



Juris Hofmanis

**INFLAMMATORY AND  
NON-INFLAMMATORY  
RISK FACTORS IN ACQUIRED  
AORTIC VALVE STENOSIS**

Doctoral Thesis  
for obtaining the degree of a Doctor of Medicine  
Specialty - Internal Medicine

Riga, 2019

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## ANNOTATION

Calcific stenosis of the aortic valve (AVS) manifests with fibro-calcific remodeling (transformation) of the aortic valve (AV), which is a slow process of chronic inflammation and calcification with completely unexplored and ambiguous etiology and pathogenesis.

Currently, there is no medical treatment to stop or delay the progression of the disease. The only treatment available is a surgical replacement of AV or transcatheter aortic valve implantation. With increasing people's survival the number of patients with clinically relevant AVS is increasing.

Almost in 25% of people after the age of 65 echocardiographically AV sclerosis is found and about 17% of these people further develop AVS. The time between diagnosis of AV sclerosis and development of severe AVS is on average 6–8 years.

The aim of the research work is to analyze and find out which factors involved in the inflammation and calcification process, cell-produced regulatory molecules (cytokines) affect AVS development in all three AVS severity degrees and to what extent. As a result, some biomarkers could be isolated to predict the rate of progression of AVS. Cell-produced regulatory molecules (cytokines) were detected in blood serum and plasma (thioredoxin reductase-1, myeloperoxidase).

The interaction of oxidative stress and inflammation in all three AVS severity degrees was analyzed during the research work. The direct and indirect effects of total cholesterol and its fractions on the development of AVS were also re-evaluated.

The conducted clinical-analytical study is a prospective case-control study of mixed type. 102 patients were selected voluntarily according to inclusion and exclusion criteria and divided into two basic groups: the control group and the AVS group. Individuals in the control group were included according to an echocardiographically approved healthy aortic valve between the age of 50 to 80 years, which corresponds to the AVS patients' age at the 2012 European Society of Cardiology and the European Association for Cardio-Thoracic Surgery Guidelines for the management of valvular heart disease. Patients of the AVS group were divided into three subgroups according to the degree of the AV stenosis, based on the 2012 European Society of Cardiology and the European Association for Cardio-Thoracic Surgery Guidelines for the Management of Valvular Heart Disease.

In the study for the first time in the Latvia population, inflammatory and non-inflammatory factors, cell-produced regulatory molecules (cytokines) in blood serum and plasma were analyzed. The obtained results were compared between the control group and all three AVS grades.

Knowing recent studies on etiopathogenesis of the calcific AVS, inflammatory and non-inflammatory factors (chemerin, FGF-21, TrxR1 and MPO) that have not been studied in AVS patients so far were identified.

The obtained results provide more complete information on the pathogenesis of AVS and the correlation between the analyzed inflammatory and non-inflammatory factors. The relationship between chemerin and TrxR1 shows that inflammation and oxidative stress are already present in the mild AVS grade. The results obtained in the study allow to suggest that chemerin in AVS patients promotes inflammation. Chemerin can be used AVS a good diagnostic marker for the mild AVS. Hereafter, a follow-up program for the AV sclerosis patients should be developed and chemerin should be determined in dynamics to find out whether this biomarker can predict the development of AVS.

Analyzing level of FGF-21 in AVS severity grades and its correlation with other factors, it can be concluded that it has anti-inflammatory activity at the beginning of AVS development. FGF-21 role in the subsequent AVS severity grades cannot be unequivocally define AVS it may be associated with both progressive calcification process and counter-action to myocardial hypertrophy.

When analyzing MMP and TIMP, we obtained the result of MMP-1 that suggests the role of genetic polymorphism in the progression process of AVS. In the future, it would be useful to study MMP-1 polymorphism to clarify whether 1G allele carriers have slower AVS progression.

Analysis of the MPO level and its association with HDL-C shows that HDL-C is indirectly associated with the AVS process, AVS ox-LDL-C levels increase under oxidative stress conditions, resulting in HDL-C dysfunction and formation of ox-HDL-C. The results of MPO and TrxR1 suggest that oxidative stress and progressive calcification are prevalent in moderate to severe AVS.

The conducted study shows that AVS is an active process in all degrees of severity regulated by inflammation, oxidative stress, and extracellular remodeling and progressive calcification which depends on them. The results of the study suggest that special attention should be paid to the patients with AV sclerosis and mild AVS, their dynamic monitoring (echocardiography, analysis) should be done to find other biomarkers that can predict disease progression.

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## ABBREVIATIONS USED IN THE WORK

5'-NT	5'-nucleotidase
5-LO	5-lipoxygenase
A2CH	apical two-chamber view
A4CH	apical four-chamber view
A5CH	apical five-chamber view
AA	arachidonic acid
ABI	ankle-brachial index
ACE	angiotensin converting enzyme
ACS	acute coronary syndrome
ALP	alkaline phosphatase
Ang I	angiotensin I
Ang II	angiotensin II
apoA-1	apolipoprotein A-1
apoB	apolipoprotein B
AVS	aortic valve stenosis
ASK1	apoptosis signal-regulating kinase 1
ASV	United States of America
ATP	adenosine triphosphate
ATX	autotaxin
AUC	area under the ROC curve
AV	aortic valve
bFGF	basic fibroblast growth factor
BH4	tetrahydrobiopterin
BMI	body mass index
BPM-2	bone morphogenetic protein
ChemR23	hemerin receptor 23
Cl <sup>-</sup>	chlorine
cMGP	carboxylated matrix Gla-protein form
COX2	cyclooxygenase 2
CRP	C-reactive protein
CVD	cardiovascular diseases
DNA	deoxyribonucleic acid

EF	ejection fraction
Ehokg	echocardiography
EIDL3	Discoidin I-Like Domains 3
ESR	erythrocyte sedimentation rate
FGF-21	fibroblast growth factor-21
FGFR1c	fibroblast growth factor receptor1c
GPR1	G- protein-bound receptor 1
H <sub>2</sub> O <sup>2</sup>	hydrogen peroxide
HDL-C	high density lipoprotein cholesterol
HOCl	hypochlorous acid
hs-CRP	highly sensitive C-reactive protein
IGF1	insulin-like growth factor 1
IL-1 $\beta$	interleukin-1 $\beta$
IL-2	interleukin-2
IL-6	interleukin-6
IL-8	interleukin-8
IMT	intima-media thickness
KLB	cofactor $\beta$ -Klotho
LDL	low-density lipoprotein
Lp(a)	lipoprotein a
Lp-PLA-2	Lp-linked phospholipase A2
LRP5	protein 5
LV	left ventricle
LV EDV	left ventricular end-diastole volume
LV ESV	left ventricular end-systole volume
LVOT	Left ventricle outflow tract
LysoPA	lysophosphatidic acid
LysoPC	lysophosphatidylcholine
MGP	matrix Gla-protein
MI	myocardial infarct
MMP	matrix metalloproteinase
MMP-1,2,3,7,9,11	matrix metaloproteinases-1,2,3,7,9,11
MnSOD	mitochondrial superoxide dismutases
MPO	myeloperoxidase

MV	mitral valve
NADPH oxidase	nicotinamide adenine dinucleotide phosphate oxidase
NF- $\kappa$ B	nuclear factor kappa $\beta$
NO	nitrogen oxide
NOS	nitrogen oxide synthase
NPP1	ectonucleotide pyrophosphatase / phosphodiesterase 1
NPV	negative prognostic value
O <sub>2</sub>	oxygen
O <sub>2</sub> <sup>-</sup>	superoxide
ONOO <sup>-</sup>	peroxynitrite
OPG	osteoprotegerin
ox-HDL-C	oxidized high density lipoprotein cholesterol
ox-LDL	oxidized low density lipoprotein
ox-LDL-C	oxidized low density lipoprotein cholesterol
ox-PLs	oxidized phospholipid
P2Y2	purine receptors
PAD	peripheral arterial disease
PAR1	protease activated receptor-1
PG	pressure gradient
PON-1	paraoxonase-1
PPAR $\alpha$	peroxisome proliferator-activated receptor $\alpha$
PPV	positive prognostic value
Pro-MMP	proenzymes
PW	pulse wave dopplerography
RAA	renin-angiotensin-aldosterone
RANK	nuclear factor kappa $\beta$ receptor activator
RANKL	nuclear factor kappa $\beta$ ligand receptor activator
ROC Curve	Receiver-Operating Characteristic Curve
ROS	reactive oxygen particles
Se	sensitivity
SOD	superoxide dismutase
Sp	specificity
SV	stroke volume
TAVI	transcatheter aortic valve implantation

TC	total cholesterol
TG	triglycerides
TGF	transforming growth factor
TGF-1 $\beta$	transforming growth factor-1 $\beta$
TGF- $\beta$	transforming growth factor- $\beta$
TIMP	matrix metalloproteinase tissue inhibitor
TIMP-1,3	matrix metalloproteinase tissue inhibitor-1,3
TNF- $\alpha$	tumor necrosis factor- $\alpha$
Trx	thioredoxin
TrxR	thioredoxin reductase
TrxR1,2,3	thioredoxin reductase-1,2,3
ucMGP	non-carboxylated matrix Gla-protein form
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VIC	valvular interstitial cells
VLDL-C	very low density lipoprotein cholesterol
VTI	velocity time integral

# INTRODUCTION

## Topicality of Doctoral Thesis

When reading the currently available literature on the formation and development of AVS, one can think that everything is well-known and clear. But despite the fact that many biochemical, molecular and gene studies have been carried out over the last 10 years, there is no clear answer about the unequivocal etiology and pathogenesis of the disease. All publications contain the words: "supposedly, possible"; many studies have been performed on histological material obtained during surgery. For ethical reasons, it is difficult to make control groups.

Highly developed countries are also aware of the current prevalence and eventual morbidity in the future of aortic valve stenosis, but there is no means to limit disease development. It encourages to explore the situation in the Latvian population and to conduct a research work to look for the different and common in different AVS degrees; to look for the biomarkers in blood plasma and serum to predict the disease development.

Calcific aortic valve stenosis (AVS) manifest with fibrocalcific remodeling (transformation) of the aortic valve (AV) cusps, which is a slow process of chronic inflammation and calcification with completely unexplored and ambiguous etiology and pathogenesis (Lindman et al., 2016; Pawade et al. , 2015; Zeng et al., 2017; Kanwar et al., 2018).

AVS has become a growing public health burden. Studies on the etiopathogenesis of the disease and the possibility to pharmacologically change the course of the disease are still ongoing. Nevertheless, there is no medical treatment to stop or delay the progression of the disease. The only available treatment is the surgical replacement of AV or transcatheter aortic valve implantation (TAVI) (Pawade et al., 2015).

Several registers and studies of the populations groups show a significant link between calcific AVS and age. Therefore, with increasing survival, the number of patients with clinically relevant AVS is increasing too (Bonow et al., 2015).

AV sclerosis is found echocardiographically (Ehokg) in almost 25% of people after 65 years of age find sclerosis, and about 17% of these people develop AVS later. AV sclerosis means thickening of the AV cusps without any flow disturbance. The mean time from diagnosis of the AV sclerosis to the development of moderate and severe AVS is 6–8 years. The prevalence of AVS increases with age: at the age of 50–59 it is found in 0.2%; at the age of 60–69 – in 1.3% and at the age of 80–89 in up to 9.8% (Eveborn et al., 2013).

According to the data of two large studies of the United States of America (USA) and one Finnish study, 2% of people of the age 70–80 years and 3–9% after the age of 80 years suffer from moderate AVS (Eveborn et al., 2013). AVS is the most common valvular disease in the US and Europe, and is the second most common cause of heart surgery (Eveborn et al., 2013). The number of patients hospitalized for AV surgical replacement grow from 24568 patients in 1989 to 31380 in 2011 (patients over 65 years of age; USA). These data which are based solely on surgical procedures do not reflect the complete frequency of AVS, as they mainly include patients with symptomatic, severe AVS. Asymptomatic patients and those in whom for some reason surgery cannot be performed remain unaccounted (Bonow et al., 2015).

Considering the demographic trends of the current global population, by 2050 about 2.1 million Europeans and 1.4 million North American people will have symptomatic severe AVS. One of the main consequences of AVS is the development of heart failure. In the future, AVS can become a major health and economic burden (Thaden et al., 2014).

### **Aim of Doctoral Thesis**

Analyze and find out, which of cell-produced regulatory molecules (cytokines), involved in inflammatory and calcification process, importantly affects the process of occurrence and development of the aortic valve stenosis in all three (each of the three) severity grades.

To identify potential biomarkers that could potentially be used for diagnosing the mild AVS.

### **Tasks of Doctoral Thesis**

1. Selection of AVS patients (all three severity grades) and control group persons by Ehokg parameters according to the study criteria.
2. Conduction of the laboratory tests for all subjects involved in the study by determining: cholesterol with fractions, fibrinogen, C-reactive protein (CRP), highly sensitive C-reactive protein (hs-CRP), chemerin, fibroblast growth factors-21 (FGF-21), matrix metalloproteinases-1,3,9 (MMP-1,3,9), tissue inhibitor of metalloproteinases-1,3 (TIMP-1,3), transforming growth factors (TGF), thioredoxin reductase 1 (TrxR1), myeloperoxidase (MPO).

3. To evaluate the results of the analysis between the control group and the AVS group and in all three severity grades.
4. To find a sufficiently informative and good biomarker for diagnosing the mild aortic valve stenosis.
5. To evaluate the relationship of the determined lipid fractions with aortic stenosis at its various severity grades.
6. To evaluate the level of new biomarkers of oxidative stress in different AVS severity grades.

### **Hypotheses of Doctoral Thesis**

1. Inflammatory and non-inflammatory factors, cell-produced regulatory molecules (cytokines) determine the development and prognosis of the calcific AVS.
2. Changes in the level of lipid fractions in AVS patients shall not be unambiguous evaluated AVS an absolute risk criterion.
3. Oxidative stress is associated with AVS in all severity grades.

### **Scientific novelty of Doctoral Thesis**

In the study, for the first time in the Latvian population, we analyze the biochemical parameters of the blood plasma and serum of the calcific AVS patients of different ages, compare the results with the control group and between all three degrees of AVS severity.

Knowing the latest studies on the etiopathogenesis of the calcific AVS, we selected in the study to identify those potential biomarkers that have not been studied in AVS patients (chemerin, FGF-21, thioredoxin reductase-1, myeloperoxidase).

All results of all the biomarkers obtained in the study are compared in different severity grades of AVS so that the pathogenetic processes prevailing at each degree of stenosis can be discussed in more detail.

Relationships between biomarkers, as well as between lipid fractions and biomarkers are sought. When analyzing lipid fractions, it is found that dyslipidemia is not unequivocal in the AVS patients. The role of oxidative stress in the development of AVS is substantiated.

# 1. LITERATURE REVIEW

## 1.1 Insight into the aortic valve anatomy

The AV consists of three cusps (the right coronary cusp, the left coronary cusp and the non-coronary cusp) that are smooth, thin (< 1mm), flexible and mobile. AV is located between the left ventricular outflow tract and the ascending aorta. The valve cusps consist of three layers: fibrous (*lamina fibrosa*), spongy (*lamina spongiosa*) and ventricular (*lamina radialis*); see Figure 1.1.

The fibrous layer is directed towards the aorta, consists mainly of circularly (rounded) oriented collagen fibers (of type I and type III) that transfer the load to the walls of the aortic root and provide tensile strength. The spongy layer is medium and consists mainly of proteoglycans and collagen fibers. The valvular interstitial cells are of entire cusp thickness, but the proteoglycans are the predominant component of the middle layer and help resist compressive forces. The ventricular layer is the thinnest one facing the left ventricle, extending beyond the cusps as an endocardial layer. This layer contains radially oriented elastin and promotes flexibility, allowing the cusps shape to change during opening and closing of the valve. Between the collagen fibers, there are interstitial cells with properties of fibroblasts and smooth muscle cells. These cells are also called myofibroblasts. During cardiac cycles, they change geometrically and actively works along with the aortic valve.

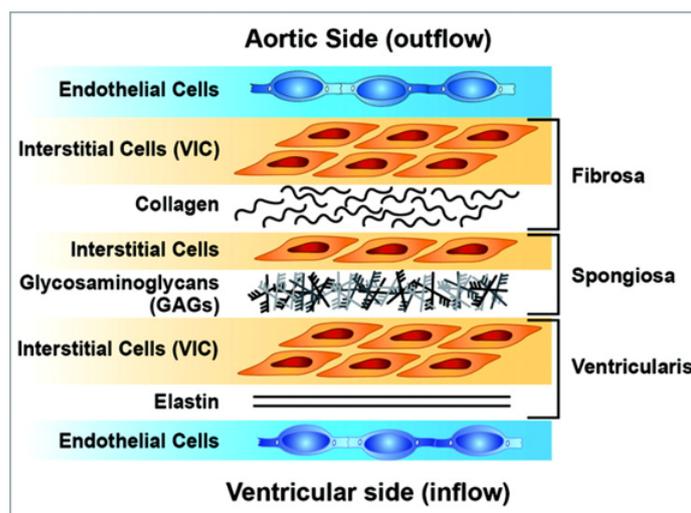


Figure 1.1 Structure of the aortic valve cells (Rajamannan et al., 2011)

The aortic valve is part of the aortic root and connects the heart to the systemic circulation. The action of the valve is subject to shear, elasticity and tension processes during the cardiac cycles. Flexibility corresponds to the active opening and closing of the valve. Shear occurs when blood flows through the open valve. The tension is observed when the valve is closed. As a result of these processes, the valve tissue is constantly deformed and moving during the cardiac cycle. (Hinton et al., 2011; Garg, 2016; Misfeld et al., 2007; Lerman et al., 2015).

In the case of AVS, the valve cusps become thicker, sclerotic and calcified that result in reduced mobility and progression of the AV obstruction (Pawade et al., 2015).

## **1.2 Risk factors for the aortic valve stenosis**

Among all types of AVS, up to the age of 70 years, 18% are of degenerative etiology but after the age of 70 years – 48% (Ren, 2017). Risk factors for degenerative calcific AVS are increasing age, smoking, diabetes mellitus, hypercholesterinemia, arterial hypertension (Pawade et al., 2015).

The role of lipoprotein a [Lp(a)] in the development and progression of AVS has been investigated and proven. Lp (a) is a lipoprotein rich in cholesterol. It differs from low-density lipoprotein (LDL) because it contains additional protein, apolipoprotein (a). Like LDL, Lp (a) particle also contains one apolipoprotein B molecule. There are several mechanisms how Lp (a) can cause and promote AVS:

- It precipitates on the AV cusps similar to LDL, causing the valve cusps to thicken.
- It promotes deposition of fibrin on the AV cusps.
- It may bind to fibrin and produce high cholesterol levels at the site of tissue damage and contribute to calcinosis.
- Genetic studies demonstrate the association of the presence of LPA gene with the causal role of Lp (a) in the development of AVS (Vassiliou et al., 2017; Mathieu et al., 2017; Torzewski et al., 2017).

Many of the mentioned risk factors also contribute to the progression of already diagnosed AVS: female gender, age, degree of the AV calcinosis, smoking, arterial hypertension, obesity, metabolic syndrome, secondary hyperparathyroidism, renal failure and increased plasma Lp (a) level ( $> 50$  mg/dL; norm  $< 30$  mg/dL).

Arterial hypertension contributes to the progression of AVS both due to increased mechanical load to the left ventricle and to increased RAA (renin-angiotensin-aldosterone) system activity. There is evidence of a relationship between AVS and elevated LDL levels,

but the fact that statin therapy is ineffective in the case of AVS suggests that LDL induces AV calcinosis but does not affect the disease progression (Lindman et al., 2016).

Genetic studies conducted so far are in small groups of patients. It is currently difficult to determine whether mentioned risk factors contribute to the development of AVS or the progression of already existing disease.

### **1.3 Clinical manifestations of the aortic valve stenosis**

Like AVS pathogenesis can be divided into early and late stage, also clinically the aortic valve stenosis has an asymptomatic period and symptomatic AVS.

The symptoms of AVS appear gradually after about 10–20 years–long latent period. The main symptoms are chest pain (usually associated with exercises), dyspnea, presyncope or syncope. Dizziness or syncope occurs due to reduced brain perfusion. It can also be induced by systemic vasodilation caused by the exercises, because the blood flow is not compensated due to the fixed cardiac output, baroreceptor dysfunction or rarely due to atrial or ventricular arrhythmias.

Narrowing of the AV area, a systolic pressure gradient occurs between the left ventricle and the aorta. As the compensatory mechanism occurs the left ventricular wall hypertrophy and diastolic dysfunction. By increasing of the left ventricular mass and increasing left ventricular systolic pressure, myocardial oxygen consumption increases, especially in the subendocardial layer. Although the coronary blood flow may be normal, the flow reserve is often inappropriate for the left ventricle mass. Thus, myocardial perfusion is decreased due to relatively lower myocardial capillary density and reduced diastolic pressure of the coronary perfusion. Left ventricular hypertrophy also causes intra–myocardial compression of the coronary artery. Patients with AVS often have tachycardia, which also shortens the diastolic time of the coronary perfusion. It results in myocardial ischemia which is manifested by chest pain. Repeated myocardial ischemias cause apoptosis of the myocytes and myocardial fibrosis.

In the more severe stages of the disease, the high left ventricular filling pressure leads to secondary pulmonary hypertension and further promotes manifestation of the heart failure and dyspnea (Ren, 2017; Kanwar et al., 2018).

However, most of patients with maintained left ventricular ejection fraction (EF) have partial left ventricular systolic dysfunction. This occurs due to the reduced longitudinal function of the left ventricle (radial and circumferential function are preserved). This is

explained by the fact that in the subendocardial layer ischemia and fibrosis are more pronounced AVS it is more exposed to pressure overload (Lindman et al., 2016). The appearance of symptoms in patients with AVS is a poor prognostic indicator. If surgical treatment is not applied, about 50% of patients die within the next 12–18 months (Perera et al., 2011; Otto et al., 2014).

There is no correlation between the AVS severity and the appearance of symptoms. Usually the symptoms appear in patients with severe AVS and preserved EF of the left ventricle. With the onset of symptoms, only 30–50% of patients do not have cardiovascular events within the next 2 years. The most frequent complication of surgically untreated and severe aortic stenosis is sudden death (Kanwar et al., 2018).

#### **1.4 Pathogenesis of the aortic valve stenosis**

The development of AVS can be divided into two stages:

- In the early phase when endothelial trauma, lipid deposition, and inflammation dominate. There are many similarities with the atherosclerosis process in this phase.
- In the late phase, when calcification and pro-osteogenic factors lead to disease progression (Pawade et al., 2015).

AVS and atherosclerosis have different rheology – coronary arteries are subjected to prolonged laminar flow, while AVS is characterized by pulsating pressure on the left ventricular walls and low reversible pressure from the aorta (Lerman et al., 2015).

Although mechanical stress is currently considered to be the main AVS onset process, there are a number of new indications that AVS is a chain of complex and highly regulated processes (Pawade et al., 2015).

Mechanical stress causes endothelial damage and dysfunction. Lipid infiltration occurs and inflammatory cytokines are released. Lipids and cytokines further contribute to the endothelial damage, amplify the inflammatory process.

