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

Summary of the Doctoral Thesis
for obtaining the degree of a Doctor of Medicine

Specialty – Internal Medicine

Riga, 2019

The Doctoral Thesis was carried out at Rīga Stradiņš University

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Defence of the Doctoral Thesis will take place at the public session of the Doctoral Council of Medicine on 12 september 2019 at 12.00 in Hippocrates Lecture Theatre, 16 Dzirciema Street, Rīga Stradiņš University

The Doctoral Thesis is available in the RSU library and at RSU webpage: www.rsu.lv

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CONTENT

ABBREVIATIONS USED IN THE WORK

5'-NT	5'-nucleotidase	MMP	matrix metalloproteinase
ABI	ankle-brachial index	MMP-1,2,3,7,9,11	matrix metaloproteinases-1,2,3,7,9,11
ACE	angiotensin converting enzyme	MPO	myeloperoxidase
ALP	alkaline phosphatase	MV	mitral valve
Ang I	angiotensin I	NADPH oxidase	nicotinamide adenine dinucleotide phosphate oxidase
Ang II	angiotensin II	NF-kβ	nuclear factor kappa β
apoA-1	apolipoprotein A-1	NO	nitrogen oxide
AVS	aortic valve stenosis	NOS	nitrogen oxide synthase
ASK1	apoptosis signal-regulating kinase 1	NPP1	ectonucleotide pyrophosphatase/ phosphodiesterase 1
AUC	area under the ROC curve	NPV	negative prognostic value
AV	aortic valve	O_2^-	superoxide
bFGF	basic fibroblast growth factor	$ONOO^-$	peroxinitrite
BH4	tetrahydrobiopterin	ox-LDL	oxidised low density lipoprotein
BMI	body mass index	PAD	peripheral arterial disease
BPM-2	bone morphogenetic protein	PG	pressure gradient
ChemR23	chemerin receptor 23	PPV	positive prognostic value
Cl^-	Chlorine anion	Pro-MMP	proenzymes
cMGP	carboxylated matrix Gla-protein form	PW	pulse wave dopplerography
CRP	C-reactive protein	RAA	renin-angiotensin-aldosterone
DNA	deoxyribonucleic acid	ROC curve	Receiver-Operating Characteristic curve
EF	ejection fraction	ROS	reactive oxygen particles
Ehokg	echocardiography	Se	sensitivity
ESR	erythrocyte sedimentation rate	SOD	superoxide dismutase
FGF-21	fibroblast growth factor-21	Sp	specificity

GPR1	G-protein-bound receptor 1	SV	stroke volume
H_2O^2	hydrogen peroxide	TC	total cholesterol
HDL-C	high density lipoprotein cholesterol	TG	triglycerides
HOCI	hypochlorous acid	TGF	transforming growth factor
hs-CRP	highly sensitive C-reactive protein	TGF-1β	transforming growth factor-1β
IGF1	insulin-like growth factor 1	TGF-β	transforming growth factor-β
IL-1β	interleukin-1β	TIMP	matrix metalloproteinase tissue inhibitor
IL-2	interleukin-2	TIMP-1,3	matrix metalloproteinase tissue inhibitor-1,3
IL-6	interleukin-6	TNF-α	tumor necrosis factor-α
IMT	intima-media thickness	Trx	thioredoxin
LDL	low-density lipoprotein	TrxR	thioredoxin reductase
Lp(a)	lipoprotein a	TrxR1,2,3	thioredoxin reductase-1,2,3
LV	left ventricle	VEGF	vascular endothelial growth factor
LV EDV	left ventricular end-diastole volume	VIC	valvular interstitial cells
LV ESV	left ventricular end-systole volume	VTI	velocity time integral
LVOT	Left ventricle outflow tract		

INTRODUCTION

Topicality of the scientific study

When reading the currently available literature on the formation and development of aortic valve stenosis (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 such words AVS "supposedly" and "possible". Moreover, 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 are no means to limit disease development. It encourages to explore the situation in the Latvian population and to conduct a research that highlights differences and commonalities in different AVS degrees, looks for biomarkers in blood plasma and serum thus predicting the development of the disease.

