uptake of cholesterol from lipoproteins as well as the removal of cholesterol from macrophages (Han et al., 2010). However, excessive levels of Il-10, as in some lymphoproliferative diseases, can lead to a decrease in high and low density cholesterol and an increase in triglycerides (Moraitis et al., 2015; Lucero et al., 2020).

In this study, we found a moderate statistically significant negative correlation between the relative number of IL-10 positive endothelial cells in the right atrial blood vessels and plasma C-reactive protein (CRP) levels. CRP is an acute phase protein that increases in response to inflammation (Pepys and Hirschfield, 2003). A similar situation - decreased IL-10 levels and increased CRP levels - was observed in patients with acute coronary syndrome and heart failure (Stumpf et al., 2003; Van Haelst et al., 2004). This finding is explained by the fact that CRP reduces the secretion of IL-10 mRNA, intracellular Il-10 and Il-10 in macrophages by inhibiting adenylate cyclase activity (Singh et al., 2006).

3.8 Beta defensins

The most striking finding in our study was the rich expression of antimicrobial peptides, such as human β defensins 2 and 3, in the right atrial tissues from all specimens.

Beta defensins are antimicrobial peptides that are part of the defence mechanism against bacterial infections. The production of βD2 and βD3 in cells can be stimulated by bacterial infection and inflammatory cytokines such as Il-1α, Il-1β, TNFα, interferon γ, and others (Harder et al., 1997, 2001; Hiratsuka et al., 1998; Singh et al., 1998; O'Neil et al., 1999; Liu et al., 2002; Pazgier et al., 2006). In addition, for example, the concentration of βD2 depends on the stage of infection – in the active phase there is a high concentration of βD2, which gradually normalizes during the recovery process (Yanagi et al., 2007). There are several studies in the literature on the role of bacteria and viruses both in the process of atherosclerotic plaque formation and its instability (McKechnie and Rubenfire, 2002). Although bacteria and viruses have been found in the walls of blood vessels affected by atherosclerosis, there is no direct evidence that the infection causes atherosclerosis, but an inflammatory response to a chronic infection could play a role (Kuo et al., 1993; Chiu et al., 1997). This is also evidenced by several studies that have shown an association of an increased inflammatory acute phase marker CRO with cardiovascular events (Koenig et al., 1999; Kaptoge et al., 2012). In addition, patients with periodontal infection or elevated antibody levels to various chronic infections, such as herpes simplex virus type 1 and Chlamydia pneumoniae, are at increased risk of developing CHD (Roivainen et al., 2000; Dietrich et al., 2008). It should be noted that, similar to atherosclerosis, Chlamydia pneumoniae has been found in the leaflets of stenotic aortic valves, and degenerative AoV stenosis is more common and more severe in patients with a high antibody levels to Chlamydia pneumoniae (Pierri et al., 2006; Turgeman et al., 2006). The possible role of infection in the

development of CHD and degenerative AoV stenosis could also be indicated by the rich βD2- and βD3-positive cell finding in all samples of our study and the fact that we found a statistically significant moderate positive correlation between the relative number of βD3-positive cardiomyocytes and plasma CRO levels. In the CHD group, we observed a moderate positive correlation between the relative number of Il-1α-positive endocardial endothelial cells and βD3-positive cardiomyocytes and connective tissue cells in right atrial tissue. This shows the inflammatory process locally in the right atrial tissue and is probably related to the ability of Il-1α to stimulate cells to produce beta defensins.

It should be noted that βD2 induces the production of the inflammatory cytokine interleukin 6 and chemokine 8, as well as the anti-inflammatory cytokine Il-10 (Boniotto et al., 2006). In our study, we observed a moderate and strong correlation between Il-10-positive connective tissue cells and the relative numbers of βD2 and βD3 positive cardiomyocytes and connective tissue in right atrial tissue.

Interestingly, in all study groups, we observed a statistically significant moderate correlation between the number of βD2-positive cells and the age of patients, but we did not observe such association with the number of βD3-positive cells. This could suggest an effect of age in patients with CHD and degenerative AoV stenosis on the ability of cells to produce βD2 but not βD3. Observations in older healthy subjects suggest that the ability of cells to produce βD2 is similar to that in younger subjects, but, for example, reduced levels of βD3 have been observed in aging skin (Castañeda-Delgado et al., 2013; Pilkington et al., 2018).

Another risk factor for cardiovascular disease is dyslipidemia (Mach et al., 2020). In both patients with CHD and patients with degenerative AoV stenosis, we observed a moderate or strong negative correlation between the number of βD2- and βD3-positive cells in the right atrial tissue and total or low-density cholesterol (LDL) plasma levels. A similar tendency was observed

between alpha defensins and total and low density cholesterol plasma levels (López-Bermejo et al., 2007). One of the explanatory mechanisms could be the ability of alpha defensins to bind to LDL receptors, thus competing with and inhibiting LDL binding (Higazi et al., 2000). We do not find such data on beta defensins in the literature, therefore additional studies are needed to evaluate the relationship between beta defensins and cholesterol fractions.