Oxidative transformation of the low-density lipoproteins (LDL) into ox-LDL produces lysophosphatidic acid (LysoPA), which is a lipid mediator and promotes mineralization and osteogenesis of the valvular interstitial cells (VIC). Due to endothelial damage, the synthesis of nitric oxide (NO) is reduced, which further contributes to the lipid oxidation and cytokine release. LDL receptor-associated protein 5 (LRP5) and osteocalcin regulate bone formation. Under hypercholesterolemia conditions, LRP5 binds to glycoprotein Wnt, which activates  $\beta$ -catenin and causes bone formation (Rajamannan, 2008; Lerman et al., 2015). Ox-LDL

promotes the release of MMP (matrix metalloproteinases) resulting in the production of osteoblastic gene markers using the Wnt pathway (Gao et al., 2015).

Lp(a) is a genetically determined, independent risk factor and possible cause of the AVS progression. Lp(a) can promote inflammation and cause calcification (Torzewski et al., 2017). Recently, the relationship between the presence of AVS and Lp(a) 1-a nucleotide polymorphism has been demonstrated (Pawade et al., 2015).

Lp (a) binds to the damaged endothelium, which can contribute to the formation and of the foam cells and inflammation. Lp(a) and its associated phosphocholine-containing oxidized phospholipid (ox-PLs) causes apoptosis in macrophages which may contribute to the progression of the valvular damage. Lp(a) is the main carrier of oxPLs in plasma. Lp(a) can promote inflammation using the apoB component. ApoB peptides can activate cytokines IL-6, IL-8 (interleukin-6,8) (Thanassoulis, 2016). Lp(a), using oxPLs, is a source of oxidative stress, promotes calcification (osteogenic differentiation of the endothelial cells) (Thanassoulis, 2016).

Enzymes transported by LDL and Lp(a), such AVS Lp-PLA-2 (Lp-linked phospholipase A2) and autotaxin (ATX), produce derivatives of the lysophospholipids. ATX, which is also secreted by VIC, converts lysophosphatidylcholine (LysoPC) into lysophosphatidic acid (LysoPA). LPA, like Wnt, contributes to the osteogenic transformation of the VIC. Accumulation and retention of the lipoproteins in the aortic valve is a decisive event, since lipids can be further used by various enzymes to produce bioactive compounds (e.g. lysophospholipids). Lp-PLA2 is transported by apoB-containing lipoproteins, enriched with LDL and Lp(a). Lp-LPA2 converts ox-PLs into lysophosphatidylcholine (LysoPC), which promotes loss of the mitochondrial membrane and VIC apoptosis. The action of the ATX depends on tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). TNF- $\alpha$  is secreted by monocytes and macrophages. Activation of the TNF- $\alpha$ -R1 causes activation of the nuclear factor kappa  $\beta$  (NF- $\kappa\beta$ ), followed by mineralization induced by activation of IL-1 $\beta$  and IL-6 (Lindman et al., 2016; Perera et al., 2011).

Due to endothelial damage, infiltration of macrophages and T-lymphocytes occurs. Using adhesion molecules, monocytes enter the endothelial layer and transform into macrophages. Activated T-lymphocytes contribute to the release of cytokines IL-1 $\beta$  and transforming growth factor-1 $\beta$  (TGF-1 $\beta$ ), followed by the release of matrix metalloproteinases (MMP) and remodeling of the extracellular space. MMP also releases tenascin-C, which promotes mineralization and bone formation (Freeman et al., 2005).

Macrophages secrete osteopontin, which is directly related to ectopic calcification (Rajamannan, 2008; Zeng, 2016).

The fact that ACE (angiotensin converting enzyme) is found together with apoB indicates that ACE can be transmitted to the lesion by LDL particles (Freeman et al., 2005).

Patients with AVS have high levels of ACE that contribute to the conversion of Ang I (angiotensin I) to Ang II (angiotensin II). Ang II has both profibrotic effects via Ang II A1 receptors and antifibrotic and anti-inflammatory activity through A2 receptors. Everything depends on the amount of A1 and A2 receptors. The A2 receptor expression pathway is regulated by the ACE-2 and Mas receptors, but the number of receptors in the calcified valve is reduced. Therefore, the increased activity of the RAA system in the calcified valve is associated with fibrosis. Increased activity of the RAA system at the systemic level is associated with arterial hypertension, which is common in patients with AVS. It further increases the mechanical load and promotes the progression of AVS (Pawade et al., 2015).

Ang II promotes collagen synthesis and fibrosis. Arachidonic acid (AA) promotes the synthesis of prostaglandins and leukotrienes through cyclooxygenase 2 (COX2) and 5 lipoxygenases (5-LO) pathways. Prostaglandins and leukotrienes promote inflammation and mineralization (Lindman et al., 2016). Microcalcification begins already at the beginning of the AVS development from microvesicles secreted by macrophages and VIC. In addition, the pronounced release of ectonucleotidases (NPP1, 5'-NT, ALP) promotes both apoptosis and mineralization; see Figure 1.2. Mineralization is accompanied by fibrosis and angiogenesis, promoted by vascular endothelial growth factor (VEGF). Angiogenesis increases the activity of inflammation and preosteogenic processes (Lindman et al., 2016).

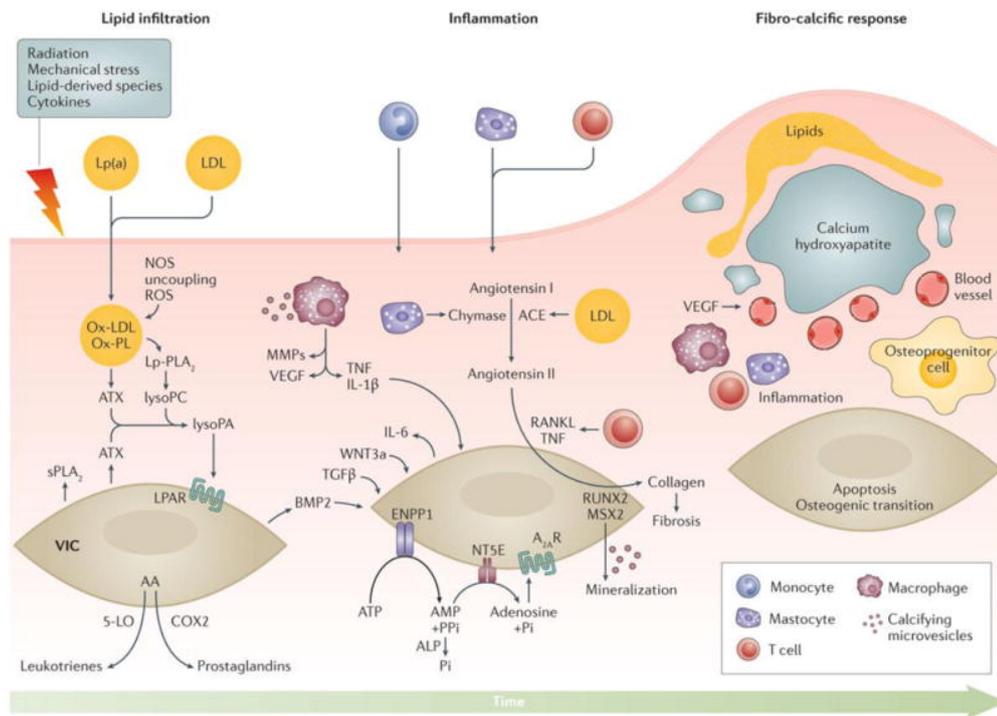


Figure 1.2 Scheme of the pathogenesis of calcific aortic valve (Lindman et al., 2016)

Already initially, the transformation of osteoblast-like cells into an osteogenic phenotype is observed. Therefore, there is an increased level of several proteins (including Cbfa-1 / Runx-2 transcription factor) which are specific for osteoblastic differentiation and regulation of the osteoblastic function. The source of osteoblast-like cells in the valve is most likely to be myofibroblasts (VIC). There is *in vivo* evidence that macrophages are involved in the early stage of the AVS development in this osteoblastic phenotype using inflammatory cytokines (IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ , insulin-like growth factor-1, TGF- $\beta$ ). However, at the later stages, calcification, including the pathways of Notch, Wnt/  $\beta$ -catenin and  $\beta$  ligand-receptor activator of the nuclear factor kappa - RANKL/ osteoprotegerin-OPG is dominating.

Notch belongs to the family of cell surface receptors (Notch 1-4) that are found in large amount in AV and play an important role in the morphological development of the valve. People with a loss of Notch-1 mutation function have a higher risk of AVS. Notch-1 is important for the formation of the osteogenic cells by using bone morphogenic protein (BMP-2). BMP-2 is a potent osteogenic differentiation factor. BMP-2 belongs to the multifunctional cytokines from the TGF- $\beta$  family. BMP-2, BMP-4 releases VIC. BMP-2 increases calcification and contributes to the osteoblastic transformation of VIC (Pawade et al., 2015).

Similarly, TGF- $\beta$ 1 is capable to induce translocation of the cell nuclei to  $\beta$ -catenin and promote osteogenic transformation pathway of Wnt ( $\beta$ -catenin promotes the osteoblastic transformation of VIC). TGF- $\beta$  may increase the deposition of calcium and collagen. 45% of

circulating TGF- $\beta$ 1 is found in platelets. Mechanical stress can lead to activation of the latent circulating TGF- $\beta$ 1 (Lerman et al., 2015; Pawade et al., 2015).

Equally important in the calcification is the RANK / RANKL / OPG pathway – in the bones, RANKL binds to RANK, which acts as a potent inducer of the osteoclast differentiation and activity. This process is controlled by OPG, which binds to RANKL and does not allow it to activate the RANK. RANKL appears to have the opposite effect in circulation cells, resulting in an osteoblastic phenotype of the VIC, resulting in the formation of calcium nodules and increased release of osteocalcin and alkaline phosphatase. RANKL also promotes osteogenic manifestations in the smooth muscle cells (Pawade et al., 2015).

Calcification is probably caused by cell death and release of apoptotic bodies. These apoptotic structures are similar to the matrix vesicles found in the bones and contain calcium crystals (including ions of calcium and inorganic phosphate) and contribute to the formation of needle-shaped hydroxyapatite crystals. Crystals of hydroxyapatite in the bones damage the membrane of the matrix vesicles and get into the extracellular space, where they form sites for further calcium deposition. In addition, precipitation of hydroxyapatite further contributes to inflammation (Pawade et al., 2015). Physiologically, hydroxyapatite crystal growth should be inhibited by Gla-protein (MGP), which also inhibits BMP-2.

Matrix Gla-Protein (MGP) consists of active (cMGP) and inactive (ucMGP) form. MGP requires carboxylation caused by vitamin K. Therefore, AVS was observed in animals to which vitamin K antagonist was given (Warfarin) (Peeters et al., 2018).

Calcification process is also regulated by ectonucleotidases produced by the VIC, which is regulated by extracellularly produced inorganic phosphate (promoter of calcification) and inorganic pyrophosphate. Ectonucleotide pyrophosphatase-1 (NPP-1) is markedly elevated in stenotic valves. Extracellular hydrolysis of adenosine triphosphate (ATP) with NPP-1 results in increased levels of inorganic phosphate, thereby promoting calcification and further NPP-1 production (positive feedback is generated). ATP acts as a VIC survival signal using P2Y2 receptors (purine receptors). The disappearance of P2Y2 receptors leads to VIC apoptosis and subsequent calcification process. Lack of P2Y2 signaling increases the secretion of IL-6, which promotes further osteogenic differentiation of VIC by BMP (Pawade et al., 2015).

Activated leukocytes after TNF- $\alpha$  secretion can stimulate proliferation of the valve fibroblasts and MMP secretion by actively promoting extracellular remodeling. MMP are zinc-dependent proteolytic enzymes. The proteolytic activity of MMP is regulated by tissue inhibitors of the matrix metalloproteinases (TIMP) - endogenous proteins. Also, VIC secretes MMP and TIMP. MMP-1,2,3,9 and TIMP 1-4 were more studied in relation to cardiovascular diseases, but with ambiguous results.

Endothelial damage contributes to the infiltration of lipids, especially infiltration of LDL and Lp (a) into the fibrous layer, and causes the inflammatory cells to bind to the aortic valve. Several factors can cause endothelial damage, including particles obtained from lipids, cytokines, mechanical stress, and influence of radiation. The production of reactive oxygen particles (ROS) is promoted by the decoupling of the nitric oxide synthase (NOS), which increases lipid oxidation and further enhances secretion of the cytokines. Enzymes, such as Lp-PLA2 and autotaxin (ATX), delivered to the aortic valve together with lipoproteins (LDL and LP (a)), produce lysophospholipid derivatives.

ATX, also secreted by valvular interstitial cells (VIC), converts lysophosphatidylcholine (LysoPC) into lysophosphatidic acid (LysoPA). Several factors, including LysoPA, the activator of the nuclear factor kappa-B ligand (RANKL) and Wnt3a receptors, contribute to the osteogenic transformation of VIC.

AA formed by the presence of enzyme PLA2 in the cytosol, promotes the production of eicosanoids (prostaglandins and leukotrienes) via cyclooxygenase 2 (COX2) and 5-lipoxygenase (5-LO) pathways, respectively. Eicosanoids, in turn, promote inflammation and mineralization. Chimerases and angiotensin converting enzyme (ACE) promotes formation of the angiotensin II, which increases synthesis and secretion of the collagen in the VIC. Due to the increased production of matrix metalloproteinases (MMP) and reduced synthesis of the tissue inhibitors of the matrix metalloprotein (TIMP), unorganized fibrous tissue is formed in the aortic valve.

Microcalcification begins at the onset of the disease, which is facilitated by microvesicles secreted by VIC and macrophages. In addition, excessive release of ectonucleotide (NPP1, 5'-NT, ALP) promotes both apoptosis and osteogenic mediated mineralization. Bone morphogenic protein 2 (BMP2) involves in osteogenic transdifferentiation process, which is associated with the release of transcription factors (RUNX2 and MSX2). The osteoblast-like cells, respectively, coordinate calcification of the aortic valve as part of a highly-regulated process similar to the process of skeletal bone formation. The formation of a mineralized matrix is complemented by fibrosis and the formation of new blood vessels, promoted by vascular endothelial growth factor (VEGF). In turn, neovascularization increases the involvement in the process of inflammatory cells and osteoprogenitor cells created in the bone marrow.

## 1.5 Brief description of the analyzed cell produced regulatory molecules (cytokines)

### 1.5.1 Chemerin

Chemerin also called retinoic acid receptor responder protein 2. It was discovered in 1997 as a protein associated with normal skin function (the study was conducted in connection with psoriasis). Chemerin is an adipokine known as G protein-associated chemokine-like receptor 1 ligand (CMKLR1), also known as chemerin receptor 23 (Chem23), which was first identified in macrophages and dendritic cells. (Roh et al., 2007; Wittamer et al., 2003).

Chemerin is mainly excreted in white fatty tissue, but found in high concentrations also in liver, lungs, brown fat, heart, kidney, pancreas and skeletal muscle. Chemerin is excreted inactive. Extracellularly proteolytically it is split, and active isoforms are formed (Docke et al., 2013; Weigert et al., 2010; Rourke et al., 2013).

Chemerin is activated by proteases of inflammation and tissue damage from macrophages, dendritic cells. Chemerin regulates the inflammatory process by promoting macrophage migration to certain tissues such as adipose tissue (Goralski et al., 2007). The further action of the chemerin is manifested by binding to the G-protein-coupled receptor 1 (GPR1) and the chemerin receptor 23 (ChemR23).

ChemR23 receptors are found in vascular endothelium cells, smooth muscle cells, and cardiomyocytes. ChemR23 expression in endothelial cells is enhanced by inflammatory cytokines: TNF- $\alpha$ , IL-6, IL-1 $\beta$ . Level of the circulating chemerin in humans and animals is strongly correlated with levels of inflammatory markers such as TNF- $\alpha$ , IL-6, highly sensitive C-reactive protein (hs-CRP), resistin and leptin (Eisinger et al., 2015; Gu et al. 2014, Kostopoulos et al., 2014). When chemerin binds to GPR1 receptors, mobilization of calcium occurs. Chemerin promotes endothelial cell proliferation and release of MMP-2, MMP-9, and MMP-7.

Chemerin plays an important role in angiogenesis, osteoblastogenesis, myogenesis, and regulation of glucose hemostasis; see Figure 1.3. Chemerin is associated with adipocyte differentiation and lipolysis stimulation. Chemerin reduces the release of nitric oxide into endothelial cells, promoting stiffness of the vascular wall and arterial hypertension (Zabel et al. 2014; Ferland et al., 2015).

The range of action of chemerin is not yet clear. Its role seems to depend on the type of target cells. After proteolytic activation, forms of chemerin with inflammatory or anti-

inflammatory activity are formed, depending on the class of protease and microenvironment (Parmentier, 2013; Er et al., 2018).

Chemerin has anti-inflammatory activity in endothelial cells, where it prevents the expression of the TNF- $\alpha$ -induced vascular cell adhesion molecule-1 (VCAM-1) and inhibits macrophage adhesion to the TNF- $\alpha$ -stimulated endothelial cells and, inhibits NF- $\kappa$ B (Ferland et al., 2015; Parmentier, 2013).

TNF- $\alpha$  promotes chronic low-grade inflammation. *In vitro*, TNF- $\alpha$  promotes the expression of chemerin in adipocytes. TNF- $\alpha$  increases both chemerin and ChemR23 concentrations. Chemerin can affect TNF- $\alpha$  levels and vice versa. The role of chemerin as an anti-inflammatory or inflammatory factor remains controversial. In the inflammatory environment, chemerin promotes the chemotaxis of macrophages and immature dendritic cells, but by treatment with chemerin, the action of neutrophils and macrophages and the release of inflammatory cytokines are reduced (Helfer et al., 2018).

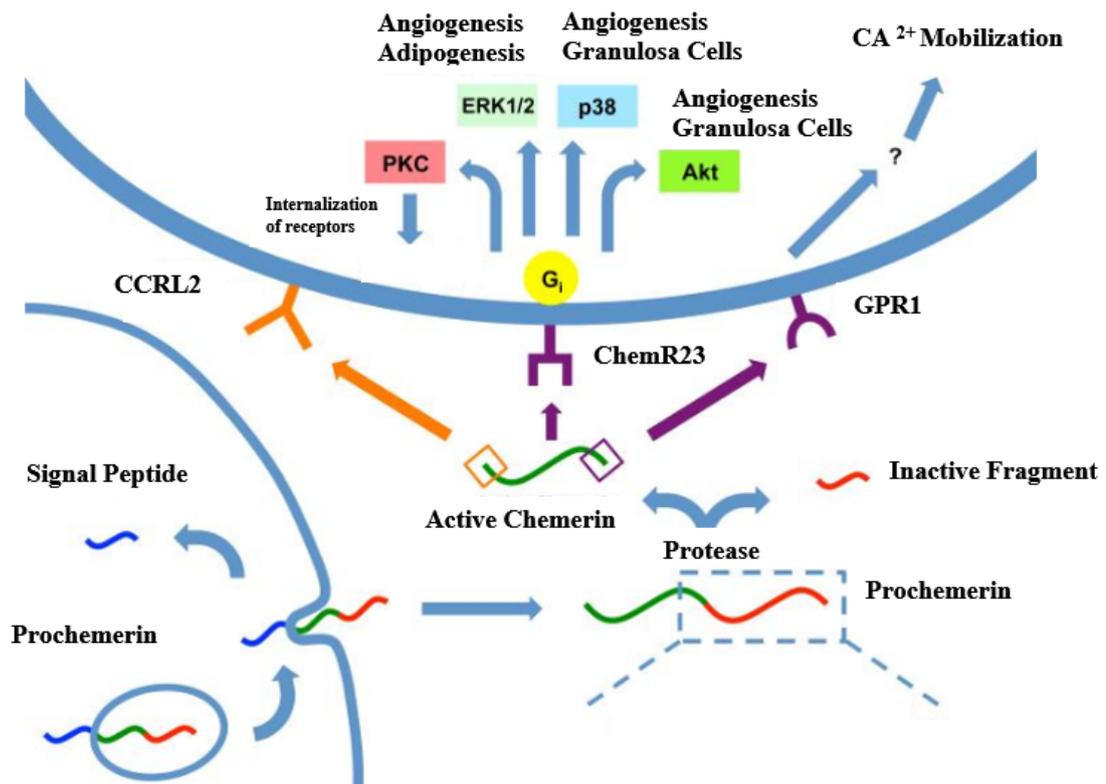


Figure 1.3 Types of chemerin action (Ferland and Watts, 2015)

Approximately 16–25% of the serum chemerin level is genetically determined (Er et al., 2018). A genome study in the human population shows that serum chemerin is inherited and

associated with a polymorphism of separate nucleotides in the Discoidin I-Like Domains 3 (EIDL3) gene that regulates angiogenesis (Helfer et al., 2018).

A positive correlation has been found between chemerin and coronary artery disease, ischemic stroke, and instability of the atherosclerotic plaque of the coronary artery. Chemerin correlates with body mass index (BMI), level of triglycerides and arterial hypertension. Elevated chemerin levels correlate with female gender and age (Zabel et al., 2014; Ferland et al., 2015; Er et al., 2018).

### **1.5.2 Fibroblast growth factor-21 (FGF-21)**

FGF-21 is a family member of the fibroblast growth factor (FGF). FGF-21 is a metabolic regulator involved in glucose and lipid metabolism. There is still no unambiguous clarity about its relation to atherosclerosis. FGF-21 has mitogen and cell survival activity. FGF-21 is involved in tissue regeneration. It has an anti-inflammatory role in myocytes and it improves oxidative capacity.

FGF-21 is a peptide hormone synthesized by liver, pancreas, brown and white adipose tissues and cardiac endothelial cells. FGF-21 regulates energetic homeostasis (Schlein et al., 2016; Zhang et al., 2015).

FGF-21 can act as a cardiomyokine, which means that it is produced at a significant level by the cardiac cells and acts on the heart in an autocrine way. The effect of FGF-21 on the heart may be systemic (from FGF-21 produced in the liver) and local.

FGF-21 protects against cardiac hypertrophy, inflammation, and oxidative stress.

The expression of FGF-21 is controlled by the peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ), which is released from the liver into the bloodstream. A permeable transmembrane protein –  $\beta$ -Klotho (KLB) is required for the FGF-21 activity. Significant amounts of both FGF-21 receptors and cofactors  $\beta$ -Klotho have been demonstrated in the heart cells (Planavila et al., 2015).

FGF-21 is considered to be a hormone of oxidative stress. Disturbances in mitochondria impair oxidative phosphorylation and lead to reduced production of adenosine triphosphate (ATP), causing an increase in serum FGF-21.

Reactive oxygen particles (ROS) are formed under oxidative stress conditions – they are chemically reactive molecules that cause damage to DNA, proteins, and enzymes. Mitochondria rapidly release FGF-21 as a stress-protecting mechanism to reduce cell death.

FGF-21 reduces oxidative stress: by reducing ROS, reducing apoptosis and suppressing NF- $\kappa$ B (Gómez-Sámano et al., 2017).

FGF-21 is elevated in patients with an unfavorable lipid profile, obesity, diabetes, metabolic syndrome, as well as coronary heart disease and carotid artery disease (Chow et al., 2013).

Increases in FGF-21 levels in blood serum in atherosclerosis, coronary heart disease, myocardial ischemia, and cardiac hypertrophy are considered as a compensatory response to protect against oxidative stress, inflammation, and apoptosis.