Calcific 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). 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; Kleinauskienė et al., 2018; Oury et al., 2019). With increasing survival, the number of patients with clinically relevant AVS is increasing AVS well (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 later develop AVS. The mean time from diagnosis of the AV sclerosis to the development of moderate and severe AVS is 6–8 years (Eveborn et al., 2013).

Aim of Doctoral Thesis

The aim of the study is to 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 each of the three severity of stenosis.

The aim of the study is 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. Conduct of 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 metalloproteases-1,3 (TIMP-1,3), transforming growth factors (TGF), thioredoxin reductase-1 (TrxR1), myeloperoxidase (MPO).
3. Evaluation of the results of the analysis between the control group and the AVS group in all three severity grades.
4. Finding a sufficiently informative and good biomarker for diagnosing mild aortic valve stenosis.

5. Evaluation of the relationship of determined lipid fractions with aortic stenosis at its various severity grades.
6. Evaluation of 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 calcific AVS.
2. Changes in the level of lipid fractions in AVS patients cannot be unambiguously evaluated as an absolute risk criterion.
3. Oxidative stress is associated with AVS in all severity grades.

Scientific novelty of the study

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

Knowing the latest studies on etiopathogenesis of the calcific AVS, only those potential biomarkers that have not been studied in AVS patients (chemerin, FGF-21, thioredoxin reductase-1, myeloperoxidase) were selected for the conduct of the study. All results of all the biomarkers obtained in the study were compared in different severity grades of AVS so that the pathogenetic processes prevailing at each degree of stenosis could be discussed in more detail. Relationships between biomarkers, as well as between lipid fractions and biomarkers were sought. When analysing lipid fractions, it was

found that dyslipidemia is not unequivocal in the AVS patients. The role of oxidative stress in the development of AVS is substantiated.

1. MATERIAL AND METHODS

1.1 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 Rīga Stradiņš University Ethics Committee on Research on Humans, the date of the meeting of the Ethics Committee was September 12, 2013.

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 the polyclinic – Zemgale Health Centre (Jelgava). A total of 102 patients were included according to the inclusion and exclusion criteria and divided into two main groups: the control group and the AV stenosis group; see Table 1.1.

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. Written informed consent to participate in the study was obtained from each individual enrolled in the current study.

Table 1.1

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	*Mdn* (*IQR*)	64 (57–75)	71 (65–75)	74 (65–79)	65 (60–74)

1.2 Inclusion and exclusion criteria for the study subjects

At the beginning of the study, disease anamnesis was collected from each person in both study groups, a questionnaire was filled on the condition of 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);
- 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 disfunction;
- 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 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. 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; ≤ 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 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.

1.3 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^2);
- indexed aortic valve area – indexed AVA (cm^2/m^2).

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

1.4 Material used in the study

Blood serum and plasma were used for the study analysis. 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. 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 μL of serum and 5 Eppendorf tubes with 200μL of plasma were prepared. The resulting serum and plasma samples were stored in a refrigerator (with temperature control) at −80 °C (in the Laboratory of Physiology and Biochemistry of Rīga Stradiņš University).

1.5 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.

1.6 Detection of cell-produced cytokines

The analysis was performed in the biochemistry laboratory of the Department of Human Physiology and Biochemistry of Rīga Stradiņš University and in the biochemistry laboratory of the Institute of Microbiology and Virology of Rīga Stradiņš 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 a 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.

1.7 Statistical analysis of the data obtained in the study

All graphic images, calculations, and statistical analysis 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–Smirnov one sample test. If the sample dispersion corresponded to the normal distribution, they were showed as the mean value (M) and standard deviation (± 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 Student's test or t–test;
- mean values between three or more separate patient groups were compared using a one-way analysis of variance (ANOVA);

Statistical analysis of the data values of which did not correspond to the normal distribution (non-parametric data):

- mean values between two separate patient groups were compared using the Mann–Whitney U–test;
- mean values between three or more separate patient groups were compared using the Kruskal–Wallis H–test;

In all cases, as a post-hoc procedure the two-stage step–up method of Benjamini, Krieger and Yekutieli was applied.