In this study, we found a statistically significant strong correlation between the number of βD2- and βD3-positive cardiomyocytes and brain natriuretic peptide (BNP) levels in the CHD group, as well as a significant moderate correlation between the relative number of βD2-positive endothelial cells and the relative number of ANUP-positive cardiomyocytes. BNP and ANUP are peptides produced by cardiomyocytes in response to increased mechanical load and wall stretch (Nakagawa, Nishikimi and Kuwahara, 2019). In clinical practice, plasma BNP levels are used to assess the decompensation of heart failure and the effectiveness of the therapy used (Ponikowski et al., 2016).

An indicator that directly shows increased pressure in the right ventricle and reflects pressure in the pulmonary arteries is the right ventricular systolic pressure (RVSP) (Thibodeau and Drazner, 2018). Interestingly, in patients with CHD, we observed a statistically significant moderate positive correlation between RVSP and the relative numbers of IL-10, βD2 and βD3 positive cells and a moderate negative correlation between RVSP and PGP 9.5-containing nerve fibers and VEGF positive endothelial cells in right atrial tissue. In turn, in the group of AoV stenosis we observed the following tendency – moderate negative correlation between RVSP and the relative number of Il-1α and βD2 positive cells in the right atrial tissue. Increased right ventricular pressure in patients with AoV stenosis is usually secondary and is associated with the decompensation of left side (Généreux et al., 2017), therefore, morphological changes in right atrial tissue are likely to be associated with increased pressure. In contrast, in the case of CHD, in the absence of left-side failure, pulmonary

hypertension can have various causes, most often it is primary (Shimony et al., 2011), therefore, changes in right atrial morphology may be associated with both the pathogenesis of CHD and the pathogenesis of pulmonary hypertension. Thus, in patients with CHD and signs of pulmonary hypertension, right atrial tissues are characterized by higher production of anti-inflammatory cytokine II-10 and antimicrobial peptides, but reduced innervation and VEGF production.

In conclusion, right atrial tissue in both CHD and degenerative AoV stenosis is characterized by non-specific degenerative morphological changes – pronounced vacuolization as well as changes in the shape and size of cardiomyocytes and their nuclei. In addition, these patients have a high proportion of apoptotic cardiomyocytes. Although there were no significant lesions in the coronary arteries in patients with AoV stenosis, connective tissue ingrowth and vascular sclerosis were observed in some patients in both groups.

In the case of CHD and degenerative AoV stenosis, activation of the right atrial endocardial endothelial cells occurs, characterized by a change of shape from flat to cubic and rich release of ChgA, ET-1, Il-1α, Il-10, βD2 and βD3.

Patients with CHD and AoV stenosis in the right atrial tissue had statistically significant higher numbers of ANUP-positive cardiomyocytes, all types of IL-10 positive cells and βD2 and βD3-positive endocardial endothelial cells, but fewer ChgA-positive cells than controls or patients with congenital heart disease. Thus, in both cases of acquired heart disease, an anti-inflammatory response prevails in the right atrial tissue, but increased activity of the neuroendocrine system is more common in patients with congenital heart disease at an early age.

Although some tendencies were observed, for example, in the CHD group, there were slightly more VEGF, ET-1, Il-1α positive endocardial endothelial cells, Il-10 positive cardiomyocytes, connective tissue and endothelial cells, but in AoV stenosis group, there were slightly more ChgA-

positive endocardial endothelial cells, however, these differences did not reach statistical significance.

The most striking finding in our study was the rich expression of antimicrobial peptides, such as human β defensins 2 and 3, in the right atrial tissues in patients with CHD, degenerative AoV stenosis, and congenital heart disease or in the control group.

Conclusions

1. In patients with both CHD and AoV stenosis, the right atrial tissues are characterized by non-specific morphological changes: nuclear shape change and vacuolization, which correlate with the presence of apoptosis in cardiomyocytes. In turn, focal inflammatory cell infiltration is characteristic of young patients with acquired heart diseases, and their correlation with IL- 10 release is an example of an intensifying anti-inflammatory response in this group of patients.
2. In patients with both CHD and AoV stenosis, right atrial endocardial endotheliocytes change shape from flat to cubic and abundantly secrete ChgA, ET-1, Il-1α, Il-10, βD2 and βD3, indicating plasticity of these cells under uneven ischemic conditions (VEGF variable increase).
3. Right atrial tissue in patients with both CHD and AoV stenosis are generally characterized by abundant innervation of neuropeptide-containing nerve fibers. Its decrease in CHD patients with more pronounced production of anti-inflammatory cytokine II-10 and antimicrobial peptides βD2 and βD3 indicates compensatory blockade of nerve fiber formation under inflammatory conditions.
4. CHD patients with increased ET-1 production in the right atrial tissue are also characterized by increased ANUP secretion, which provides protection of the myocardium against the adverse effects of ET-1.
5. In the case of acquired heart disease, the amount of VEGF positive structures in the right atrial tissue varies individually. In patients with AoV stenosis, the increased number of VEGF-positive cells correlates with the intensification of apoptosis, whereas in CHD patients, VEGF-maintained ischemia stimulates the production of local anti-inflammatory factors IL-10 and βD3.