The fact that FGF-21 is not only a biomarker of heart diseases but has a protective effect has been shown by a positive effect of treatment with FGF-21 in animal models.

Cardiac hypertrophic remodeling is a cardiac adaptive response to certain tensions, including in case of valvular diseases. Under stress conditions, cardiac hypertrophy is characterized by enlargement of cardiomyocytes rather than cell division.

Mechanical studies show that FGF-21 reduces cardiac hypertrophy by activating the fibroblast growth factor receptor 1c (FGFR1c)/ $\beta$ -Kloth pathway and promoting expression of multiple antioxidant genes, including SOD-2, and inhibiting ROS formation in the autocrine pathway (Cheng et al., 2016).

Biopsy of the left ventricle from donors with hypertensive heart showed significantly higher levels of FGF-21 compared to normotensive controls. High levels of FGF-21 were thought to be associated with cardiac hypertrophy and fibrosis (Ferrer-Curriu et al., 2018).

FGF-21 reduces plasma triglyceride levels: decreases the plasma concentration of unesterified fatty acids and increases the catabolism of triglyceride-rich lipoproteins in white and brown fatty tissues (Schlein et al., 2016).

FGF-21 levels correlate with the thickness of intima-media and carotid artery disease in women but not in men (Chow et al., 2013).

Similarly, elevated levels of FGF-21 in women correlate with diabetes mellitus (the study was conducted in China) (Zhang et al., 2015).

FGF-21 deficiency increases the level of angiotensin II and promotes arterial hypertension (Pan et al., 2018).

### **1.5.3 C-reactive protein (CRP)**

CRP is a low-level inflammatory biomarker released by the liver. The increase in CRP is more thought to be associated with signs of systemic inflammation – heart failure, obesity (Clavel et al., 2014).

Highly sensitive C-reactive protein (hs-CRP) is a good biomarker for coronary atherosclerosis, but AVS patients have no unequivocal results. Hs-CRP could be useful for identifying patients with initial calcific AVS.

Increased CRP levels are associated with age, female gender, increased BMI, high blood pressure, dyslipidemia, diabetes mellitus, and smoking. The increase in CRP is found in patients with AVS and also with peripheral artery disease (Cho et al., 2016). There are studies that demonstrate the relationship between CRP genetic polymorphism and AVS.

The polymorphism of the CRP gene rs1205C>T is associated with increased levels of CRP and severe AV calcification. Patients with rs1205C>T allele have more severe AVS. CRP may be a marker of progression of aortic stenosis.

There have been reports on patients with severe symptomatic AVS and high levels of CRP in whom CRP levels decreased after surgical therapy. Also, the patients with asymptotically severe AVS were studied in which high CRP levels indicated the rapid progression of the disease (Wypasek et al., 2015).

There is a genetic study that reveals that the presence of IL-6R 358Ala allele in AVS patients are associated with a weakened systemic inflammatory condition and a 22% lower level of CRP (Wypasek et al., 2014).

### **1.5.4 Matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP)**

MMP is zinc-dependent endopeptidases: collagenases, gelatinases, and stromelysin, and plays an important role in remodeling the extracellular space and tissue transformation. The process of tissue remodeling depends on the balance between MMP and TIMP.

Matrix metalloproteinases (MMP) function in the extracellular space and degrade proteins. MMP participates in morphogenesis, tissue repair, and remodeling.

In the case of heart failure, left ventricular hypertrophy and other diseases, structural changes occur in myocardium called myocardial remodeling, which involves the summing up of both cellular and extracellular processes. Cellular processes include the growth, apoptosis,

and necrosis of myocytes. Extracellular processes include transformation by induction and activation of proteolytic enzymes, MMP, etc.

Patients with aortic valve stenosis often have left ventricular hypertrophy. Pressure and volume overload result in changes in the left ventricular walls. MMP is excreted in the extracellular space as proenzymes (pro-MMP). Pro-MMP binds to specific proteins of the extracellular space (Spinale, 2007).

MMP activity is regulated by tissue inhibitors (TIMP). Human has 24 MMP genes. MMP expression is controlled by inflammatory cytokines, growth factors, cell-cell, and matrix-cell interaction. MMP activity increases by inhibiting TIMP. In the remodeling of extracellular space, the balance between MMP and TIMP is essential (Nagase et al., 2006).

Angiotensin II is an important mediator in the myocardial remodeling process associated with myocardial infarction, left ventricular hypertrophy or dilatation cardiomyopathy. Potentially Ang II activation can cause MMP activation. However, there are indications that the MMP increase caused by Ang II may not be long-lasting. There is evidence that the activation of Ang II causes the release of TGF, which in turn affects the transcription of MMPs in various ways.

Ang II stimulates the production of aldosterone, which promotes the production of fibrotic proteins such as collagen. Whether and to what extent aldosterone affects transcription of MMPs is not known. In any case, Ang II and aldosterone promote myocardial fibrosis.

Endothelin is another bioactive molecule that possibly promotes myocardial remodeling. Endothelin activates protein kinase C, which in turn activates NF- $\kappa$ B and further MMP. Endothelin increases MMP-1 levels in vascular endothelial cells.

In the case of left ventricular hypertrophy, TGF is released, which is considered to be a profibrotic molecule, assuming that TGF inhibits MMP transcription and reduces changes in the extracellular space. However, it has been observed that TGF promotes the activity of some types of MMP (e.g. MMP-13). Thus, different effects of TGF on the transcription of MMP is possible, which may depend on concentration, cell and time.

Oxidative stress and ROS formation also stimulate MMP transcription (Spinale, 2007).

In patients with the aortic valve stenosis, remodeling of the extracellular space includes disorganization of the collagen bundles and fragmentation of the elastic fibers. Calcific zones are found between disorganized collagen bundles.

In a previous study on remodeling of the extracellular space in patients with AVS using histological material, MMP-9 and MMP-3, MMP-2 activity in the tissue samples of the valves affected by aortic stenosis (n = 49) was found to be higher comparing to tissue samples (n =

8) obtained during transplantation, and they were used as control. This was thought to be mainly due to inflammation in the valves affected by aortic stenosis. TIMP-1 and TIMP-2 were found in both normal and abnormal aortic valves. There was no difference in TIMP-2 concentration, but TIMP-1 levels were significantly higher in the aortic stenosis group. The MMP-9/ TIMP-1 ration tended to be lower in the AVS group than in the control group. This was mainly due to a significant increase in TIMP-1 in the stenotic AV tissues. Valvular interstitial cells *in vitro* were exposed to IL-1 $\beta$ -and TNF- $\alpha$ , but only a variation of pro-MMP-2 activity was observed; neither MMP-9 nor MMP-3 and MMP-7 were detected. Likewise, no changes in TIMP-1 and TIMP-2 synthesis and secretion were detected. In the valves affected by aortic stenosis, histological and biochemical analyses showed that abnormal transformation of the valvular extracellular space, including the MMP/ TIMP system, was occurred. (Fondard et al., 2005).

During a study in which compared the objective changes of the left ventricle and MMP-3 changes between gender and expression of clinical symptoms, higher MMP-3 levels were found in man than women; worse systolic and diastolic left ventricular function and higher indexed left ventricular mass. It was concluded that women have less pronounced left ventricular remodeling and fibrosis. However, the results could be influenced by the uneven distribution of the population, i.e. 133 out of 174 patients included in the study were men (76.4%) (Singh et al., 2019).

Table 1.1 shows MMP activity that may affect migration, differentiation, growth, inflammatory processes, apoptosis, etc. of the cells.

MMPs are extracellular proteins, but studies show that MMP-1, MMP-2, and MMP-11 are also intracellular and can affect intracellular proteins.

By chemical structure MMP-1 is an interstitial collagenase; MMP-3 is a stromelysin and MMP-9 is a gelatinase.

Table 1.1

**Brief description of the matrix metalloproteinases (MMP) analyzed during the work**

MMP-1	MMP-3	MMP-9
promotes keratinocyte migration	promotes cellular migration	improves collagen affinity
promotes keratinocyte reepithelization	promotes apoptosis of the epithelial cells	pro-inflammatory and anti-inflammatory activity
promotes cellular migration	improves collagen affinity	reduces IL-2 response

MMP-1	MMP-3	MMP-9
promotes platelet aggregation	releases bFGF	forms resistance of the tumor cells
pro-inflammatory and anti-inflammatory activity	increases IGF1 bioavailability and cell proliferation	increases TGF- $\alpha$ bioavailability
	pro-inflammatory and anti-inflammatory activity	creates neovascularization of the thymus
	increases TGF- $\beta$ bioavailability	promotes hypertrophic chondrocytes apoptosis and osteoclast formation

Collagenases split interstitial collagen I, II, III and these fragments can digest other molecules of the extracellular space and soluble proteins. MMP-1 activates the protease-activated receptor-1 (PAR1) (Nagase et al., 2006).

MMP-1 has high specificity against fibrillar collagens and other proteins of the extracellular space such as proteoglycans (Spinale, 2007).

Gelatinases easily digest gelatin using three repetitions of the fibronectin of type II. Gelatinases also digest several other molecules of the extracellular space, including collagen of type IV, V, XI, and laminin.

Stromelysins have a similar domain layout as collagenases, but they do not split the interstitial collagens (Nagase et al., 2006).

MMP-9 has an affinity to denatured fibrillar collagen, mainly to membrane proteins. MMP-9 also has proteolytic activity against elastin, and proteoglycans (Perrotta et al., 2016).

MMP-3 acts on all basal membrane proteins, elastin and proteoglycans. MMP-3 has the ability to induce proteolytic activation of another MMP. (Spinale, 2007). MMP-3 digests several molecules of the extracellular space and participates in proMMP activation (Nagase et al., 2006).

MMP-3 degrades collagen of type II, III, IV, IX and X, proteoglycans, fibronectin, laminin, and elastin. In addition, MMP-3 can activate other MMP, such as MMP-1, MMP-7, and MMP-9, playing an important role in tissue remodeling (Ye et al., 1996). In addition to the classical activity, MMP-3 can enter cell nuclei and control transcription (Eguchi et al, 2008).

The MMP-3 gene has polymorphism, caused by fluctuations in the number of adenosines. *In vitro*, functional analyses have shown that the 5A allele has a higher promoter activity compared to the 6A allele (Ye et al., 1996). In some studies, individuals with 5A allele have been shown to be hypersensitive to acute myocardial infarction and abdominal

aortic aneurysm (Terashima et al., 1999; Yoon et al., 1999). In turn, it has been found that the 6A allele is associated with diseases characterized by lower expression of MMP-3, such as coronary atherosclerosis (Ye et al., 1996; Humphries et al., 1998; de Maat et al., 1999).

MMP activity is regulated by 2 major types of endogenous inhibitors:  $\alpha$ 2-macroglobulin and TIMP. MMP activity in the liquid phase is mainly regulated by  $\alpha$ 2-macroglobulin and related proteins. The protein is linked to the macroglobulin and this complex is rapidly eliminated by low-density lipoprotein receptor-related protein-1.

TIMP consists of 184–194 amino acids; they are divided into N-terminal and C-terminal subdomains. TIMP inhibits all MMP tested so far (Solache-Berrocal et al., 2016).

In a previous study, patients with aortic valve stenosis were evaluated and it was found how the MMP-1 polymorphism affects the development of AVS. Analyzing the calcium content of the aortic valves obtained during surgery and deoxyribonucleic acid (DNA) in the peripheral blood, it was determined that the 1G allele would have a protective effect against calcium deposition. Individuals with 2G allele (homozygous and heterozygous) had higher levels of calcium in the stenotic valves. Further studies could show whether 1G>2G MMP-1 polymorphism can be used to predict possible development of the aortic valve stenosis (Solache-Berrocal et al., 2016).

Researching the valves obtained during surgery from the patients with calcific aortic valve stenosis it was found that MMP-9 was almost completely localized in or around the calcification regions. MMP-9 may be thought to play an important role in promoting fibrotic and calcification remodeling of the extracellular space (Perrotta et al., 2016).

### **1.5.5 Thioredoxin reductase-1 (TrxR1)**

Superoxide (one of the most active ROS molecules) inactivates NO and promotes endothelial dysfunction. NO with "uncoupled" NO synthase (NOS) is split from L-arginine. There are 3 forms of NO involved in the homeostasis: inhibits contraction and growth of the smooth muscle cells; reduces platelet aggregation; reduces the adhesion of inflammatory cells to the endothelium. Patients with aortic stenosis have been shown to have elevated levels of endogenous inhibitor of NOS (Chester, 2011).

Interaction between ROS, endothelium, and antioxidants is important in the disease developmental process. Superoxide reacts with NO and forms peroxynitrite (ONOO<sup>-</sup>) - a powerful oxidant that further promotes apoptosis or necrosis. Mitochondrial superoxide dismutases (MnSOD) should suppress this binding. This formation of the peroxynitrite occurs more quickly than superoxide dismutase (SOD) is able to convert superoxide into hydrogen

peroxide ( $H_2O_2$ ) or normal oxygen. Peroxynitrite promotes loss of the bioactive NO and acts directly cytotoxically. Peroxynitrite inactivates SOD, thus further increasing superoxide levels. Normally superoxide is produced in mitochondria as a by-product of oxygen metabolism. SOD plays an important role as antioxidants. There are three forms of SOD: SOD1 is found in the cytoplasm, SOD2 in mitochondria and SOD3 - extracellularly.

Also, xanthine oxidase, NADPH oxidases, and cytochromes are capable to produce superoxide. In order to maintain homeostasis, a balance must be achieved between the production and consumption of ROS.

Superoxide levels may be increased by enzymes such as nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), a component of ROS stimulated by Ang II, xanthine oxidase, cyclooxygenase, “uncoupled” NO synthase. In the inflammatory conditions, leukocytes can also increase superoxide levels. Ang II causes oxidative stress and endothelial dysfunction, its action can be reduced by angiotensin-converting enzyme 2 (ACE 2), which converts Ang II into vasoprotective peptide angiotensin (1-7) (Heistad et al., 2009; Silva et al., 2012).

There is a hypothesis that SOD by forming  $H_2O_2$  can contribute to vasodilatation. In the conditions of oxidative stress, the SOD level is usually elevated. In histological materials, it can be found that the superoxide in the stenotic valves is around the calcification zones. The key role in the development of aortic valve stenosis is played by “uncoupled” NOS and not by NADPH; see Figure 1.4. In the calcific valve, the release and activity of all three SOD isoforms are markedly reduced, contributing to oxidative stress. Oxidative stress contributes to fibrosis and in addition to MMP activity (Heistad et al., 2009).

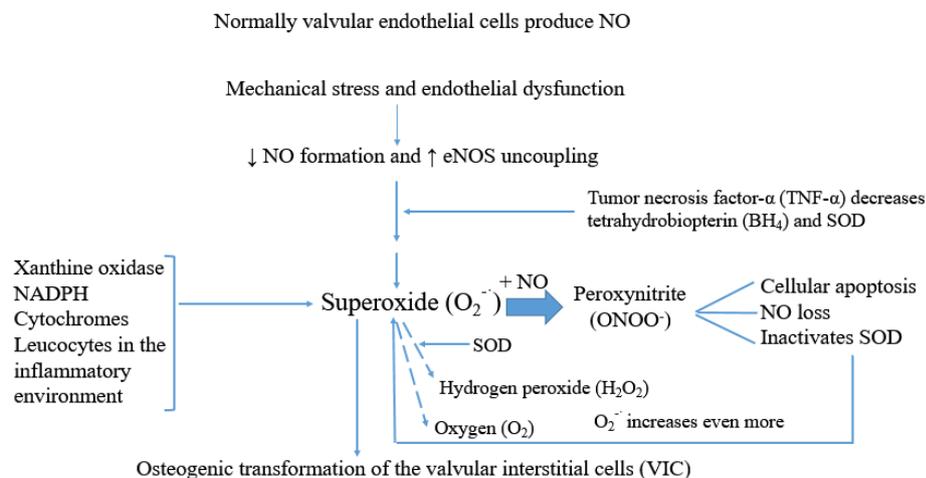


Figure 1.4 Role of eNO and  $O_2^{\cdot -}$  in the process of aortic valve stenosis (Heistad et al., 2009; Farrar et al., 2015)

Inflammatory cytokines, TNF- $\alpha$  and IL-6 reduce the release of NOS (Hulin et al., 2018). TNF- $\alpha$  causes loss of the protective function of the valvular interstitial cells, chronic inflammation, and osteogenic activation by reducing SOD and tetrahydrobiopterin (BH4) (Farrar et al., 2015).

Already publication of 2008 proves the role of oxidative stress in calcified AV, but with a different mechanism than in atherosclerosis. In the tissue histological examinations, high levels of superoxide around calcific zones are found, which is associated with the myofibroblasts activation. Unlike in atherosclerosis, in the case of aortic valve stenosis, the increase in oxidative stress is not associated with NADPH activity. In the case of aortic valve stenosis, oxidative stress is thought to be associated with a decrease in the protection of antioxidant mechanisms and an increase of NOS uncoupling. In healthy, intact valves, superoxide was in low concentration and evenly distributed, but in calcific valves, superoxide was localized around calcified zones and in twice high concentration. There were no NADPH activities in the calcified regions. The antioxidant mechanisms of the calcified areas were attenuated, SOD activity and expression of all three SOD isoforms were significantly reduced (Miller et al., 2008).

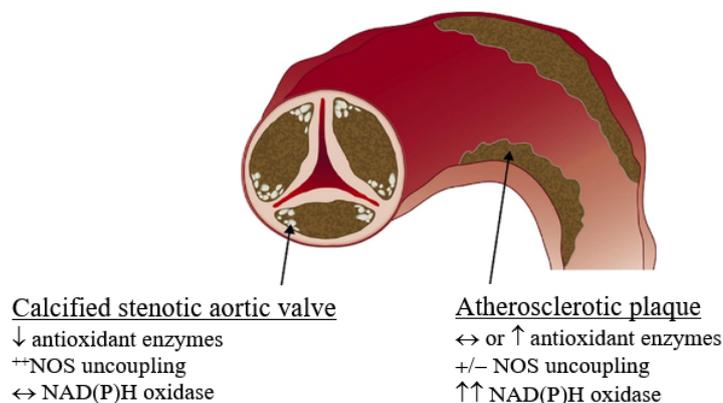


Figure 1.5 Changes caused by oxidative stress in the process of aortic valve stenosis and atherosclerosis (Heistad et al., 2009; Miller et al., 2008)

There is a histological study in which in the case of aortic valve stenosis changes of two types in the valves are found – in one part the calcinosis affects more the valvular base whereas the other – the free part of the cusps; see Figure 1.5. The second form is characterized by very high ROS activity (Takata et al., 2016).

The thioredoxin system consists of NADPH, TrxR, and Trx, it protects against oxidative stress. The key role of thioredoxin (Trx) is to protect cells from the oxidative stress. Trx depends on the action of thioredoxin reductase (TrxR) to reduce peroxide, thus affecting metabolism, reducing inflammation, proliferation, and apoptosis.

Humans have three TrxR isomers: TrxR1 (cytosol), TrxR2 (mitochondrial) and TrxR3. TrxR is a protein that needs selenium to work. Selenium deficiency reduces the antioxidant protection of TrxR. In non-stressed cells, the reduced form of Trx may bind to apoptosis signal-regulated kinase 1 (ASK1) and directly inhibit apoptosis-promoting kinases and acting as a negative kinase activity regulator. Under oxidative stress conditions, Trx is oxidized and unable to bind to and inhibit the activity of apoptosis-promoting kinases, and as a response to stress is kinase-induced cell apoptosis (Lee et al., 2013).

Trx interacting proteins (TXNIP) act as a physiological inhibitor of Trx. In addition, TXNIP effects are partially independent of Trx – they include direct activation of the inflammation and inhibition of glucose uptake. TXNIP interacts with the NO signaling system and suppresses NO effects. TXNIP production is modulated by redox stress, glucose levels, hypoxia and several inflammatory cytokines (Chong et al., 2014).

### **1.5.6 Myeloperoxidase (MPO)**

Myeloperoxidase is associated with both inflammation and oxidative stress as it is located in leukocytes. MPO is encoded by the MPO gene of the 17th chromosome. MPO is stored in the azophilic granules of the neutrophil leukocytes and is excreted in the extracellular space during degranulation. MPO contains a calcium binding agent (Strzepa et al., 2017).

Myeloperoxidase – a pro-oxidant enzyme is release from activated neutrophils and macrophages and aggravates HDL-C protection. The antioxidant properties of HDL-C are mainly related to paraoxonase-1 (PON-1), an enzyme associated with HDL-C. Significantly lower PON-1 concentrations and higher MPO levels are found in the patients with aortic valve stenosis. Patients with aortic valve stenosis have an inverse MPO and PON-1 ratio that confirms the imbalance (disbalance) between prooxidants and antioxidants and can contribute to the development of aortic valve stenosis (Matsushita et al., 2018).

MPO catalyzes the conversion of hydrogen peroxide ( $H_2O_2$ ) into hypochlorous acid. MPO from  $H_2O_2$  and chlorine ( $Cl^-$ ) produces hypochlorous acid (HOCl), which oxidizes tyrosine. HOCl and tyrosine radicals are cytotoxic. HOCl can cause oxidative damage. MPO,

by oxidizing apoA-1, reduces HDL-C-induced inhibition of inflammation and cellular apoptosis.

In conditions of sterile inflammation, it is believed that MPO and the oxidants produced by it contribute to inflammation and tissue damage; see Figure 1.6. HOCl increases endothelial permeability. MPO accumulates in the extracellular space, MPO reduces NO bioavailability, and it promotes endothelial dysfunction and remodeling of the extracellular space (Strzepa et al., 2017).

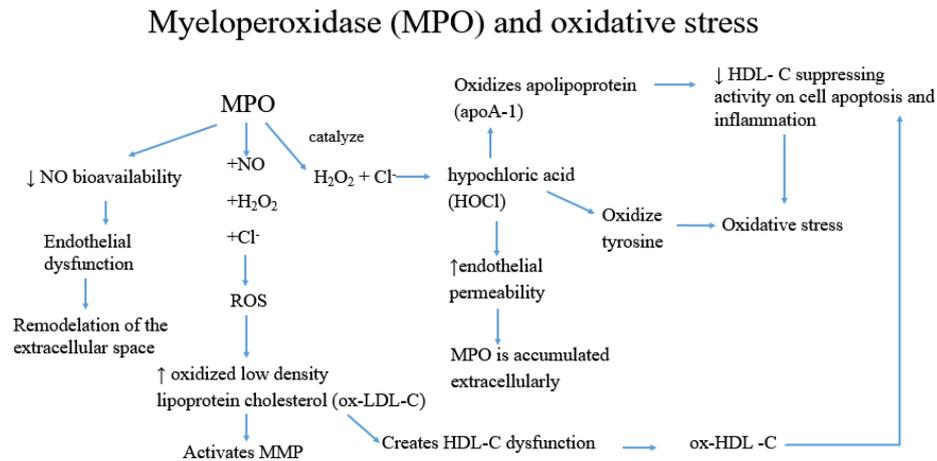


Figure 1.6 **Myeloperoxidase (MPO) and the role of oxidative stress in the process of aortic valve stenosis (Strzepa et al., 2017; Schindhelm et al., 2009; Matsushita et al., 2018)**

The undesirable effect of MPO in the blood vessels is that macrophages uptake ox-LDL and form foam cells. Ox-LDL by the help of ROS is made from LDL-C.