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.

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, a parametric (*Pearson*) or non-parametric (*Spearman*) correlation analysis was used.

The following parameters were used to characterise 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.

2. RESULTS

2.1 Baseline characteristics of study subjects

The basic data of the subjects included in the study are presented in Table 5.1. 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 strict clinical and echocardiography exclusion criteria allowed 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 $p < 0.05$ to $p < 0.0001$.

The average age of patients in all aortic stenosis groups and in the control group was similar, and the mean body mass index (BMI) did not differ between groups. The mean values of triglycerides and lowdensity lipoprotein cholesterol (LDL-C) are not statistically different between stenosis groups and the 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; see Table 2.1.

Table 2.1

Basic data of the subjects in control and AVS groups

		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	*Mdn*	62	71	74	65
	(IQR)	(57–75)	(65–75)	(65–79)	(60–74)
[a] BMI	*M (± SD)*	26.04 (4.31)	27.39 (3.10)	25.81 (4.58)	27.40 (3.18)
	p value vs control		*p = 0.399*	*p = 0.682*	*p = 0.869*
[b] LDL-C, mmol/l	*M (± SD)*	3.28 (1.18)	3.05 (0.97)	2.59 (0.92)	3.10 (1.12)
	p value vs control		*p* > 0.999	*p* = 0.057	*p* > 0.999
[c] TG, mmol/l	*M (± SD)*	1.47 (0.71)	1.64 (0.84)	1.11 (0.56)	1.27 (0.57)
	p value vs control		*p = 0.406*	*p = 0.178*	*p = 0.406*
[d] TC, mmol/l	*M (± SD)*	5.49 (1.28)	5.01 (1.34)	4.21 (1.18)	4.68 (1.08)
	p value vs control		*p = 0.056*	***p = 0.001***	***p = 0.016***
[e] CRP, mg/l	*Mdn*	0.95	3.00	1.75	1.2
	(IQR)	(0.50–2.55)	(1.50–3.70)	(0.37–2.97)	(0.70–5.00)
	p value vs control		***p = 0.016***	*p = 0.37*	*p = 0.17*
[f] SV, ml	*Mdn*	96.5	100.0	96.0	90.0
	(IQR)	(90.0–106.3)	(90.0–110.0)	(88.0–100.0)	(88.0–95.0)
	p value vs control		*p = 0.716*	*p = 0.375*	*p = 0.103*
[g] EF %	*Mdn*	63.5	60.0	61.0	60.0
	(IQR)	(57.7–68.0)	(57.5–63.5)	(58.0–66.0)	(57.0–64.0)
	p value vs control		*p = 0.347*	*p = 0.981*	*p = 0.347*
[h] SVI, ml/m^2	*Mdn*	52.2	53.6	49.4	49.7
	(IQR)	(46.3–59.1)	(49.6–60.2)	(47.4–52.1)	(42.9–52.7)
	p value vs control		*p = 0.767*	*p = 0.288*	*p = 0.157*

[a] BMI (body mass index); weight in kilograms divided by the square of the height in meters, (kg/m^2);
[b] LDL-C; low density lipoprotein cholesterol;
[c] TG; Triglycerides;
[d] TC; Total cholesterol;
[e] CRP; C-reactive protein;
[f] SV; stroke volume, measured by left ventricular outflow method;
[g] EF; ejection fraction, measured by Simpson's method;
[h] SVI (stroke volume index); the relation between the stroke volume (SV) and the size of the person body surface area (BSA), ml/m^2

2.2 Results of cellular produced cytokines

2.2.1 Chemerin

Circulating biomarkers are widely used to determine risks of many diseases, including cardiovascular disease. Assuming that chemerin may affect inflammatory and calcification processes, its significance and potential diagnostic value was studied by comparing persons of the control group with patients with different severity degrees of AVS. Comparing subjects of the control group with all AVS patients, a statistically significant ($p < 0.0001$) higher chemerin level was revealed in the AVS patient group, see Figure 2.1.