6. Increased ChgA production in the right atrial tissue and, consequently, the activity of the neuroendocrine system is more common in patients with congenital heart disease at an early age. In the case of acquired heart diseases, the variable relative number of ChgA-positive cells correlates mainly with IL-10, βD2 and βD3, indicating the stimulation of the anti-inflammatory response promoted by ischemia (correlation with the relative number of VEGF-containing cells), but the negative correlation with apoptosis justifies the cardioprotective role of the factor.
7. The amount of ET-1-containing cells in patients with CHD and AoV stenosis is individually variable, but higher than in patients with congenital heart disease. ET-1 is secreted mainly by endocardial endotheliocytes, indicating a more intense involvement of these cells in factor expression.
8. The right atrial tissue of patients with acquired heart diseases is characterized by a small presence of Il-1α and more pronounced presence of Il-10-containing cells, indicating a correct inflammatory / anti-inflammatory cytokine ratio, especially in CHD patients.
9. Increased numbers of βD2- and βD3-containing cells are observed in the right atrial tissue of patients with acquired heart disease, which correlates with increased IL-10 and CRO levels, indicating that the entire local defense system is intensified and is associated with an inflammatory response. Aging has a selective ability to stimulate an increase the number of βD2-containing cells, emphasizing the importance of degenerative processes in the release of certain defensins. Increased RVSP is an additional factor that promotes the activation of the local antimicrobial system.

List of publications

Scientific publications included in international data databases (Web of Science, SCOPUS, ERIH PLUS)

1. **Edīte Vārtiņa**, Māra Pilmane, Romans Lācis. Homeostasis Regulating Factors, Innervation, Ischemia and Inflammatory Markers in the Right Atrial Tissue from Patients with Degenerative Aortic Valve Stenosis and Coronary Heart Disease. *Proceedings of the Latvian Academy of Sciences. Section B*. 2021; 75(3): 186.–193. doi: 10.2478/prolas-2021-0028.d
2. **Edīte Vārtiņa**, Māra Pilmane, Romans Lācis. Inflammatory cytokines and antimicrobial peptides in acquired heart diseases. *Histol Histopathol.* 2019 Aug;34(8):889–897. doi: 10.14670/HH-18-091.

Scientific articles in peer-reviewed local journals

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2. **Edīte Vārtiņa**, Māra Pilmane, Romans Lācis. Common and Different Homeostasis Regulating Factors, Innervation, Ischemia and Inflammatory Markers in the Right Atrial Tissue from Patients with Degenerative Aortic Valve Stenosis and Coronary Heart Disease. Rīga Stradiņš University International Conference on Medical and Health Care Sciences. *Abstracts*, 2019: 581. (Poster presentation).
3. **Edīte Vārtiņa**, Māra Pilmane, Emīls Šmitiņš, Romans Lācis. Homeostasis Regulating Factors, Innervation and Ischemia Markers in the Right Atrial Tissue from Different Acquired and Congenital Heart Diseases. 9th Baltic Morphology Conference, Tartu, Estonia. *Abstracts*, 2017: 93. (Oral presentation).
4. **Edīte Vārtiņa**, Māra Pilmane, Romans Lācis. Distribution of PGP 9.5 Immunoreactive Nerves in Right Atrial Tissue from Patients with Coronary Heart Disease. 26th Nordic-Baltic Congress of Cardiology, 2017, Vilnius, Lithuania. *Medicina*, 2017; 53(1):67 (Poster presentation).

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6. **Edīte Kulmane**, Māra Pilmane, Romans Lācis. Antiinflammatory Cytokines and Antimicrobial Peptides in Acquired Heart Diseases. 8th Baltic Morphology Scientific Conference, 2015. Vilnius, Lithuania. *Abstracts*, 2015: 53. (Best PhD Student Oral Presentation Award).

7. **Edīte Kulmane**, Māra Pilmane, Romans Lācis. Right Atrial Tissue Morphology in Acquired Heart Diseases. International Conference on Microscopic and Macroscopic Anatomy, Barcelona, Spain. *Abstracts*, 2015: 1335. (Oral presentation).

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2. **Edīte Vārtiņa**, Māra Pilmane, Romans Lācis. PGP 9.5 saturošu nervu šķiedru izplatība labā priekškambara audos koronāras sirds slimības pacientiem. Rīga Stradiņš University Conference on Medical and Health Care Sciences. *Abstracts*, 2018:149. (Oral presentation).

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