In inflammatory conditions, macrophages release MPO, which forms ROS using  $H_2O_2$ ,  $Cl^-$ , NO and it even more enhances the formation of ox-LDL, as the result HDL-C becomes dysfunctional, ox-HDL forms and decreases the protective effect of HDL-C (Schindhelm et al., 2009).

MPO has been studied in the patients with acute coronary syndrome (ACS). MPO levels are independent of troponin levels and increase even before troponin levels – this may help in the selection of therapy tactics for patients with unstable angina. Unlike CRP and fibrinogen levels that remain high relatively longer for ACS patients, MPO levels return to normal after about one week. MPO can be a marker of acute period (instability of the atherosclerotic plaque). MPO is likely to not be specific to cardiovascular diseases since activation of neutrophils and macrophages can occur in any process of infection, inflammation, and infiltration (Loria et al., 2007).

## 2. MATERIAL AND METHODS

### 2.1 Study population

The clinically–analytical study “Inflammatory and Non-Inflammatory Risk Factors in Acquired Aortic Valve Stenosis” is a mixed prospective case–control study. The study was carried out with the permission of the Riga Stradins University Ethics Committee on Research on Humans, the date of the meeting of the Ethics Committee 12.09.2013. (See Appendix No. 4).

The study protocol conforms to the Ethical Guidelines of the 1975 Declaration of Helsinki. From January 1, 2013 to December 31, 2016, patients were selected in various hospitals and outpatient institutions of Latvia – Vidzeme Regional Hospital (Valmiera), P. Stradins Clinical University Hospital (Riga) and at the polyclinic – Zemgale Health Centre (Jelgava). A total of 102 patients were volunteered according to the inclusion and exclusion criteria and divided into two main groups: the control group and the AV stenosis group. In the control group, patients without AV stenosis aged 50 to 80 years were included, corresponding to the average age of AV stenosis patients according to the 2012 European Society of Cardiology and the European Association for Cardio–Thoracic Surgery Guidelines for the Management of Valvular Heart Disease (Vahanian et al., 2012). The control group was created to determine reference values of cytokines and to compare them with the results of patients with aortic stenosis. 28 (27%) men and 74 (73%) women were included in the study; see Table 2.1. Written informed consent to participate in the study was obtained from each individual enrolled in the current study.

Table 2.1

**Baseline characteristics of study subjects**

		Control, n = 50	AV mild stenosis, n = 18	AV moderate stenosis, n = 19	AV severe stenosis, n = 15
Gender, (%)	Male	11 (22.0)	2 (11.1)	8 (42.1)	7 (46.7)
	Female	39 (78.0)	16 (88.9)	11 (57.9)	8 (53.3)
Age, years	<i>Mdn</i> ( <i>IQR</i> )	64 (57–75)	71 (65–75)	74 (65–79)	65 (60–74)

## 2.2. Inclusion and exclusion criteria for the study subjects

At the beginning of the study, a disease anamnesis was collected from each person in both study groups, a questionnaire was filled on the condition of the cardiovascular system and questions related to inclusion and exclusion criteria, used medications and performed examinations (see Appendix No. 3). Before inclusion in the study, the following data were obtained/tests performed and analyzed:

- patient's general / demographic data,
- data of the subjective status (history of cardiovascular diseases, targeted questions for exploration of cardiovascular diseases),
- laboratory data [complete blood count + erythrocyte sedimentation rate (ESR), blood chemistry test],
- the “ankle–brachial’ index (ABI) was determined,
- bilaterally measured intima-media thickness (IMT) in the common carotid artery (*arteria carotis communis*),
- echocardiography that confirms or excludes aortic valve stenosis.

Individuals in the control group were included according to the echocardiographically confirmed healthy aortic valve. Exclusion criteria for both groups – the control and the AVS group – were the following:

- obesity,
- connective tissue diseases, infectious diseases, oncological diseases,
- diabetes mellitus
- thyroid dysfunction
- severe, moderate and uncontrolled arterial hypertension,
- history of acute coronary syndrome and manifested coronary heart disease,
- left ventricular systolic dysfunction with reduced EF below 50%,
- cerebral infarction and transient ischemic attack,
- echocardiographically confirmed cardiomyopathy,
- visual AV sclerosis,
- pathologies of other valves,
- no lipid lowering therapy is used

The exclusion criterion in the patient group with aortic valve stenosis was congenital (for example, bicuspid aortic valve) and rheumatic aortic valve damage.

ABI (ankle–brachial index) was determined for all study subjects before inclusion in the study. Initially, this index was proposed for non-invasive diagnostics of peripheral arterial

disease (PAS) of the lower limbs. Later it was proved that ABI is an indicator of atherosclerosis in other blood vessels and can serve as a prediction marker for cardiovascular events and functional disorders. ABI is the ratio of systolic pressures measured at ankle level on *a. tibialis posterior* or *a. dorsal pedis* and on the brachial arteries. The obtained results were evaluated according to the recommendations of the American Heart Association (Aboyans et al., 2012): 1.4 and > indicates calcified, non-compressible arteries; 1.0–1.39 normal ABI; if there is claudication, then an exertional test is performed; 0.91–0.99 possibly, there is a peripheral arterial disease; < 0.9 there is a peripheral arterial disease;  $\leq 0.5$  severe ischemia and < 0.4 critical ischemia.

The intima-media thickness (IMT) in the common carotid artery was determined in all individuals involved in the study, which was considered normal if < 0.9 mm.

For all subjects involved in the study, the ejection fraction (EF) was determined during the echocardiography examination using the Simpson method – the Biplane disc method (optional method), dividing the LV cavity into many elliptical discs (usually 20) and analyzing changes in their total volume. One diameter of each disc is set from the A4CH view (apical four-chamber view), the second from the A2CH view (apical two-chamber view). Manual tracing of the LV cavity endocardium at end-systole and end-diastole is done, excluding papillary muscles. Basal level is a straight line connecting the lateral and septal edges of the MV ring at the A4CH view and the anterior and inferior edges at the A2CH view.

The stroke volume (SV) was also determined. The stroke volume (norm: 55–100 ml) is the amount of blood pumped by the left ventricle of the heart in one contraction. SV is determined by subtracting left ventricular end systole volume (LV ESV) from left ventricular end diastole volume (LV EDV) by applying the left ventricular outflow tract (LVOT) method. Using zoom of the LVOT, measure its diameter. The diameter is measured during the systole 5–10 mm from fibrous ring of the aortic valve. The LVOT area is calculated:  $\text{LVOT area} = \pi \times (\text{LVOT diameter})^2/4$ . The LVOT VTI (velocity time integral) is measured in the A5CH view using pulse wave Doppler. The ultrasonic beam must go parallel to the blood flow and then tracing the flow. SV is calculated using this formula:  $\text{SV} = \pi \times (\text{LVOT diameter})^2/4 \times \text{LVOT VTI}$ .

## 2.3 Diagnosis and evaluation of the aortic valve stenosis

Echocardiography with data saving was done to all persons prior to inclusion in the study, using the echocardiography devices *GE VIVID 7 Dimension* and *Philips IE 33*. Each echocardiography examination was evaluated by two echocardiography specialists. Patients with aortic valve stenosis were divided into three subgroups (mild, moderate and severe), depending on the severity of the AVS, according to the criteria of the 2012 European Society of Cardiology and the European Association for Cardio–Thoracic Surgery Guidelines for the management of valvular heart disease criteria:

- aortic jet velocity – Vmax (m/s),
- mean pressure gradient – PG mean (mmHg),
- aortic valve area – AVA (cm<sup>2</sup>),
- indexed aortic valve area – indexed AVA (cm<sup>2</sup>/m<sup>2</sup>).

Severe AVS: Vmax > 4 m/s, PG mean > 40 mmHg, AVA < 1.0 cm<sup>2</sup>, indexed AVA < 0.6 cm<sup>2</sup>/m<sup>2</sup>; moderate AVS: Vmax 3.0–4.0 m/s, PG mean 20–40 mmHg, AVA 1.0–1.5 cm<sup>2</sup>, indexed AVA 0.60–0.85 cm<sup>2</sup>/m<sup>2</sup>; mild AVS: Vmax 2.5–2.9 m/s, PG mean < 20 mmHg, AVA > 1.5 cm<sup>2</sup>, indexed AVA > 0.85 cm<sup>2</sup>/m<sup>2</sup>.

## 2.4 Study scheme

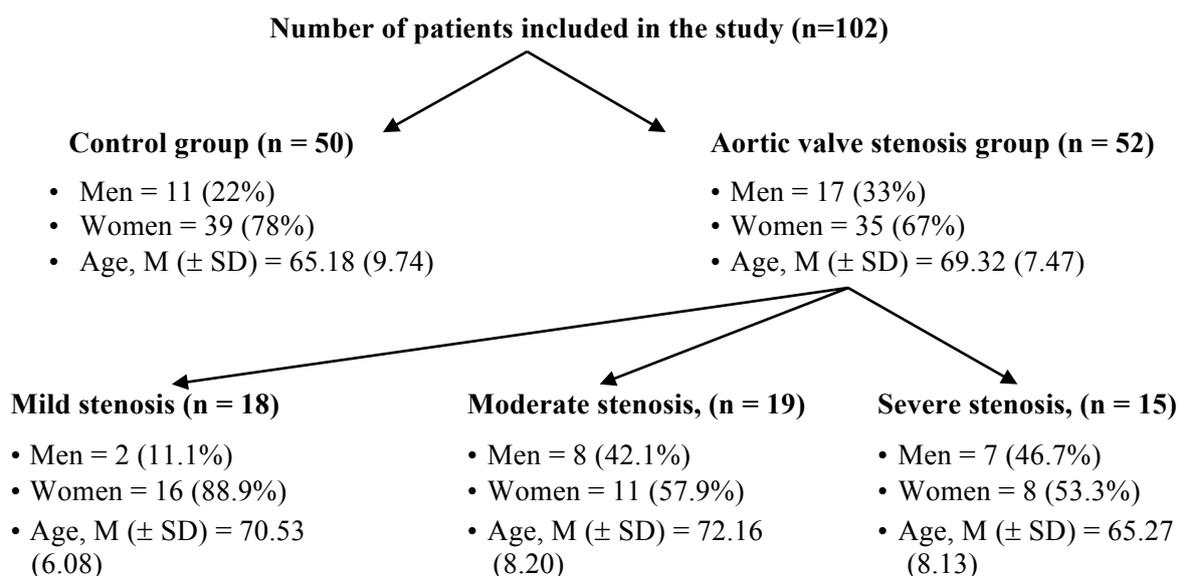


Figure 2.1 Study scheme

## **2.5 Material used in the study**

Blood serum and plasma were used for the study analyses. Samples of peripheral blood were collected from all subjects involved in the study. Venous blood was taken on an empty stomach in the morning. Plasma and serum were obtained from the blood samples. Blood for the serum collection was taken in vacutainer without an anticoagulant with a special blood coagulant. One hour or 60 minutes after the blood was collected, serum was obtained by centrifuging the blood samples for 10 minutes at  $\sim 1000\text{--}2000 \times g$ . Vacutainers with EDTA was used for plasma collection. Cells were separated from plasma by centrifugation at  $1000\text{--}2000 \times g$  for 15 minutes using a cooled centrifuge and collecting the supernatant of the centrifuge – plasma was collected.

The obtained serum and plasma were divided into conical Eppendorf tubes (their volume was 1.5 ml). For each study subject, 7 Eppendorf tubes with 200  $\mu\text{L}$  of serum and 5 Eppendorf tubes with 200 $\mu\text{L}$  of plasma were prepared, Eppendorf tubes were placed in a special Eppendorf box and frozen immediately after the material was collected at  $-20^{\circ}\text{C}$  (Eppendorf tubes were labeled with "short-accurate identification") – for each patient specific and different for the control and aortic stenosis groups, while creating an identification deciphering – list on paper and electronic format. The resulting serum and plasma samples were stored in a refrigerator (with temperature control) at  $-80^{\circ}\text{C}$  (in the Laboratory of Physiology and Biochemistry of Rīga Stradiņš University).

## **2.6 Determination of the clinical laboratory parameters**

For all subjects enrolled in the study a complete blood count (erythrocyte count, hemoglobin level, platelet count, leukocyte count, hematocrit) + erythrocyte sedimentation rate (EGA) and blood biochemical analysis (glucose, fibrinogen, cholesterol with its fractions, C-reactive protein) were analysed via standard methods at the certified laboratory of the Pauls Stradins Clinical University Hospital (Riga, Latvia); see Table 2.2.

Total cholesterol (TC) was determined using a commercially available test (CHOL\_2, Siemens Healthcare Diagnostics Inc., Tarrytown, USA) based on an enzymatic method. Triglycerides (TG) were determined using a commercially available test (TRIG\_2, Siemens Healthcare Diagnostics Inc., Tarrytown, USA) based on the Fossati three-step enzymatic reaction with a Trinder endpoint. High density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) were determined using commercially available tests (D-HDL and DLDL, Siemens Healthcare Diagnostics Inc., Tarrytown, USA) using the direct

method. Total cholesterol, triglycerides, HDL-C and LDL-C were determined using the Siemens Advia 1800 analyzer (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) according to the manufacturer's protocol.

The C-reactive protein was determined using the particle enhanced turbidimetric method. Human CRP was determined using a commercially available assay where CRP was agglutinated with latex particles coated with monoclonal anti-CRP antibodies (CRPLX, Roche Diagnostics, Basel, Switzerland). The precipitate was determined turbidimetrically at 552 nm. The method was performed using the Roche Cobas Integra 400 Plus analyzer (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol.

Table 2.2

**Reference intervals for determined laboratory parameters**

Determined parameter	Testing method	Reference interval / measure unit
red blood cell count	Determination of the cell number by hydrodynamic focusing DC (Direct Current) method	for male 4.50–5.90 $\times 10^{12}/L$ for female 3.90–5.00 $\times 10^{12}/L$
hemoglobin level	SLS-Hb method	for men 13.5–17.5 g/dL for women 12.0–16.0 g/dL
hematocrit	Calculation with RBC pulse height determination method	for men 39–50% for women 35–45%
leucocyte count	flow cytometry method	4.0–10.0 $\times 10^9/L$
platelet count	Determination of the cell number by hydrodynamic focusing DC method	150–400 $\times 10^9/L$
ESR	capillary photometric method	1–30 mm/h
glucose	hexokinase method	4.1–5.9 mmol/l
CRP	turbidimetry method	0–5.0 mg/l
triglycerides	Fossati three-step enzymatic reaction	< 1.7 mmol/l
Total cholesterol	enzymatic reaction	< 5.0 mmol/l
HDL-C	direct method	$\geq 1.2$ mmol/l for female $\geq 1.0$ mmol/l for male
LDL-C	direct method	< 1.8 mmol/l if very high risk < 2.5 mmol/l, if high risk < 3.0 mmol/l if low or moderate risk
fibrinogen	Modification of the Klaus method	1.8–3.6 g/l

Changes in the results beyond these reference intervals were interpreted as decreased or increased levels, respectively.

## 2.7 Detection of cell-produced regulatory molecules (cytokines)

The analyzes were determined in the biochemistry laboratory of the Department of Human Physiology and Biochemistry of Riga Stradins University and in the biochemistry laboratory of the Institute of Microbiology and Virology of Riga Stradins University. Chemerin (ng/ml), FGF-21 (pg/ml), MMP-1 (pg/ml), MMP-3 (ng/ml), MMP-9 (pg/ml), TIMP-1 (pg/ml), TIMP-3 (pg/ml), TGF (pg/ml) was determined in the subject's blood serum and MPO (ng/ml), TrxR1 (ng/ml) was determined in the plasma by enzyme-linked immunosorbent assay (ELISA) but hs-CRP (mg/l) was determined in the blood serum using Luminex xMAP technology.

For the determination of MMP-1 in the blood serum the human MMP-1 ELISA Assay Kit, Cat. # EHMMP1, *Pierce (Thermo Fisher Scientific)*, USA was used; for the determination of MMP-3 in the blood serum the human MMP-3 ELISA Assay Kit, Cat. #ELH-MMP3, *RayBiotech*, USA was used; for the determination of MMP-9 in the blood serum the human MMP-9 ELISA Assay Kit, Cat. #KHC3061, *Invitrogen (Thermo Fisher Scientific)*, USA was used; for the determination of TIMP-1 in the blood serum the human TIMP-1 ELISA Assay Kit, Cat. #ab100651, *Abcam*, UK was used; for the determination of TIMP-3 in the blood serum the human TIMP-3 ELISA Assay Kit, Cat. #ab119608, *Abcam*, UK was used; for the determination of chemerin in the blood serum the human chemerin ELISA Assay Kit, Cat. #EZHCMRN-57 K, *Merck Millipore*, USA was used; for the determination of FGF-21 in the blood serum the human FGF-21 ELISA Assay Kit, Cat. #EZHFGF21-19k, *Merck Millipore*, USA was used; for the determination of TrxR1 in the blood plasma the human thyredoxin-1 ELISA Assay Kit, prod. #RAB1756/Lot #0522F2032, *Sigma-Aldrich, Inc.*, USA was used; for the determination of MPO in the blood plasma the human myeloperoxidase ELISA Assay Kit, Item No. 501410, *Cayman chemical*, USA was used; for the determination of hs-CRP in the blood serum the Luminex xMAP technology (*Luminex TM 200; Austin, Texas*) and Assay Kit, Cat. # HCVD3MAG-67K, *Milliplex MAP*, USA) were used.

The results were obtained using *Infinite 200 PRO multimode* reader (*Tecan Group, Mannedorf, Switzerland*) and *Multiskan Ascent* microplate reader (*Thermo Labsystems, Helsinki, Finland*). The procedures were performed according to the protocol of the ELISA kit manufacturer.

## 2.8 Statistical analysis of the data obtained in the study

All graphical images, calculations, and statistical analyzes included in the study were performed using IBM SPSS (Statistical Package for the Social Sciences) Statistics 23 (*IBM Corp.*, Armonk, NY, USA) and GraphPad Prism 7.0 software (*GraphPad Software*, San Diego, CA, USA) as well as Microsoft Excel 2013 (*Microsoft*, Redmond, WA, USA).

Normal distribution of data was tested by Brown-Forsythe and Bartlett tests or by Kolmogorov-Smyrnov one sample test. If the sample dispersion corresponded to the normal distribution, they were showed as the mean value (M) and standard deviation ( $\pm$  SD). Otherwise, the data is displayed as a median and interquartile range (IQR).

Statistical analysis of the normal distribution data (parametric data): the mean values between two separate patient groups were compared using the Student's test or t-test; mean values between 3 and more separate patient groups were compared using a one-way analysis of variance (ANOVA).

Statistical analysis of data that which values did not correspond to the normal distribution (non-parametric data): the mean values between two separate patient groups were compared using the Mann-Whitney U-test; the mean values between 3 and more separate patient groups were compared using the Kruskal-Wallis H-test.

In all cases, the Benjamin, Krieger and Yekutieli statistical analysis method was used AVS a post-hoc analysis.

The geometric mean and geometric standard deviation were used to reflect the results and data which by their distribution were more in line with logarithmic data distribution.

The P value of less than 0.05 ( $p < 0.05$ ) was considered statistically significant for all used statistical tests. The method of correlation analysis was used to study the relationship of quantitative variables. Depending on the distribution of the data, we used a parametric (*Pearson*) or non-parametric (*Spearman*) correlation analysis.

The following parameters were used to characterize the diagnostic markers of the AV stenosis: ROC curves, area under the ROC curve, cutoff value obtained from them, sensitivity and specificity, positive and negative predictive value, reaching the relevant cutoff values. The accuracy of the diagnostic test was evaluated by the area under the ROC curve values, according to this classification: 0.90–1 = excellent; 0.80–0.90 = good; 0.70–0.80 = fair; 0.60–0.70 = poor; and  $< 0.60$  = no diagnostic value.

### 3. RESULTS

#### 3.1. Baseline characteristics of study patients and control group individuals

The basic data of the subjects included in the study are presented in Table 3.1. A total of 102 patients were enrolled in the study – 50 persons without AVS in the control group and 52 patients in the AVS group. Patients in the AVS group were divided into three groups of severities of the AV stenosis: 18 patients with mild AVS, 19 with moderate AVS and 15 patients with severe AVS. Focusing on the strict clinical and echocardiography exclusion criteria allowed us to choose the most appropriate study groups. Although the number of patients in the subgroups was limited, the results of statistical analysis of the data revealed significant p values ranging from  $p < 0.05$  –  $p < 0.0001$ .

The average age of patients in all aortic stenosis groups and in the control group is similar, and the mean body mass index (BMI) does not differ between groups. The mean values of triglycerides and low density lipoprotein cholesterol (LDL-C) are not statistically different between stenosis groups and control group. The groups are similar for the mean values of the ejection fraction (EF) determined by the Simpson's method and the stroke volume (SV) measured by the left ventricular outflow method as well as according to the inclusion and exclusion criteria.

Table 3.1

**Basic data of individuals in the control group and patients in the AVS group**

		Control, n = 50	AV mild stenosis, n = 18	AV moderate stenosis, n = 19	AV severe stenosis, n = 15
Gender, (%)	Male	11 (22.0)	2 (11.1)	8 (42.1)	7 (46.7)
	Female	39 (78.0)	16 (88.9)	11 (57.9)	8 (53.3)
Age, years	<i>Mdn</i> ( <i>IQR</i> )	62 (57–75)	71 (65–75)	74 (65–79)	65 (60–74)
<sup>a</sup> BMI	<i>M</i> ( $\pm$ <i>SD</i> )	26.04 (4.31)	27.39 (3.10)	25.81 (4.58)	27.40 (3.18)
<i>P value vs control</i>			<i>p</i> = 0.399	<i>p</i> = 0.682	<i>p</i> = 0.869

		Control, n = 50	AV mild stenosis, n = 18	AV moderate stenosis, n = 19	AV severe stenosis, n = 15
<sup>b</sup> ZBL-H, mmol/l, <i>P value vs control</i>	<i>M (± SD)</i>	3.28 (1.18)	3.05 (0.97) <i>p &gt; 0.999</i>	2.59 (0.92) <i>p = 0.057</i>	3.10 (1.12) <i>p &gt; 0.999</i>
<sup>c</sup> TG, mmol/l, <i>P value vs control</i>	<i>M (± SD)</i>	1.47 (0.71)	1.64 (0.84) <i>p = 0.406</i>	1.11 (0.56) <i>p = 0.178</i>	1.27 (0.57) <i>p = 0.406</i>
<sup>d</sup> KH, mmol/l, <i>P value vs control</i>	<i>M (± SD)</i>	5.49 (1.28)	5.01 (1.34) <i>p = 0.056</i>	4.21 (1.18) <b><i>p = 0.001</i></b>	4.68 (1.08) <b><i>p = 0.016</i></b>
<sup>e</sup> CRP, mg/l, <i>P value vs control</i>	<i>Mdn (IQR)</i>	0.95 (0.50–2.55)	3.00 (1.50–3.70) <b><i>p = 0.016</i></b>	1.75 (0.37–2.97) <i>p = 0.37</i>	1.2 (0.70 – 5.00) <i>p = 0.17</i>
<sup>f</sup> SV, ml, <i>P value vs control</i>	<i>Mdn (IQR)</i>	96.5 (90.0–106.3)	100.0 (90.0–110.0) <i>p = 0.716</i>	96.0 (88.0–100.0) <i>p = 0.375</i>	90.0 (88.0–95.0) <i>p = 0.103</i>
<sup>g</sup> EF %, <i>P value vs control</i>	<i>Mdn (IQR)</i>	63.5 (57.7–68.0)	60.0 (57.5–63.5) <i>p = 0.347</i>	61.0 (58.0–66.0) <i>p = 0.981</i>	60.0 (57.0–64.0) <i>p = 0.347</i>
<sup>h</sup> SVI <i>P value vs control</i>	<i>Mdn (IQR)</i>	52.2 (46.3–59.1)	53.6 (49.6–60.2) <i>p = 0.767</i>	49.4 (47.4–52.1) <i>p = 0.288</i>	49.7 (42.9–52.7) <i>p = 0.157</i>

<sup>a</sup> BMI (body mass index) – weight in kilograms divided by the square of the height in meters, (kg/m<sup>2</sup>);

<sup>b</sup> LDL-C – low density lipoprotein cholesterol;

<sup>c</sup> TG – Triglycerides;

<sup>d</sup> TC – Total cholesterol;

<sup>e</sup> CRP – C-reactive protein;

<sup>f</sup> SV – stroke volume, measured by left ventricular outflow method;

<sup>g</sup> EF – ejection fraction, measured by Simpson's method;

<sup>h</sup> SVI (stroke volume index) – the relation between the stroke volume (SV) and the size of the person body surface area (BSA), ml/m<sup>2</sup>

## 3.2 Results of cellular produced regulatory molecules (cytokines)

### 3.2.1 Chemerin

Circulating biomarkers are widely used to determine the risk of many diseases, including cardiovascular disease. Assuming that chemerin may affect inflammatory and calcification processes, we studied its significance and potential diagnostic value by comparing persons of the control group with patients with different severity degrees of AVS.