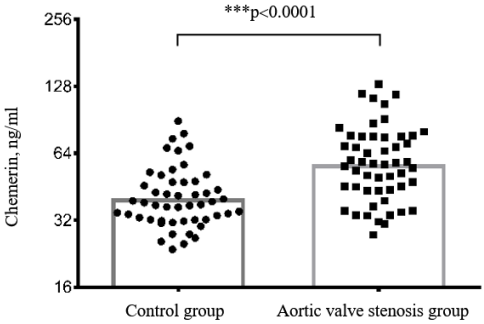

Figure 2.1 **Serum chemerin level in the control and AVS groups**

Analysing chemerin levels across all three AVS severity levels (Figure 2.2), statistically reliable differences were found comparing to the control group. For mild stenosis, the highest level of chemerin ($p = 0.0001$) was obtained, but for severe stenosis the lowest chemerin level ($p = 0.042$) was 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 was observed. A statistically reliable difference ($p < 0.05$) was obtained between chemerin levels in patients with mild and severe AV stenosis.

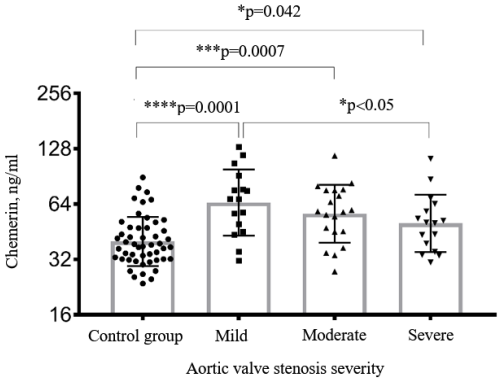

Figure 2.2 **Serum chemerin level in the control group and in all AVS severity grades**

A linear regression analysis shows a statistically significant reduction in the chemerin levels as the AVS severity grade increases ($p = 0.047$); see Figure 2.3.

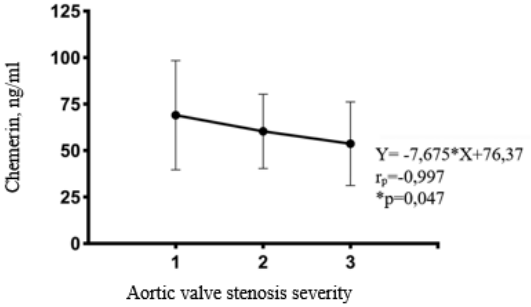

Figure 2.3 **Regression line of serum chemerin level in connection with AVS severity grade**

When evaluating the potential importance of the chemerin as a biomarker, 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 aortic stenosis versus subjects of the control group; see Table 2.2 and Figure 2.4.

Table 2.2

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

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

[a] AUC; area under the curve; [b] Sp; specificity; [c] Se; sensitivity; [d] NPV; negative predictive value; [e] PPV;positive predictive value

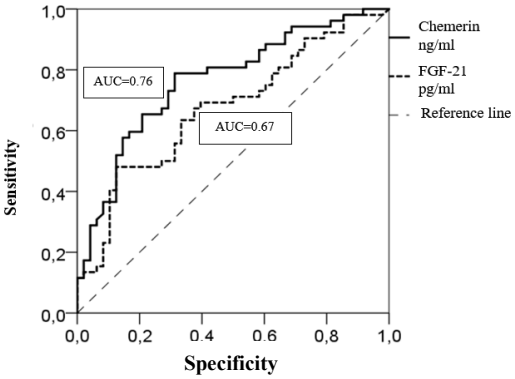

Figure 2.4 **ROC analysis of chemerin and FGF-21 as diagnostic markers in AVS diagnostics** (included all AVS severity grades) **vs. control group**

The prognostic value of the serum chemerin as a potential biomarker was assessed in patients with mild AVS vs. the subjects of the control group; see Table 2.3 and Figure 2.5.