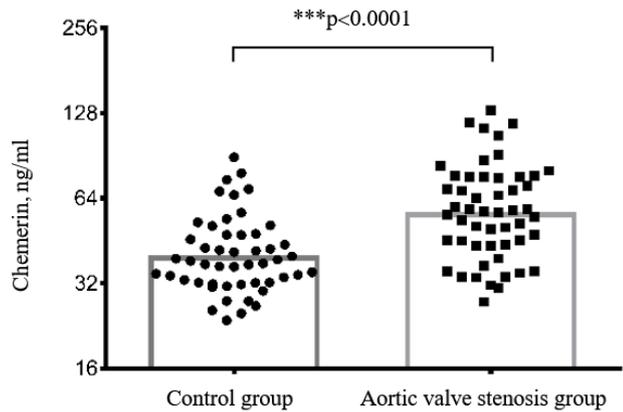


Figure 3.1 Serum chemerin level in the control and AVS groups

Comparing subjects of the control group with all AVS patients, reveal a statistically significant ( $p < 0.0001$ ) higher chemerin level in the AVS patient group; see Figure 3.1.

Analyzing chemerin levels across all three AVS severity levels (see Figure 3.2), statistically reliable differences were found comparing to the control groups. For mild stenosis, the highest level of chemerin ( $p=0.0001$ ) is obtained, but for severe stenosis the lowest chemerin level ( $p = 0.042$ ) is found comparing to the control group. Chemerin levels decrease as the severity of stenosis increases, and in patients with severe stenosis the lowest level of chemerin in the blood serum is observed. A statistically reliable difference ( $p < 0.05$ ) is obtained between chemerin levels in patients with mild and severe AV stenosis.

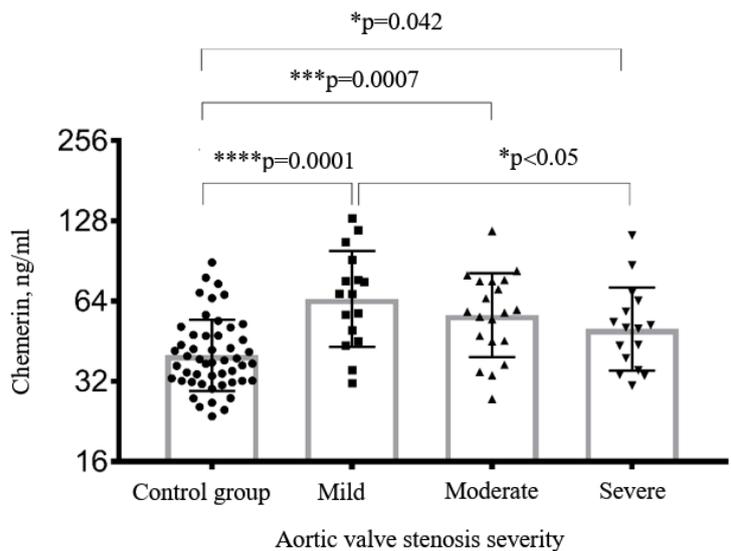


Figure 3.2 Serum chemerin level in the control group and in all AVS severity grades

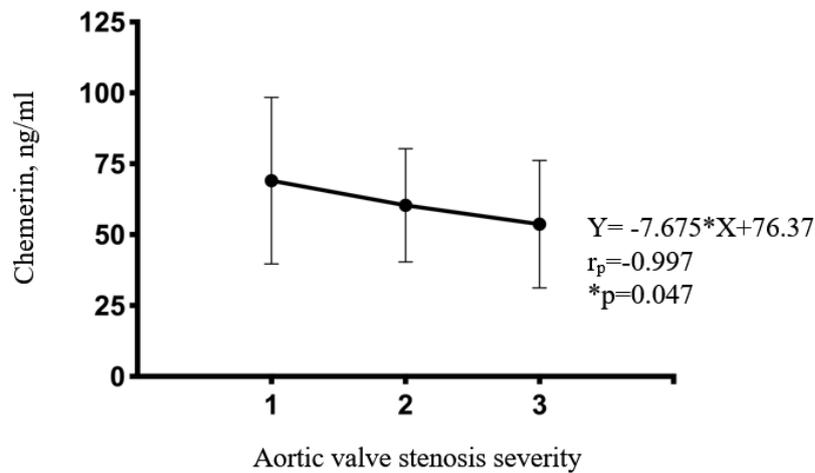


Figure 3.3 **Regression line of serum chemerin level in connection with AVS severity grade**

A linear regression analysis shows a statistically significant reduction in the chemerin levels AVS the AVS severity grade increases ( $p = 0.047$ ); see Figure 3.3.

When evaluating the potential importance of the chemerin AVS a biomarker, an analysis of the ROC (Receiver–Operating Characteristic Curves) was done. Initially, the diagnostic accuracy of the serum chemerin was assessed in the group of patients of all degrees of the aortic stenosis versus subjects of the control group; see Table 3.2 and Figure 3.4.

Table 3.2

**Sensitivity and specificity of chemerin (ng/ml) in aortic valve stenosis patients of all severity grades (mild, moderate, severe)**

<sup>a</sup> AUC (95% CI)	P value	Cutoff value	<sup>b</sup> Sp %	<sup>c</sup> Se %	<sup>d</sup> NPV %	<sup>e</sup> PPV %	Accuracy %
0.76 (0.67–0.85)	<0.001	38.60	55	80	72.2	63.6	67.5

<sup>a</sup>AUC, area under the curve; <sup>b</sup>Sp, specificity; <sup>c</sup>Se, sensitivity; <sup>d</sup>NPV, negative predictive value;

<sup>e</sup>PPV, positive predictive value

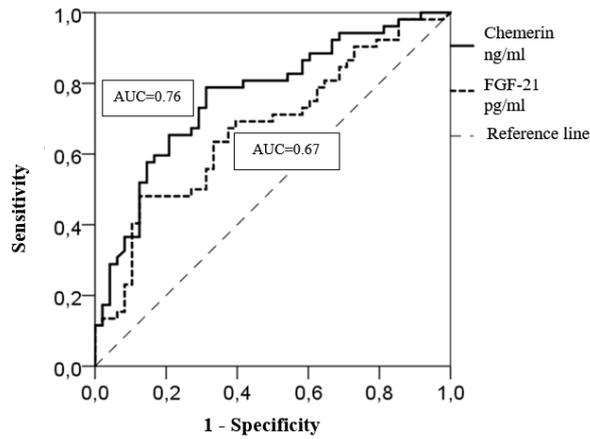


Figure 3.4 ROC analysis of chemerin and FGF-21 as diagnostic markers in AVS diagnostics (included all AVS severity grades) vs. control group

The prognostic value of the serum chemerin AVS a potential biomarker was assessed in the patients with mild AVS vs. the subjects of the control group; see Table 3.3 and Figure 3.5.

Table 3.3

**Sensitivity and specificity of chemerin (ng/ml) in mild aortic valve stenosis**

<sup>a</sup> AUC (95% CI)	P value	Cutoff value	<sup>b</sup> Sp %	<sup>c</sup> Se %	<sup>d</sup> NPV %	<sup>e</sup> PPV %	Accuracy %
0.82 (0.70–0.95)	<0.001	43.12	69	87	75.5	71.9	78

<sup>a</sup>AUC, area under the curve; <sup>b</sup>Sp, specificity; <sup>c</sup>Se, sensitivity; <sup>d</sup>NPV, negative predictive value; <sup>e</sup>PPV, positive predictive value

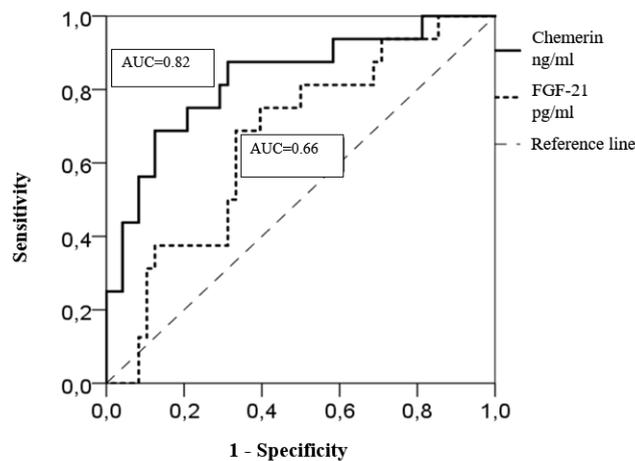


Figure 3.5 ROC analysis of chemerin and FGF-21 as diagnostic markers in mild AVS diagnostics vs. control group

When evaluating the potential importance of chemerin as a biomarker for the entire AVS patient group (including all severity grades), we obtained results that chemerin is a medium diagnostic marker: AUC = 0.76; 0.70–0.80 = fair;  $p < 0.001$ ; Sp – 55% and Se – 80%. At the same time, the ROC analysis showed that serum chemerin is a sufficiently specific and sensitive biomarker for the diagnostics of mild aortic stenosis: AUC = 0.82; 0.80–0.90 = good;  $p < 0.001$ ; sensitivity is 87% and the specificity is 69%.

### 3.2.2. Fibroblast growth factor-21

For all subjects, included in the study, in the serum FGF-21 were tested. Its significance and potential diagnostic value were studied by comparing subjects of the control group with all severity grades AVS patients.

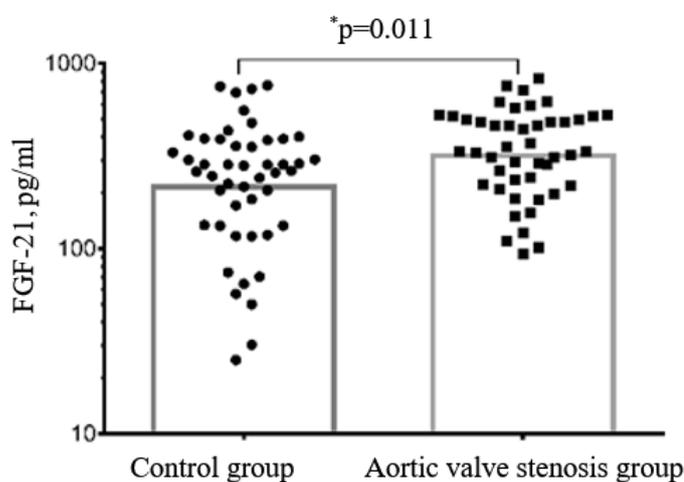


Figure 3.6 Serum FGF-21 level in the control and AVS groups

Patients with aortic valve stenosis have higher FGF-21 levels than the subjects of the control group ( $p = 0.011$ ); see Figure 3.6.

Comparing the FGF-21 serum levels in each severity degree with the subjects of the control group, a statistically significant increase in FGF-21 was observed by progression of the aortic valve stenosis: mild AVS ( $p = 0.013$ ), moderate AVS ( $p = 0.015$ ), and severe AVS ( $p = 0.003$ ); see Figure 3.7.

A linear regression analysis shows a statistically significant increase in FGF-21 serum levels from mild to severe degree of AVS ( $p = 0.0103$ ). The increase in FGF-21 in all AVS degrees confirms that FGF-21 reflects oxidative stress, tissue damage. In the severe AVS degree, when there are the most pronounced valvular tissue changes, the highest level of FGF-21 is also found; see Figure 3.8.

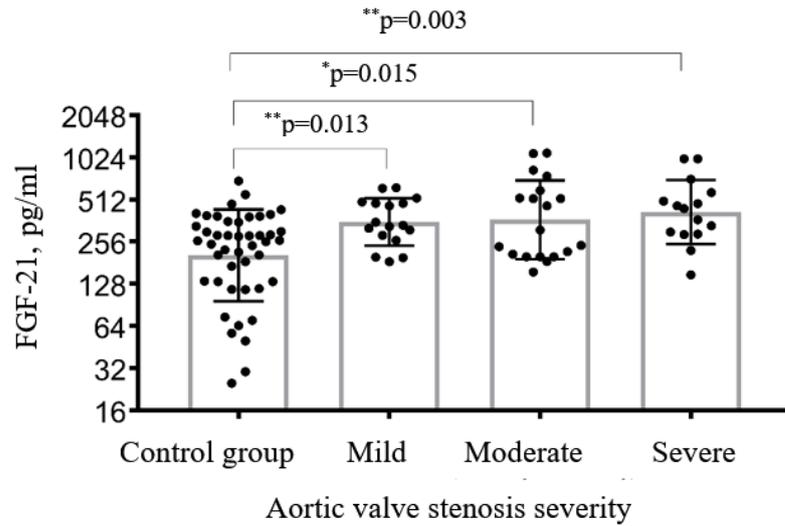


Figure 3.7 Serum FGF-21 level in the control group and in all AVS severity grades

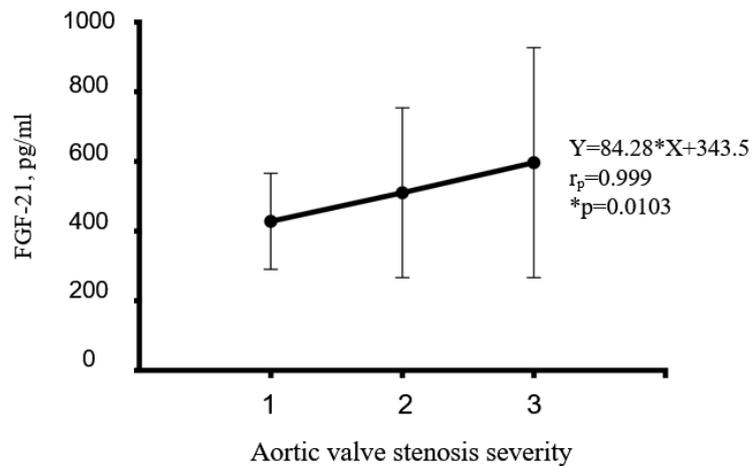


Figure 3.8 Regression line of the serum chemerin level in connection with the AVS severity grades

When evaluating the potential role of FGF-21 as a biomarker, the ROC (Receiver-Operating Characteristic Curves) analysis was performed. Initially, the diagnostic accuracy of the serum FGF-21 was assessed in the group of patients of all severity degrees of the aortic stenosis versus the subjects of the control group (see Table 3.4 and Figure 3.4).

Table 3.4

**Sensitivity and specificity of FGF–21 (pg/ml) in aortic valve stenosis patients of all severity grades (mild, moderate, severe)**

<sup>a</sup> AUC (95% CI)	P value	Cutoff value	<sup>b</sup> Sp %	<sup>c</sup> Se %	<sup>d</sup> NPV %	<sup>e</sup> PPV %	Accuracy %
0.67 (0.56–0.77)	0.003	309.83	67	61.5	61.5	66.6	64.2

<sup>a</sup> AUC, area under the curve; <sup>b</sup> Sp, specificity; <sup>c</sup> Se, sensitivity; <sup>d</sup> NPV, negative predictive value; <sup>e</sup> PPV, positive predictive value

The prognostic value of the serum FGF–21 as a potential biomarker was evaluated in the patients with mild AVS versus the subjects of the control group (see Table 3.5 and Figure 3.5).

Table 3.5

**Sensitivity and specificity of FGF–21 (pg/ml) in patients with mild aortic valve stenosis**

<sup>a</sup> AUC (95% CI)	P value	Cutoff value	<sup>b</sup> Sp %	<sup>c</sup> Se %	<sup>d</sup> NPV %	<sup>e</sup> PPV %	Accuracy %
0.66 (0.51–0.81)	0.04	283.78	61	75	64.4	65.4	68

<sup>a</sup> AUC, area under the curve; <sup>b</sup> Sp, specificity; <sup>c</sup> Se, sensitivity; <sup>d</sup> NPV, negative predictive value; <sup>e</sup> PPV, positive predictive value

When evaluating the potential role of FGF–21 as a biomarker for the entire AVS patient group (including all severity grades), we found that FGF–21 is a poor diagnostic marker: AUC = 0.67 (0.56–0.77);  $p = 0.003$ ; Sp – 67% and Se – 61.5%. The ROC analysis also showed that the serum FGF–21 has poor diagnostic marker for mild aortic stenosis: AUC = 0.66 (0.51–0.81);  $p=0.04$ ; sensitivity is 61% and the specificity is 75%. In general, FGF–21 is assessed as a poor AVS biomarker.

When analyzing the correlation between chemerin, FGF–21, and HDL–C:

- It is found in the control group that higher levels of FGF–21 are associated with higher chemerin levels ( $p = 0.019$ ;  $r_p = 0.337$ ); there is no correlation between chemerin and HDL–C, see Figure 3.9 (A).
- Also in the patient group with aortic valve stenosis a similar correlation – it was found, that higher FGF–21 level correlate with higher chemerin level ( $p = 0.012$ ;  $r_p = 0.348$ ), there was no correlation between chemerin and HDL–C, see Figure 3.9 (B).

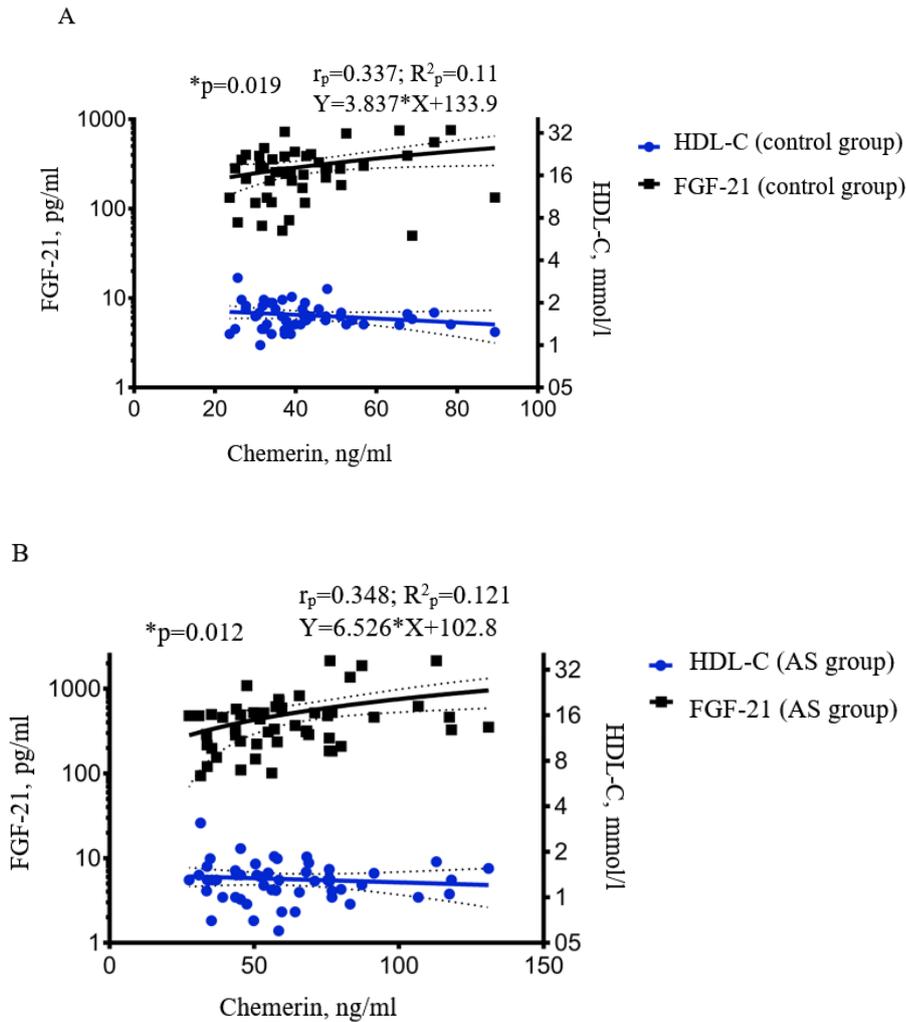


Figure 3.9 (A) Correlation between serum FGF-21 and chemerin, chemerin and HDL-C concentrations in the control group (A) and AVS group (B)

### 3.2.3 C-reactive protein

In our study, a statistically significant difference between the control group and the mild AVS group ( $p = 0.016$ ) was obtained by analyzing the CRP values between the control group and all three severity grades; see Figure 3.10.

In the mild AVS group, serum CRP levels are elevated compared to the control group, as well as to the moderate and severe AVS group. Although in our study, as much as possible, we exclude all possible causes that could cause elevated levels of CRP, we did not observe any increase in CRP by progression of AVS.

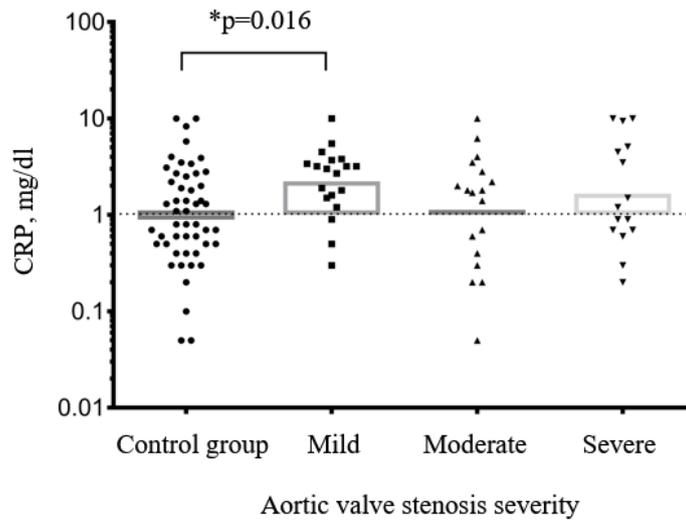


Figure 3.10 Serum CRP level in the control and AVS groups

### 3.2.4 Matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases

Up to now, MMP and TIMP in AVS patients are studied in the tissue histological materials after aortic valve replacement surgery.

We determined the serum levels of MMP-1, MMP-3, MMP-9, and TIMP-1 and TIMP-3, respectively. We can evaluate not only the control group against severe AVS, but also analyze the levels of MMP and TIMP in all grades of aortic valve stenosis.

When comparing the MMP-1 levels in the control group and AVS group. We found statistically significantly higher ( $p = 0.0043$ ) MMP-1 levels in the patient group of aortic valve stenosis, but observed multimodal distribution of the results; see Figure 3.11 and 3.12.

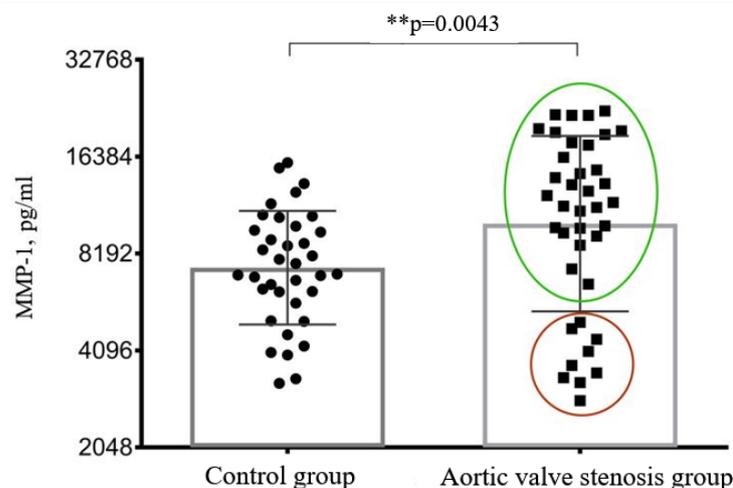


Figure 3.11 Serum MMP-1 level in the control and AVS groups

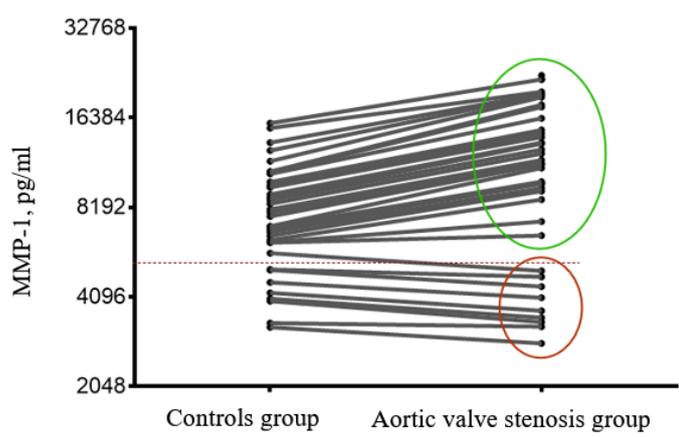


Figure 3.12 Serum MMP-1 level in the control and AVS stenosis groups, ovals in green and red represent AVS patient clusters of data

In order to determine more precisely the differences between the control and stenosis groups, distribution analysis was performed; see Figure 3.13 and 3.14

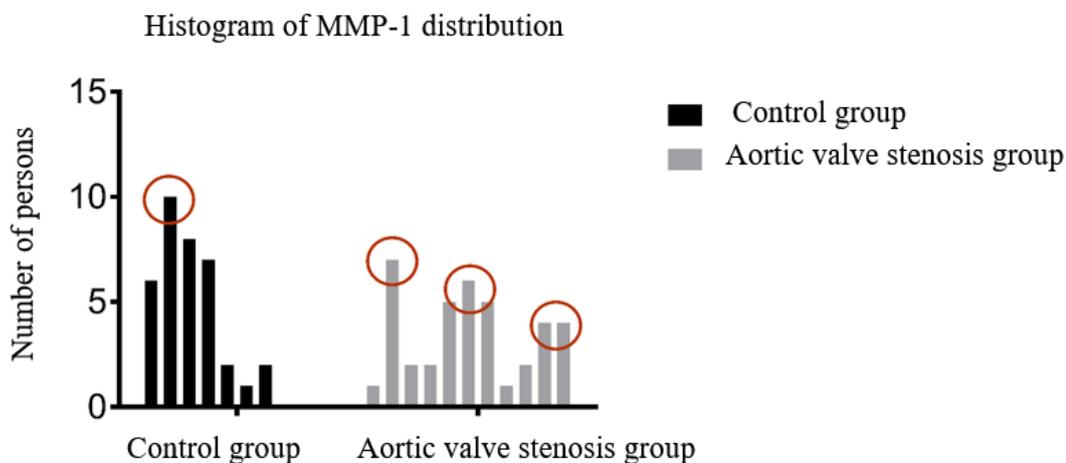


Figure 3.13 Histograms of MMP-1 distribution, modes are represented by color circles

Data distribution histograms determined that the distribution of MMP-1 results in the AVS patient group is trimodal, and in the group of control individuals – monomodal.

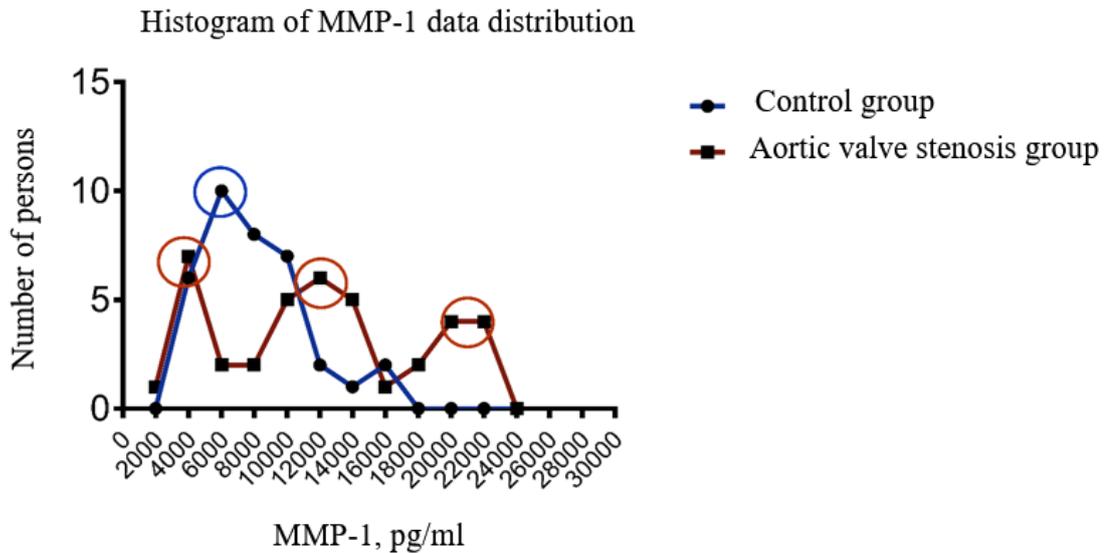


Figure 3.14 **Comparative MMP-1 data distribution between control individuals and AVS patient groups**

80% of the patients in the group of aortic valve stenosis have a statistically reliably higher ( $p < 0.0001$ ) MMP-1 level than in the control group, while 20% of the patients with aortic valve stenosis have MMP-1 levels at the level of the control group; see Figure 3.15.

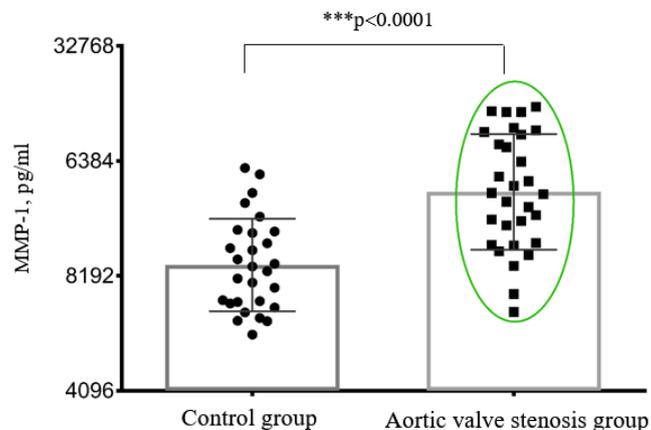


Figure 3.15 **Serum MMP-1 (upper cluster corresponding to 80% of patients, see Figure 3.11) in the control and AVS patient group**

When analyzing differences in MMP-1 levels between the control group and three severity grades of the aortic valve stenosis, we found that the highest MMP-1 level is in the moderate AVS grade ( $p < 0.0001$ ), lower MMP-1 serum level is in severe AVS grade ( $p = 0.012$ ) and even lower levels are in the mild grade of the aortic valve stenosis ( $p = 0.031$ ); see Figure 3.16.

During the study, matrix metalloproteinases such as AVS MMP-3 and MMP-9 were detected and analyzed in the blood serum in the control group and AVS patients. Tissue inhibitors corresponding to these matrix metalloproteinases were also analyzed – the corresponding TIMP-1 and TIMP-3.

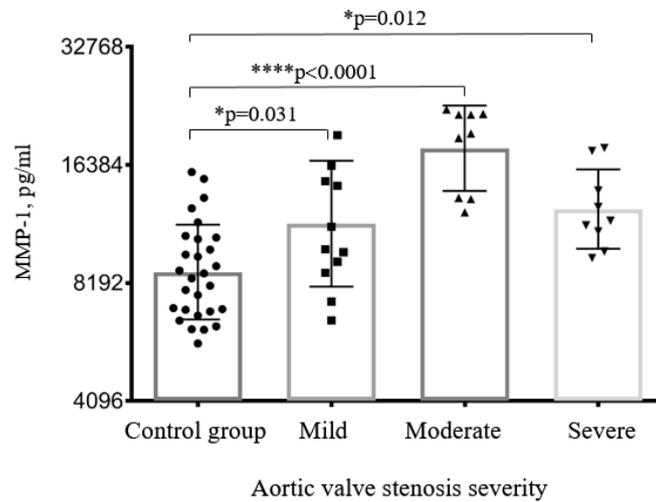


Figure 3.16 **MMP-1 serum levels in the control group and all AVS severity grades**

No statistically significant differences were found in the analysis of the MMP-3 serum levels in the control group and in the aortic valve stenosis group; see Figure 3.17).

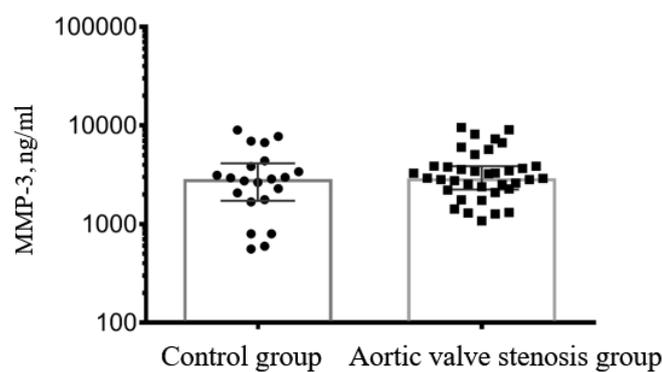


Figure 3.17 **Serum MMP-3 level in the control and AVS groups**

Likewise, no statistically significant differences were found between the individuals of the control group and patients with aortic valve stenosis, when analyzing the MMP-9 results during the study; see Figure 3.18.

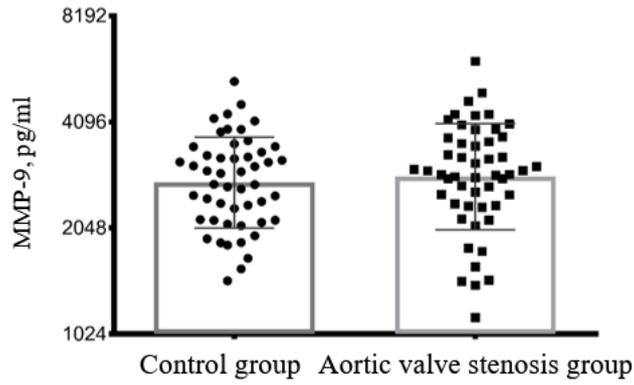


Figure 3.18 Serum MMP-9 level in the control and AVS groups

Analyzing the matrix metalloproteinases appropriate tissue inhibitors TIMP-1 and TIMP-3 serum levels in the control group and the aortic valve stenosis group, no statistically significant differences were found between the study groups; see Figures 3.19. and 3.20.

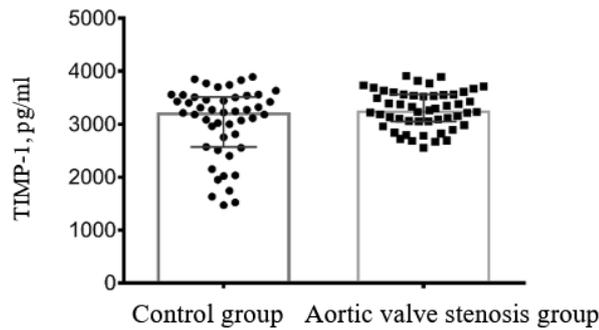


Figure 3.19 Serum TIMP-1 level in the control and AVS groups

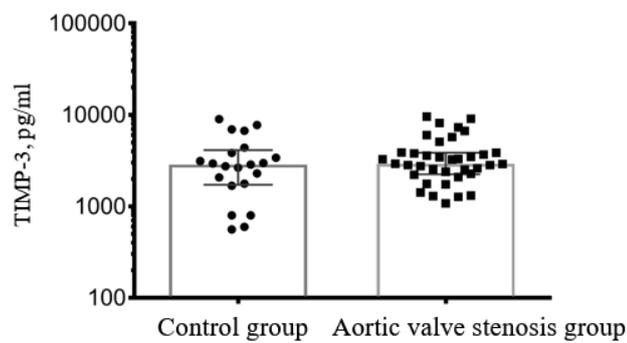


Figure 3.20 Serum TIMP-3 level in the control and AVS groups

Possible correlations between MMP-1 and TIMP-1 as well as MMP-3, MMP-9, and TIMP-3 were analyzed during the study. Correlation analyses were performed to determine the potential associations between matrix metalloproteinases, tissue inhibitors of matrix metalloproteinase; see Figure 3.21.

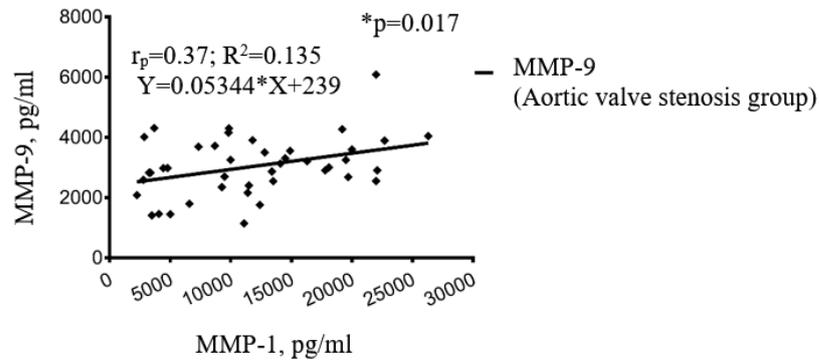


Figure 3.21 **Correlation between MMP-1 and MMP-9 concentrations in AVS patient group**

By performing correlation analysis between MMP-1 and MMP-9, we obtained an association ( $p = 0.017$ ;  $r_p = 0.37$ ): by increase in the MMP-1 serum levels in patients with AVS increases also the MMP-9 serum level.

The correlation between MMP-1 and MMP-3, as well as MMP-1 and TIMP-1 was analyzed during the study. No associations between MMP-1 and MMP-3, as well as between MMP-1 and TIMP-1 were found; see Figures 3.22 and 3.23.

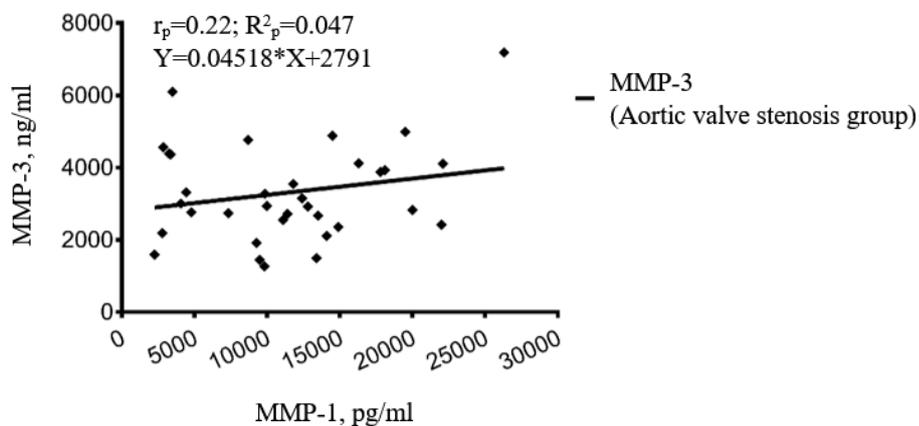


Figure 3.22 **Correlation between MMP-1 and MMP-3 concentrations in AVS patient group**

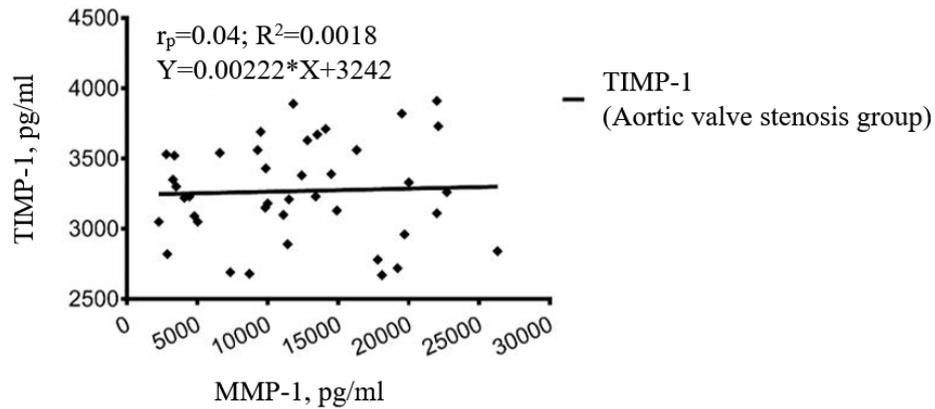


Figure 3.23 Correlation between MMP-1 and TIMP-1 concentrations in AVS patient group

In our study, we did not find the associations between MMP-9 and TIMP-1 in the blood serum; see Figure 3.24.

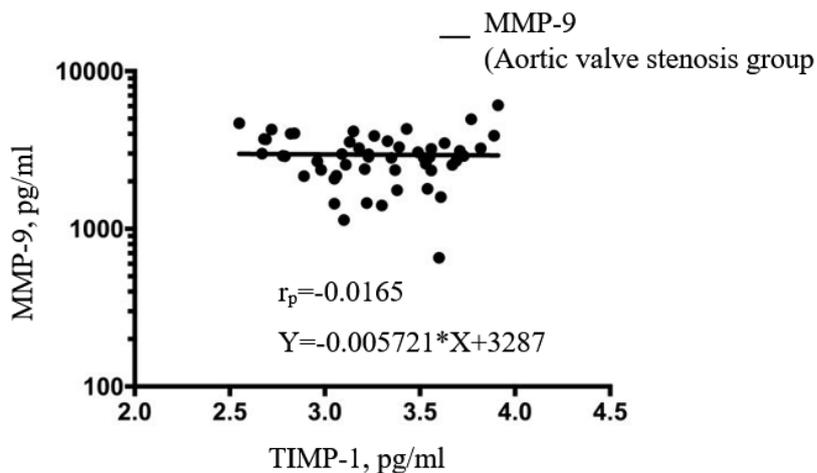


Figure 3.24 Correlation between TIMP-1 and MMP-9 concentrations in AVS patient group

There was also a correlation analysis between MMP-9, MMP-3, MMP-1, and chemerin; see Figures 3.25, 3.26 and 3.27.

We obtained a correlation ( $p = 0.0084$ ;  $r_p = 0.362$ ) that higher chemerin serum levels were associated with higher MMP-9 serum levels. This further confirms the role and the presence of MMP-9 in the aortic valve stenosis process.

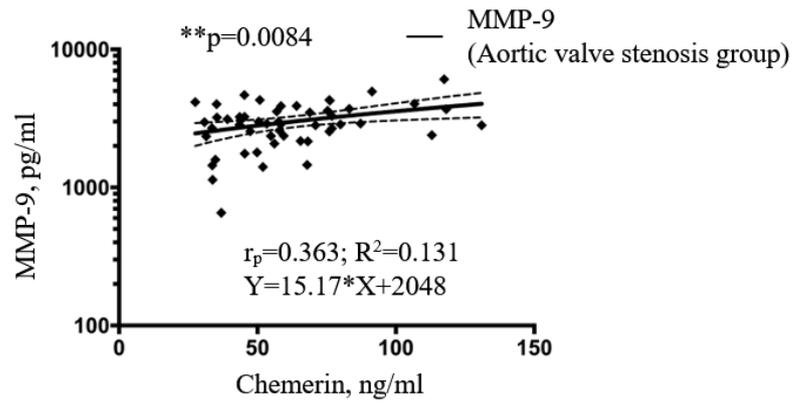


Figure 3.25 Correlation between chemerin and MMP-9 concentrations in AVS patient group

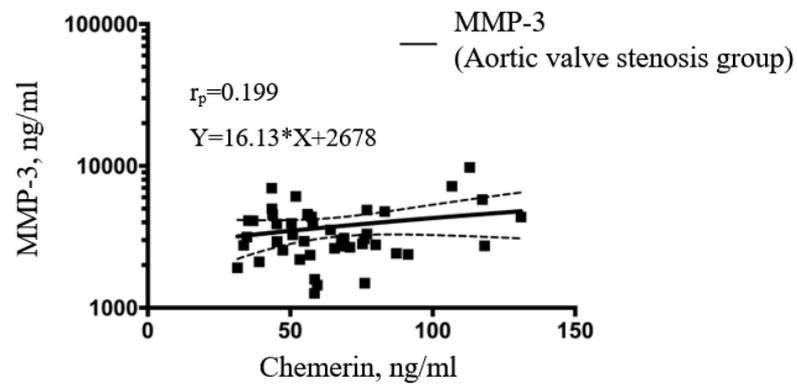


Figure 3.26 Correlation between chemerin and MMP-3 concentrations in AVS patient group

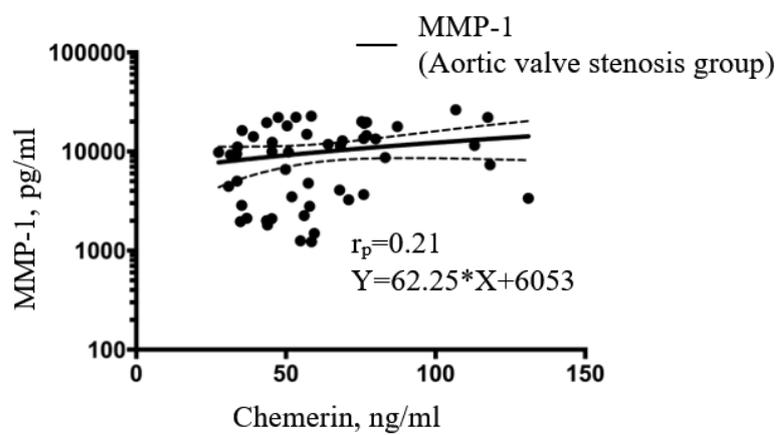


Figure 3.27 Correlation between chemerin and MMP-3 concentrations in AVS patient group

### 3.2.5. Thioredoxin reductase-1 (TrxR1)

We analyzed the antioxidant thioredoxin reductase-1 (TrxR1) in the blood plasma. When determining the level of TrxR1 in the plasma in the subjects of the control group and in the patients with aortic valve stenosis, we obtained a statistically significantly higher its level in the AVS group ( $p = 0.0016$ ); see Figure 2.28.