Table 2.3

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

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

[a] AUC; area under the curve; [b] Sp; specificity; [c] Se; sensitivity; [d] NPV; negative predictive value; [e] PPV; positive predictive value

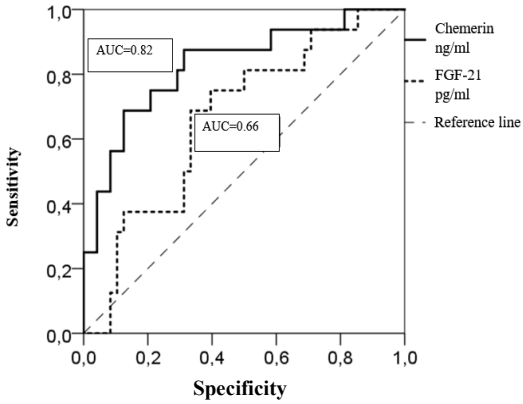

Figure 2.5 **ROC analysis of chemerin and FGF-21 as diagnostic markers in mild AVS diagnostics vs. control group**

When evaluating the potential importance of chemerin as a biomarker for the entire AVS patient group (including all severity grades), the results were obtained 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 diagnostics of mild aortic stenosis: AUC = 0.82; 0.80–0.90 = good; $p < 0.001$; sensitivity is 87 % and the specificity is 69 %.

2.2.2 Fibroblast growth factor-21

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

Patients with aortic valve stenosis have higher FGF-21 levels than subjects of the control group ($p = 0.011$); see Figure 2.6.

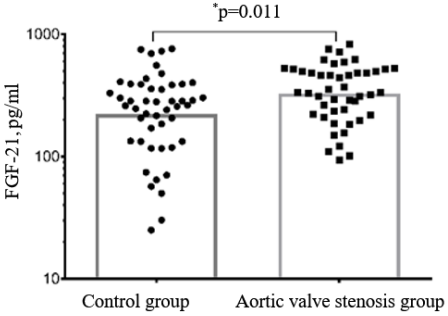

Figure 2.6 **Serum FGF-21 level in the control and AVS groups**

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 2.7.

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 2.8.

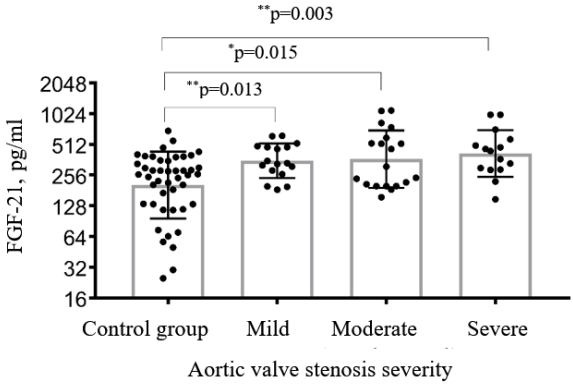

Figure 2.7 **Serum FGF-21 level in the control group and in all AVS severity grades**

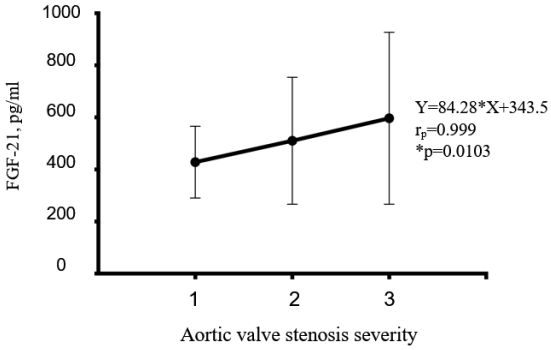

Figure 2.8 **Regression line of serum FGF-21 level in connection with AVS stenosis severity grades**

When evaluating the potential role of FGF-21 as a biomarker, 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 2.4 and Figure 2.4).

Table 2.4

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

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

[a] AUC; area under the curve; [b] Sp; specificity; [c] Se; sensitivity; [d] NPV; negative predictive value; [e] PPV; positive predictive value

The prognostic value of the serum FGF-21 as a potential biomarker was evaluated in patients with mild AVS versus the subjects of the control group; see Table 2.5 and Figure 2.5.