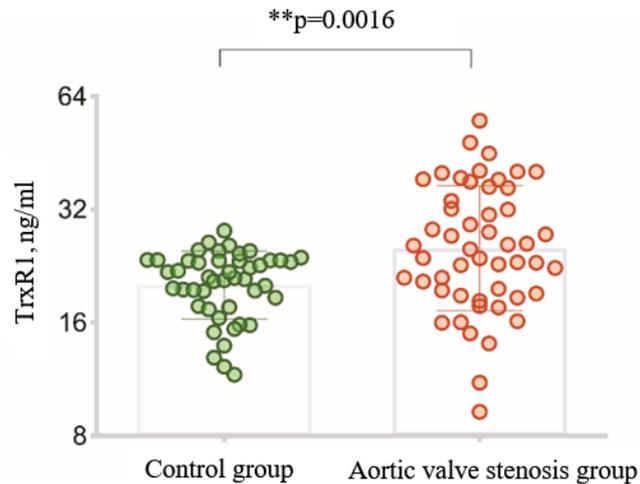


Figure 3.28 Plasma TrxR1 level in the control and AVS groups

When analyzing differences of the TrxR1 level between the control group and three severity grades of the aortic valve stenosis, we obtain statistically significant ( $p = 0.0001$ ) higher TrxR1 level in patients with mild aortic valve stenosis and severe aortic valve stenosis ( $p = 0.039$ ); see Figure 3.29.

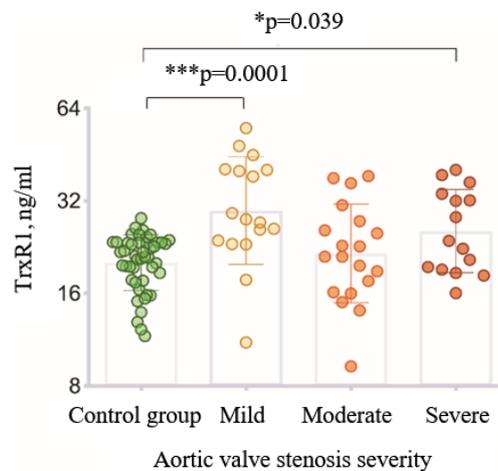


Figure 3.29 Plasma TrxR1 level in the control group and in all AVS severity grades

A correlation analysis was performed with the creation of the linear regression line to analyze and search for possible correlations between TrxR1 and other biomarkers.

In the group of aortic valve stenosis, we obtained a statistically significant positive correlation between TrxR1 and MMP-3 ( $p = 0.013$ ;  $r_p = 0.37$ ); see Figure 3.30.

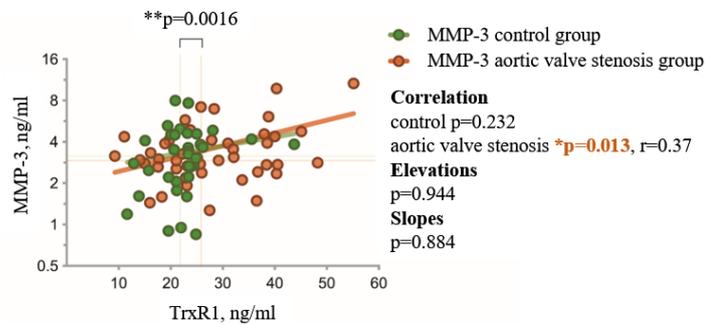


Figure 3.30 Correlation between TrxR1 and MMP-3 concentrations in the control group and AVS patients

In contrast, it was absent between TrxR1 and MMP-9, or was not statistically significant between TrxR1 and MMP-9. However, in the case of TrxR1 and MMP-1 (see Figure 3.31), a significant difference was found between the slopes of the curves characterizing the correlation ( $p = 0.026$ ) was found when comparing control group with the AV stenosis group; furthermore, the correlation in case of AV stenosis, compared to the control group, where the negative direction is observed ( $p = 0.096$ ,  $r = -0.27$ ) has a positive tendency ( $p = 0.062$ ,  $r = 0.24$ ), and the difference between the correlation coefficients of the two groups,  $\Delta r$ , reaches 0.51 or  $[0.24 - (-0.27)]$ , that is, increasing TrxR1 level, MMP-1 level also increase; see Figure 3.31.

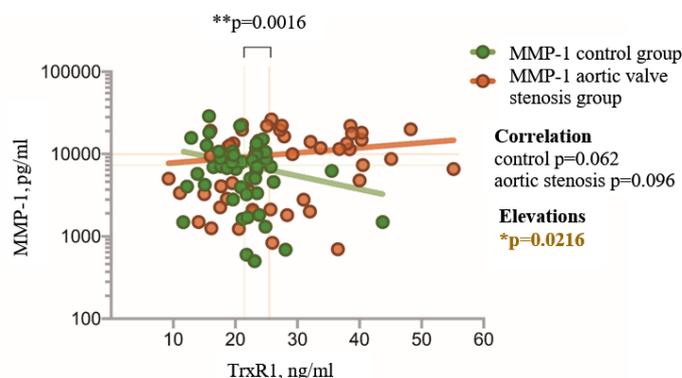


Figure 3.31 Correlation between thioredoxin reductase-1 and MMP-1 concentrations in the control group and AVS patients

We analyzed the association between TrxR1 and chemerin by the correlation method and obtained a statistically significant ( $p = 0.006$ ;  $r_p = 0.32$ ) positive correlation; see Figure 3.32.

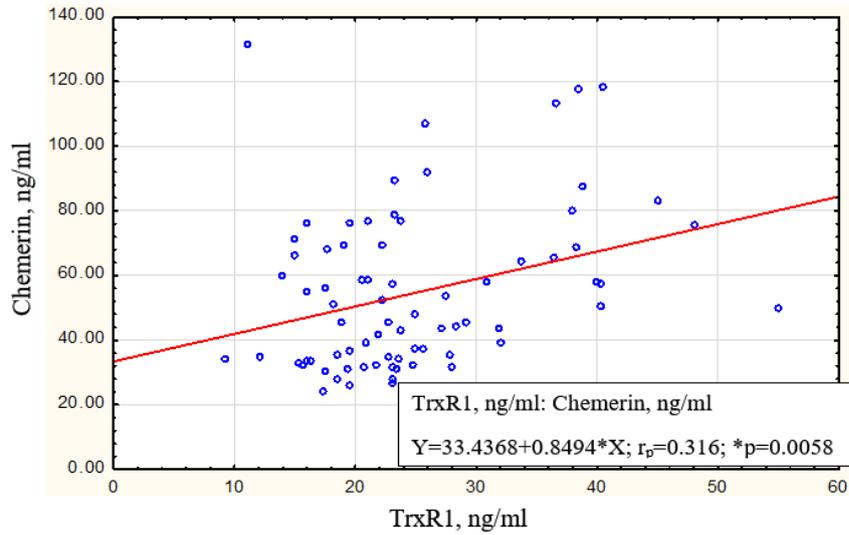


Figure 3.32 Correlation analysis between thioredoxin reductase-1 and chemerin concentrations in the control group and AVS patients

We also analyzed the correlation between TrxR1 and FGF-21 and also obtained a statistically reliable ( $p = 0.031$ ;  $r_p = 0.25$ ) positive correlation; see Figure 3.33.

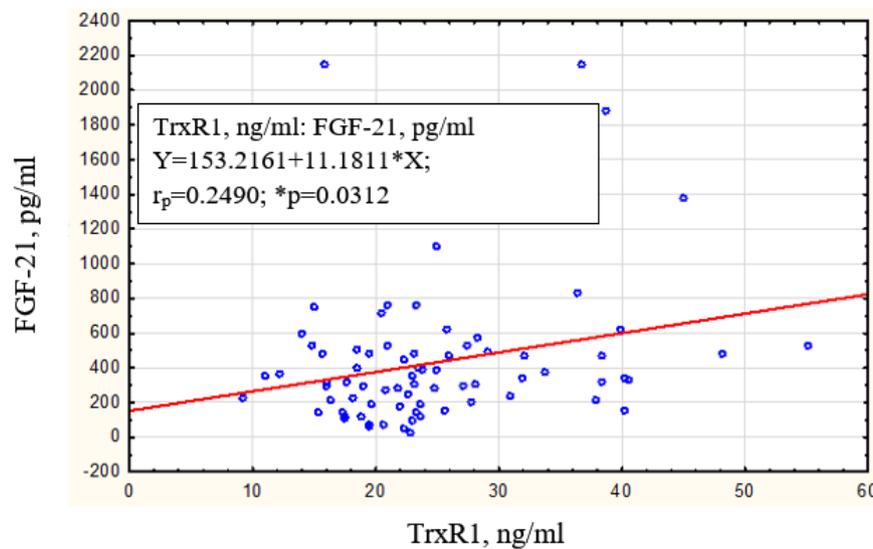


Figure 3.33 Correlation analysis between thioredoxin reductase-1 and FGF-21 concentrations in the control group and AVS patients

### 3.2.6. Myeloperoxidase (MPO)

MPO levels were determined in the control group and in the patients with aortic valve stenosis in all three severity grades of the aortic valve stenosis. We obtained a statistically significant result that the plasma myeloperoxidase levels are higher in the patients with aortic valve stenosis compared to the control group ( $p < 0.00003$ ). When performing through in-depth analysis and comparison of the myeloperoxidase plasma levels between severity grades of the aortic valve stenosis, we obtained statistically significant differences in all severity grades from the control group ( $p < 0.02$  mild stenosis;  $p < 0.001$  moderate stenosis;  $p < 0.0007$  severe stenosis); see Figure 3.34. The results show that myeloperoxidase levels increase with the increase in the severity of the aortic valve stenosis and is the highest in the patient group with severe stenosis.

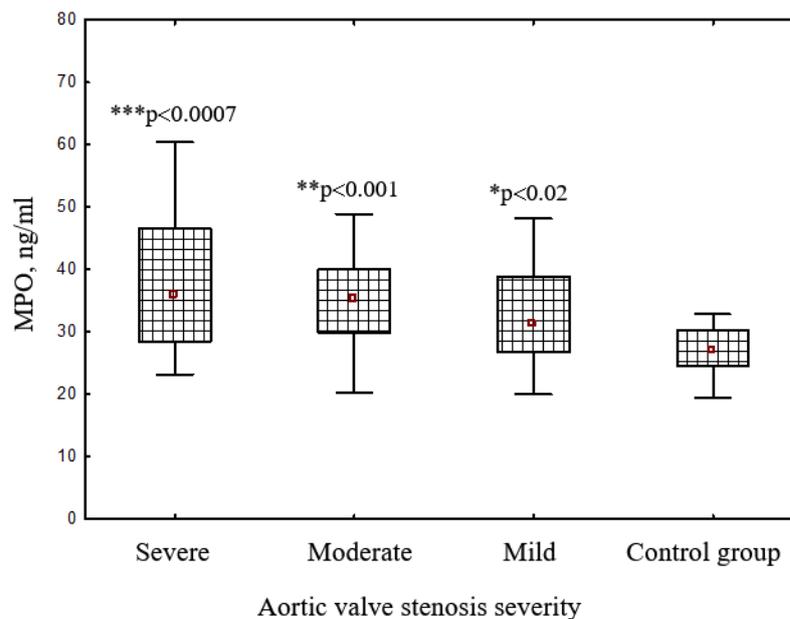
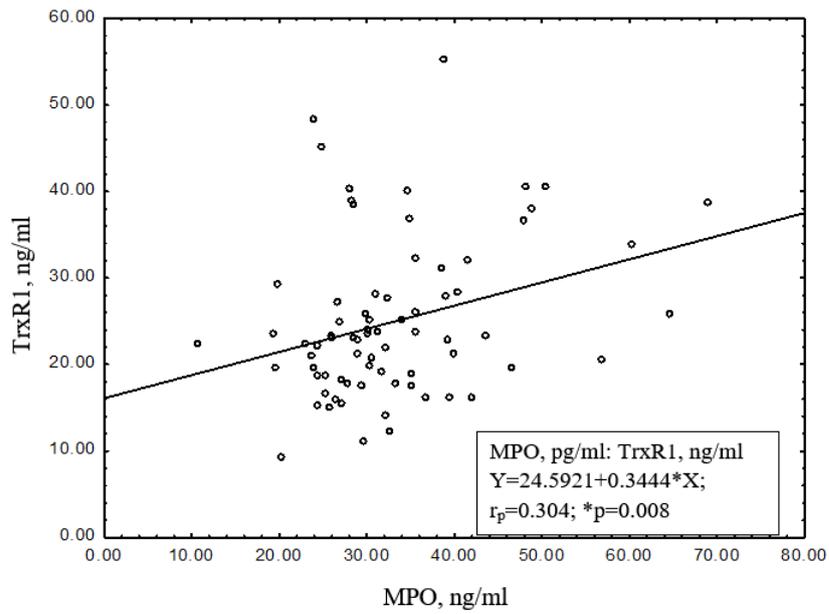


Figure 3.34 **Plasma MPO level in the control group and in all AVS severity grades**

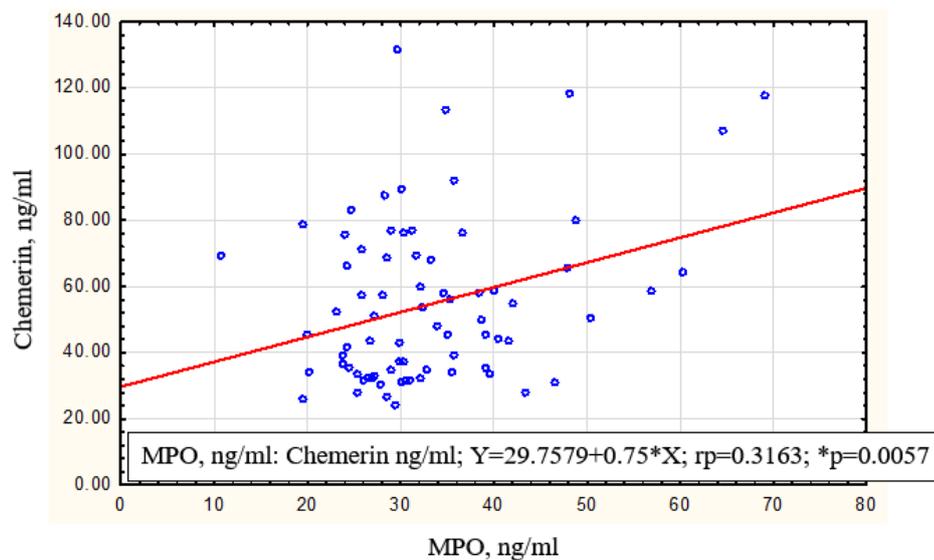
A correlation analysis was performed with the creation of the linear regression line to analyze the correlation between myeloperoxidase (MPO) and thioredoxin reductase-1 (TrxR1); see Figure 3.35.



**Figure 3.35 Correlation analysis between MPO and TrxR1 concentrations in the control group and AVS patients**

We obtained a statistically reliable positive association ( $p = 0.008$ ;  $r_p = 0.304$ ). Increases in myeloperoxidase plasma levels lead to increase in the thioredoxin reductase-1 levels.

We also investigate the association between MPO and chemerin and obtained a statistically reliable positive ( $p = 0.0057$ ;  $r_p = 0.316$ ) correlation. Increase in the serum chemerin levels increases myeloperoxidase levels; see Figure 3.36.



**Figure 3.36 Correlation analysis between MPO and TrxR1 concentrations in the control group and AVS patients**

When analyzing the possible association between MPO and FGF-21, we did not obtain statistically significant correlation ( $p < 0.05$ ) between these biomarkers neither in individuals of the control group nor in the patients with aortic valve stenosis; see Figure 3.37.

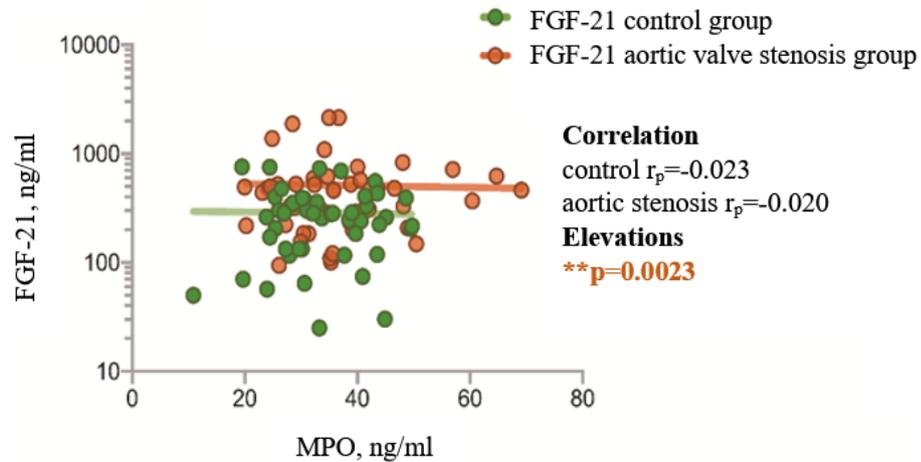


Figure 3.37 Correlation analysis between MPO and FGF-21 concentrations in the control group and AVS patients

When evaluating the associations between myeloperoxidase and matrix metalloproteinases, we obtained a statistically significant, positive ( $p = 0.007$ ;  $r_p = 0.37$ ) correlation between MPO and MMP-9, by increase in the myeloperoxidase plasma levels in the patients with aortic valve stenosis the MMP-9 level also increases; see Figure 3.38.

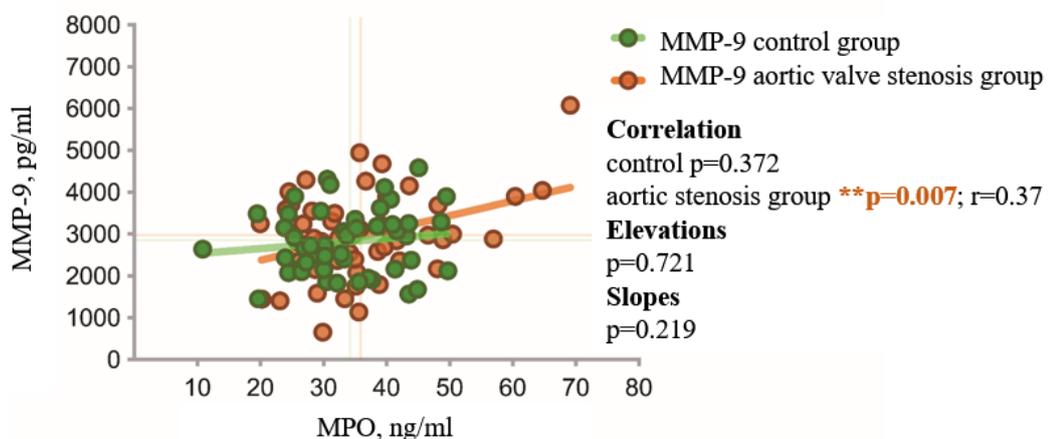


Figure 3.38 Correlation analysis between MPO and MMP-9 concentrations in the control group and AVS patients

By the correlation analysis we obtained a statistically significant, negative ( $p = 0.047$ ;  $r_p = -0.28$ ) correlation between MPO and HDL-C: the higher is the MPO level, the lower is the level of HDL-C; see Figure 3.39.

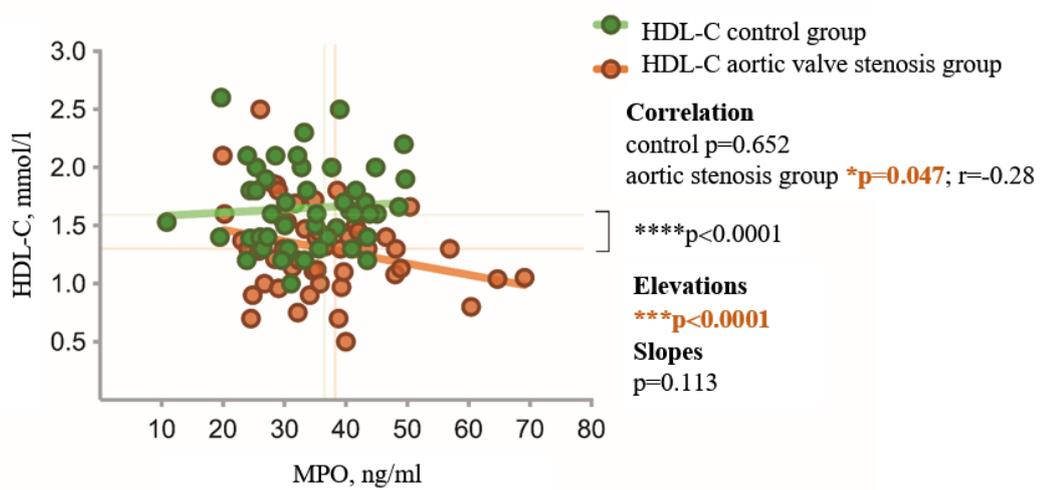


Figure 3.39 Correlation analysis between MPO and HDL-C concentrations in the control group and AVS patients

## 4. DISCUSSION

During the development of the study, 2017 European Society of Cardiology (ESC) and the European Association for Cardio–Thoracic Surgery (EACTS) Guidelines for the management of valvular heart disease were adopted (Baumgartner et al., 2017). Compared to the previous guidelines of 2012, Echocardiography is still the main diagnostic method confirming the existence of AVS, evaluates the degree of AV calcification, left ventricular function and wall thickness, and reveals the possible accompanying pathology of other valves and aortic pathology.

Our study included severe AVS patients with the high gradient AVS as the exclusion criteria were the changed function of the left ventricle (both AVS and the control group included persons with  $EF > 50\%$  and  $SVI > 35 \text{ ml/m}^2$ ).

Following the above–mentioned guidelines of 2017, the determination of the amount of calcium (calcium score) by multislice CT is a first–line additional examination in patients with  $PG \text{ mean.} < 40 \text{ mm/Hg}$ ,  $AVA \leq 1.0 \text{ cm}^2$  un  $SVI \leq 35 \text{ ml/m}^2$ . We did not have such patients in our study, therefore the determination of the calcium score was not indicated AVS patients with normal left ventricle function were initially included in order to avoid the examination, which availability is limited and during the formation of patient groups insufficiently developed to determine the precise calcium score.

The etiopathogenesis of calcific AVS by comparing guidelines and analyzing the findings of the study is still not fully known and undiscovered.

The fact that serum chemerin levels decrease with increase in the AVS severity grade and is highest in the mild AVS patients suggest that the action more happens when chemerin binds to the Chem23 receptor. If chemerin would bind to GPR-1, then its level should increase with the severity of AVS and then the serum chemerin level could indicate the degree of calcification; the more severe the AVS, the more severe calcification.

We did not find statistically reliable differences between the control group and the AVS group when determining MMP-9 serum levels, but we obtain a relationship between chemerin and MMP-9: higher levels of MMP-9 are associated with higher levels of the chemerin. It is known that chemerin promotes the release of MMP-2,9,7. Our obtained results further support the association with inflammatory processes in the AVS patients and indicate remodeling of the extracellular space already in the mild degree of the aortic valve stenosis when there is the highest level of the chemerin. This connection could also indicate that chemerin acts as an inflammatory promoter.

We also found a positive association between chemerin and antioxidant thioredoxin reductase-1 (TrxR1): the higher the level of chemerin, the higher the level of TrxR1. The main role of TrxR1 is to protect against oxidative stress, thioredoxin (Trx) directly inhibits apoptosis-promoting kinases. The correlation, obtained by us between chemerin and TrxR1 indicates that there is both oxidative stress and inflammation in the development of AVS from its beginning.

When analyzing the pro-oxidant enzyme myeloperoxidase (MPO), we obtained a positive correlation between chemerin and MPO: with increasing in the chemerin levels, MPO levels increase.

Chemerin and MPO have a known common property of influencing NO: chemerin reduces NO release; MPO reduces the bioavailability of NO and by binding NO forms ROS.

Both chemerin and MPO contribute to the release of MMP and remodeling of the extracellular space. This may indicate that chemerin in the patients with aortic valve stenosis is inflammatory-promoting.

Chemerin can be used as a good diagnostic marker for mild AVS. In the future, a group of patients with AV sclerosis without stenosis could be formed to determine if chemerin can predict the development of AVS.

By the changes in the serum FGF-21, we cannot unequivocally evaluate the role of this biomarker in the development of AVS.

FGF-21 results allow assuming that FGF-21 can have a protective role in the pathogenesis of AVS. This substantiates the presence of oxidative stress throughout the development process of AVS. However, knowing the role of FGF-21 in the process of connective tissue formation (Schumacher et al., 2016), the data from our study on the highest levels of FGF-21 in the patients with severe AVS may also indicate progressive calcification. FGF-21 has also a reversible effect on myocardial hypertrophy, which is common in AVS patients. This can also be one of the explanations why FGF-21 increases with the severity of AVS. Other biomarkers of oxidative stress should be sought and the results compared to speak more accurately on the role of FGF-21 in the development of AVS.

FGF-21 and TrxR1 are linked to oxidative stress: improves oxidative capacity, reduces ROS activity, reduces cell apoptosis, and contributes to tissue regeneration. There is a positive correlation between both factors: the higher the level of FGF-21, the higher the level of TrxR1. It shows the interaction of both these factors and the relationship between oxidative stress and inflammation. A positive association between FGF-21 and chemerin was also found. Considering that the highest level of FGF-21 is in the degree of severe aortic valve stenosis and has a correlative tightness with both TrxR1 and chemerin, it could be assumed

that chemerin acts as an inflammatory factor, while FGF-21 is associated with anti-inflammatory action at the beginning of aortic valve stenosis development. The highest level of FGF-21 in severe AVS may be associated with both progressive calcification and action against myocardial hypertrophy.

We could know more about FGF-21 by determining the level of IL-6 and TNF- $\alpha$  in the blood serum, as these biomarkers are responsible for the inflammatory process and depend on FGF-21.

The result of the statistical data of C-reactive protein suggests that the high levels of CRP may be indicative of initial calcific AVS. According to our study, CRP cannot be used to predict the rate of progression of AVS. Our data is similar to the data from the study evaluating the association between hs-CRP and AVS (Cho et al., 2016).

MMP (MMP-3, MMP-9) and TIMP (TIMP-1, TIMP-3) serum levels analyzed in the work showed no statistically significant differences between the control group and AVS patients.

Analyzing MMP-1, a significantly higher level of MMP-1 was detected in the AVS group, and with a trimodal distribution of results. 80% of the value in the AVS group is significantly higher than in the control group. The highest MMP-1 level is in the moderate AVS grade.

The obtained data on the highest MMP-1 level in the moderate aortic valve stenosis may indicate that in this grade of the aortic valve stenosis there is the most pronounced remodeling of the extracellular space with the degradation of the collagen fibers and osteoblast differentiation and calcification.

In our study, MMP-1 levels did not increase in 20% of patients with aortic valve stenosis and remained at the level of the control group. It was found when analyzing these patients that all these patients had mild aortic valve stenosis. We think this can be explained by the described MMP-1 polymorphism. The 1G allele has a protective action against calcium deposition, while 2G allele carriers (both homozygous and heterozygous) have more pronounced aortic valvular calcinosis (regardless of age, gender, and renal function) (Solache-Berrocal et al., 2016).

It would be helpful to continue to study and regularly control these 20% of patients with mild aortic stenosis without elevated MMP-1 levels, AVS these patients could theoretically expect a slow progression of aortic valve stenosis.

The data obtained in our study on changes in the MMP-1 levels in different grades of aortic valve stenosis (see Figure 3.16), the lowest MMP-1 levels in the patients with severe aortic valve stenosis, corresponds to the results of other studies on histologic material

collected during surgery. Lower levels of MMP-1 in severe AVS patients may indicate that the inflammatory process at this stage of the disease is inactive or of low activity, and that calcinosis has developed.

We did not find any correlation between MMP-1 and TIMP-1 serum levels. This suggests that the level and function of MMP-1 involved in remodeling the extracellular space of the aortic valve stenosis is not regulated by TIMP-1.

We did not find statistically significant differences in the TIMP-1 serum levels between the patients of the aortic valve stenosis and the control group. Also in other studies an individually variable level of TIMP-1 was found in both stenotic valves and those of the control group (without statistical significance) (Kaden et al., 2005).

By the correlation analysis between MMP-1 and MMP-9, we obtained the correlation that by the increase in the MMP-1 level in the patients with aortic valve stenosis also the MMP-9 serum levels increase. When comparing our results with the findings of other researchers, it can be assumed that MMP-9 functions locally at the cellular level.

Since MMP-9 has been shown to be a significant biomarker of atherosclerosis, but in our study it does not have statistically significant difference from the control group, it only re-confirms that the aortic valve stenosis is a different process from atherosclerosis.

The expression of MMP and TIMP has been studied in the case of non-rheumatic aortic valve stenosis. Also in these histological studies a disproportion between MMP-9 and TIMP-1 was found. Localization of MMP-9 around the calcification nodules was observed.

We found a correlation, that higher chemerin serum levels are associated with higher MMP-9 serum levels. This further substantiates the role and the presence of MMP-9 in the process of aortic valve stenosis.

The results of the analysis of thioredoxin reductase-1 show that oxidative stress is associated with calcific aortic valve stenosis. The higher level of TrxR1 in patients with mild AVS and a positive correlation with chemerin and FGF-21 shows that TrxR1 reflects well the high expressiveness of the oxidative stress in the mild degree of aortic stenosis.

Unlike FGF-21, the level of which increased with the severity of aortic valve stenosis, TrxR1 levels are variable – the highest in the mild aortic valve stenosis and lower in the severe aortic valve stenosis, the lowest in the moderate degree of stenosis. This could be explained by the fact that in the severe AVS degree, left ventricular hypertrophy which is found in many patients with severe aortic valve stenosis is observed. It has been shown that myocardial hypertrophy promotes the expression of TrxR1 (Yamamoto et al., 2003). The other reason could be heart failure because it is shown that the more pronounced is the heart failure the higher is the thioredoxin level (Jekell et al., 2004).

We found a positive correlation between TrxR1 and MMP-1 in patients with aortic valve stenosis: higher levels of TrxR1 are associated with higher levels of MMP-1. Since MMP-1 is found both extracellularly and intracellularly, it is believed to be related to oxidative stress and remodeling of the extracellular space.

Previously, when studying MMP-1, MMP-3, MMP-9, we did not find any correlations that would demonstrate the role of MMP-3 in the aortic valve stenosis. The fact that we find a statistically significant, positive correlation between the thioredoxin reductase-1 and MMP-3 in AVS patients suggest that MMP-3 also plays a role in the process of aortic valve stenosis.

We found some association between TrxR1 and MMP-9, and this can be explained by the results already obtained and the data published by other researchers showing that MMP-9 is more localized around the calcification zones.

When determining the MPO level, we obtained it statistically significantly higher in the patients with aortic valve stenosis, moreover, by increasing from mild to severe aortic valve stenosis. MPO has a positive correlation with TrxR1, chemerin, and MMP-9. MPO activity should be explained in different ways:

a) the positive correlation with TrxR1 and chemerin could be explained by endothelial dysfunction, ROS formation and active participation in inflammatory and oxidative stress processes.

b) the positive correlation with MMP-9, on the one hand, proves the role of MMP-9 in the process of aortic valve stenosis, but knowing that MMP-9 basically is found around the calcification zones and MPO contains a calcium-binding site, it may suggest that MPO could participate in the calcification process.

However, this cannot be unambiguously asserted because MPO can activate MMP by acting through ROS and ox-LDL.

The correlation between the levels of MPO and HDL-C corresponds to the adverse effects of MPO by increasing the level of ox-LDL-C, resulting in HDL-C dysfunction and formation of ox-HDL-C, thus further reducing HDL-C protection. It also occurs under the influence of hypochlorous acid produced by MPO, which oxidizes apoA-1 and reduces the protective activity of HDL-C. Since the highest MPO level is found in patients with severe aortic valve stenosis, respectively, the lowest HDL-C protection is also in this AVS severity grade. We do not have the ability to directly influence the activity of MPO, but the higher will be the level of HDL-C in patients, the better will be the protective role of HDL-C.

If we explain the increase in TrxR1 in the severe aortic stenosis by heart failure, left ventricular hypertrophy, then the association between MPO and HDL-C substantiates the oxidative stress in severe aortic valve stenosis.

MPO levels, as well as FGF-21 levels, increase with the progression of the stenosis. This indicates the presence of oxidative stress in the development of aortic valve stenosis. Neither MPO, TrxR1 nor FGF-21 levels in the patients with severe aortic valve stenosis are at a lower level than in the patients with moderate stenosis, but with a tendency to be higher, suggesting that oxidative stress is present in all AVS severity grades. Unlike chemerin, which is a good biomarker of mild aortic valve stenosis, and its level decreases by the progression of AVS, the results obtained with MPO and TrxR1 suggest that the aforementioned oxidative stress and progressive calcification are prevailing in moderate and severe aortic stenosis.

When analyzing the possible association of oxidative stress markers (MPO and TrxR1) with other cell-produced regulatory molecules (cytokines), we find a positive correlation between MPO and MMP-9, as well as MMP-3. This could indicate that both MMP-9 and MMP-3 are involved in the development of aortic valve stenosis and may participate in remodeling of the extracellular space caused by oxidative stress.

The fact that the oxidative stress level is relatively higher in the severe aortic stenosis than in the moderate could be explained by changes occurred as the result of AVS progression: heart failure, left ventricular hypertrophy and low coronary flow reserve.

## 5. CONCLUSIONS

1. Distribution of the patients with aortic valve stenosis into three severity grades and evaluation versus the control group allows a more accurate and complete assessment of the pathogenesis of the disease.
2. Chemerin is a good diagnostic biomarker for mild degree of aortic stenosis.
3. Remodeling of the extracellular space begins at the mild degree of aortic valve stenosis, and most pronounced is in the moderate severity degree of the aortic stenosis. Significant increases in MMP-1 concentrations have been identified, both in the mild AVS grade and the more pronounced in moderate grade of AVS. A positive association between MMP-1 and MMP-9, chemerin and MMP-9 has been identified.
4. The obtained results of the analyses substantiate the presence of inflammatory and oxidative stress in all three severity grades of AVS. The most active inflammatory process is in the mild AVS degree, where the chemerin levels are the highest, but the relationships between biomarkers shows that oxidative stress starts already in the mild AVS degree. The highest level of TrxR1 is in the mild AVS degree, while the MPO level increases with the AVS severity and reaches the highest level in the severe AVS degree compared to the control group. The higher levels of oxidative stress in the severe AVS degree than in the moderate AVS degree we attribute to the fact that both oxidative stress continues and most of the patients with severe AVS have developed complications such as left ventricular hypertrophy, chronic heart failure.
5. Oxidative stress and inflammation are interrelated processes which is substantiated by the positive correlation of chemerin with TrxR1 and MPO.
6. Part of first hypothesis of the role of inflammatory factors and cytokines in the development of AVS is demonstrated by the statistically significant differences between the control group and the AVS group in the results of the analyzes of chemerin, FGF-21, CRP, MMP-1, TrxR1, MPO. The correlation between MMP-1 and MMP-9 and chemerin and MMP-9 also substantiate the MMP-9 commitment with the AVS process. The correlative association between TrxR1 and MMP-3 allows the expression about MMP-3 participation in the AVS process. Whether these factors and cytokines, determined in blood serum and plasma, determine the prognosis of AVS can not be judged. This requires genetic research.
7. We also confirmed the second hypothesis: it was found that HDL-C is significant for the AVS patients. It was found a negative relationship between MPO and HDL-C, when

higher MPO levels are associated with lower levels of HDL-C. Patients with severe AVS have the highest MPO levels. This can be explained by the MPO action to form ox-HDL-C and to reduce the protective activity of HDL-C, therefore the higher there will be the level of HDL-C in the patients, and the better will be the protective role of HDL-C.

8. We also confirmed the third hypothesis that oxidative stress is at all severity degrees of AVS; we also determined its relation to the inflammatory process.

## 6. PRACTICAL RECOMMENDATIONS

1. Chemerin and its detection in the blood serum is a good diagnostic marker for the patients with mild AVS. It could be recommended to determine in patients, in whom heart murmurs are heard, till while the echocardiography is not performed. Similarly, chemerin could be detected in patients in whom due to the reduced left ventricular systolic function it is difficult to distinguish between mild and moderate degrees of stenosis, based on the maximum flow rate and mean pressure gradient.
2. It would be possible to develop a program of monitoring and dynamic examination of the patients with mild AVS to determine the rate of progression of AVS and its association with the MMP-1 1G allele, in carriers of which theoretically slow progression of the disease should be observed. This could show the role of genetic factors in the AVS patients.
3. The correlation of laboratory results with oxidative stress and inflammation and the known AVS pathogenesis requires special attention to patient with mild AVS, as from the point of pathogenesis it is possible to prevent or delay the progression of the disease. Research could be done with drugs that affect the inflammatory process. There have been attempts to use cytostatic drugs in the past, but their side effects did not overbalance the clinical benefit.
4. Although lipids and statin therapy have no direct relationship with the AVS process, which is also supported by our analyzes, however, both ox-LDL-C and HDL-C are associated with AVS. Efforts should be made to maximize the level of HDL-C in the patients of all AVS grades in order to maintain and improve the protective role of HDL-C in oxidative stress conditions. Since there is no particular possibility to directly influence the activity of MPO, special attention should be paid to the level of HDL-C in the blood of patients – because it will be higher, the stronger its protective role.

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1. Impact of several proinflammatory and degeneration factors in aortic valve stenosis. J. Lurins, D. Lurina, P. Tretjakovs, V. Mackevics, A. Lejnieks, V. Rapisarda, V. Baylon (20'). 22nd World Congress on Advances in Oncology and 20th International Symposium on Molecular Medicine. Athens, Greece, 2017, October 5–7
2. Plasma thioredoxin reductase and myeloperoxidase levels in acquired aortic valve stenosis patients. J. Lurins, D. Lurina, G. Krievina, V. Mackevics, P. Tretjakovs, A. Lejnieks, S.S. Signorelli (15'). 23rd World Congress on Advances in Oncology and 22nd International Symposium on Molecular Medicine. Athens, Greece, 2018, September 20–22

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## ANNEXES

Pētījuma nosaukums:

Nelipīdu riska faktoru ietekme uz iegūtas aortas vārstuļa stenozes attīstību.

**Informācijas lapa pacientam**Paskaidrojums

Mēs lūdzam Jūsu palīdzību pētījumā, kura mērķis ir noskaidrot, kuri no nelipīdu riska faktoriem un cik lielā mērā ietekmē aortas vārstuļa stenozes rašanos.

Mēs būtu pateicīgi, ja Jūs varētu atbildēt uz dažiem jautājumiem par savu dzīvesveidu un atļautu nomērīt Jūsu augumu, nosvērt, paņemt asins paraugus analīzēm.

Visa iegūtā informācija par Jūsu veselības stāvokli paliks augstākā mērā konfidenciāla un būs pieejama tikai Jūsu ārstējošajam ārstam, pētniekam un pētījuma vadītājam.

Nav nepieciešams piedalītais pētījumā, ja Jūs nevēlaties to darīt. Ja jūs nolemsiet nepiedalīties, Jūsu lēmums nekādā veidā neietekmēs Jūsu ārstēšanu. Jums ir tiesības izstāties no pētījuma jebkurā laikā, neminot nekādus paskaidrojumus. Tas nekādā veidā neietekmēs Jūsu turpmāko ārstēšanu.

Sīkāku informāciju Jūs varat saņemt, kontaktējoties ar

---

(ārsta vārds, uzvārds, telefona numurs)

Mēs ceram, ka Jūs varēsiet palīdzēt šajā pētījumā par nelipīdu riska faktoru ietekmi uz iegūtas aortas vārstuļa stenozes attīstību, kuru rezultāti tiks apkopoti zinātniskajās publikācijās.

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(ārsta vārds, uzvārds, telefona numurs)

## Informēta pacienta piekrišanas forma

Projekta nosaukums: Nelipīdu riska faktoru ietekme uz iegūtas aortas vārstuļa stenozes attīstību.

Pacientam jāaizpilda šī forma personīgi

(lūdzu atzīmēties atbilstoši, kas attiecas uz Jums)

Es esmu izlasījis informācijas lapu pacientam

Man ir bijusi izdevība uzdot jautājumus un apspriest pētījumu

Es esmu saņēmis apmierinošas atbildes uz visiem saviem jautājumiem

Es esmu saņēmis pietiekami informāciju par pētījumu

Pētījuma būtību man izskaidroja:

---

(ārsta vārds, uzvārds)

Es saprotu, ka man ir tiesības izstāties no pētījuma jebkurā laikā, neminot izstāšanās iemeslu un nekādi neiespaidot savu medicīnisko aprūpi nākotnē

Es piekrītu piedalīties šajā pētījumā

Paraksts.....Datums.....

(Vārds un uzvārds

ar drukātiem burtiem).....

Pētnieka paraksts.....Datums.....

(Vārds un uzvārds

ar drukātiem burtiem).....

## Pacienta aptaujas anketa

1. Vai Jums piemīt kāds no zemāk minētajiem simptomiem:
  - spiedošas, dedzinošas sāpes krūšu kurvja priekšpusē, slodzes laikā
  - elpastrūkums slodzes laikā
  - reibonis
  - samaņas zuduma epizodes
  - tūskas uz kājām
2. Vai Jums ir paaugstināts holesterīna līmenis asinīs
  - nē
  - ja jā, vai Jūs zināt tā lielumu \_\_\_\_\_
3. Vai Jums jebkad dzīvē ir bijis paaugstināts holesterīna un/vai zema blīvuma lipoproteīnu un/vai triglicerīdu līmenis asinīs:
  - nē
  - jā
4. Vai Jums ir paaugstināts asinsspiediens: jā  nē
5. Vai Jums ir glikozes vielmaiņas traucējumi vai cukura diabēts: jā  nē
6. Vai Jūs pašreiz lietojat kādu no sekojošiem medikamentiem:
7. Vai Jūs jebkad dzīvē esat lietojusi/jis kādu no holesterīnu zeminošiem medikamentiem:
 

<ul style="list-style-type: none"> <li>• <math>\beta</math> adrenoblokatorus</li> <li>• <math>\alpha</math> receptoru blokatorus</li> <li>• sirds glikozīdus</li> <li>• klopidogrelu</li> <li>• holesterīnu zeminošus medikamentus</li> <li>• F1 kanālu blokatorus</li> <li>• hormonu medikamentus vai citus</li> <li>• jā</li> <li>• nē</li> </ul>	<ul style="list-style-type: none"> <li>• kalcija kanālu blokatorus</li> <li>• AKE-I</li> <li>• diurētiskus</li> <li>• aspirīnu</li> <li>• insulīnu</li> <li>• p/o antidiabēta medikamentus</li> <li>• pretiekaisuma medikamentus</li> </ul>
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8. Vai Jums pēdējo 5 gadu laikā ir veikts kāds no sekojošiem izmeklējumiem:
 

<ul style="list-style-type: none"> <li>• koronarogrāfija</li> <li>• ehokardiogrāfija</li> <li>• glikozes analīzes</li> <li>• brahiocefālo a/v doplerogrāfija</li> </ul>	<ul style="list-style-type: none"> <li>• miokarda perfūzijas scintigrāfija</li> <li>• lipīdu analīzes</li> <li>• spirogrāfija</li> </ul>
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9. Vai pēdējo 5 gadu laikā esat ārstējies slimnīcā kādas sirds – asinsvadu slimības dēļ:
 

<ul style="list-style-type: none"> <li>• miokarda infarkts</li> <li>• sirds mazspēja</li> <li>• koronārā sirds slimība</li> </ul>	<ul style="list-style-type: none"> <li>• paaugstināts asinsspiediens</li> <li>• smadzeņu insults</li> <li>• transitora išēmiska lēkme (TIL)</li> </ul>
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## Ētikas komitejas atļauja

Veidlapa Nr. E-9 (2)

### RSU ĒTIKAS KOMITEJAS LĒMUMS

Rīga, Dzirciema iela 16, LV-1007  
Tel. 67409101

Komitejas sastāvs	Kvalifikācija	Nodarbošanās
1. Asoc. prof. Olafs Brūvers	Dr.theo.	teologs
2. Profesore Vija Sīle	Dr.phil.	filozofs
3. Docente Santa Purviņa	Dr.med.	farmakologs
4. Asoc. prof. Voldemārs Arnis	Dr.biol.	rehabilitologs
5. Profesore Regīna Kleina	Dr.med.	patalogs
6. Asoc. prof. Guntars Pupelis	Dr.med.	ķirurgs
7. Asoc. prof. Viesturs Liguts	Dr.med.	toksikologs

**Pieteikuma iesniedzējs:** Dr. Juris Lūriņš  
Medicīnas fakultāte

**Pētījuma nosaukums:** „Nelipīdu riska faktoru ietekme uz iegūtas aortas vārstuļa stenozes attīstību”

**Iesniegšanas datums:** 04.09.2013.

**Pētījuma protokols:** Izskatot augstāk minētā pētījuma pieteikuma materiālus (protokolu) ir redzams, ka pētījuma mērķis tiek sasniegts veicot ar pacientiem (bez kāda apdraudējuma veselībai) klīniski-analītisku darbu, aptauju intervijas veidā, iegūto datu apstrādi un analīzi, kā arī izsakot priekšlikumus. Personu (pacientu, dalībnieku) datu aizsardzība, brīvprātīga informēta piekrišana piedalīties pētījumā un konfidencialitāte tiek nodrošināta. Līdz ar to pieteikums atbilst pētījuma ētikas prasībām.

**Izskaidrošanas formulārs:** ir

**Piekrišana piedalīties pētījumā:** ir

**Komitejas lēmums:** piekrist pētījumam

Komitejas priekšsēdētājs Olafs Brūvers

Tituls: Dr. miss., asoc. prof.

Paraksts



Ētikas komitejas sēdes datums: 12.09.2013.