Table 2.5

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

[a] AUC (95% CI)	P value	Cutoff value	[b] Sp %	[c] Se %	[d] NPV %	[e] PPV %	Accuracy %
0.66 (0.51-0.81)	0.04	283.78	61	75	64.4	65.4	68

[a] AUC; area under the curve; [b] Sp; specificity; [c] Se; sensitivity; [d] NPV; negative predictive value; [e] 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), it was 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 %. 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.

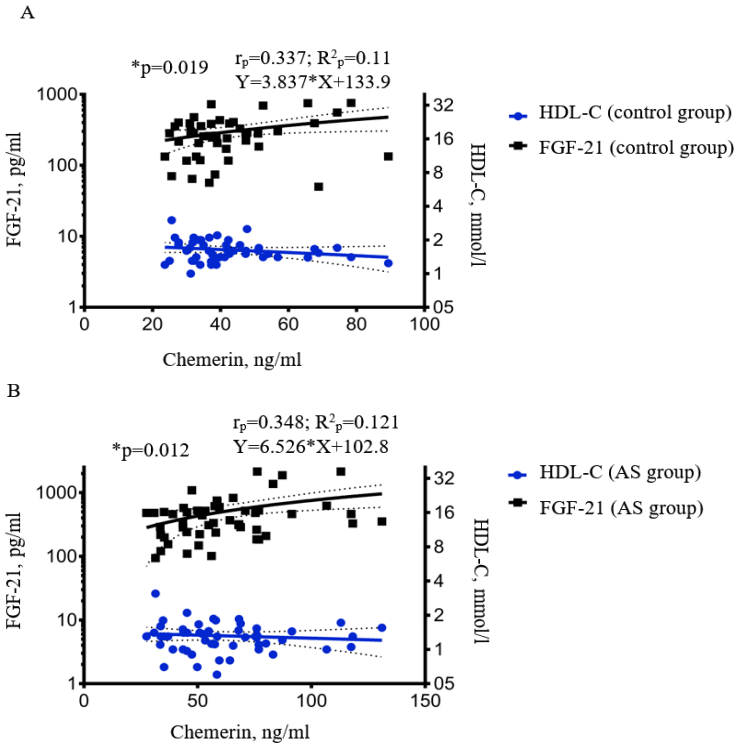

Figure 2.9 **Correlation between serum FGF-21 and chemerin, chemerin and HDL-C concentrations in the control (A) and AVS group (B)**

Analyzing the correlation between chemerin, FGF-21, and HDL-C:

- in the control group, it was found that higher levels of FGF-21 are associated with higher chemerin levels ($p = 0.019$; $r_p = 0.337$); there was no correlation between chemerin and HDL-C, see Figure 2.9 (A);
- in the patient group with aortic valve stenosis a similar correlation, it was found that higher FGF-21 level correlates with higher chemerin level ($p = 0.012$; $r_p = 0.348$); there was no correlation between chemerin and HDL-C, see Figure 2.9 (B).

2.2.3 C-reactive protein

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

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 the current study all possible causes that could cause elevated levels of CRP were exclude; no increase in CRP by progression of AVS was observed.

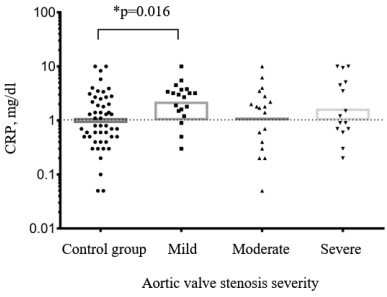

Figure 2.10 **Serum CRP level in the control and AVS groups**

2.2.4 Matrix metalloproteinases and their inhibitors

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

In the current study, serum levels of MMP-1, MMP-3, MMP-9, and TIMP-1 and TIMP-3 were determined, respectively. This allowed to evaluate not only the control group against severe AVS, but also analyse the levels of MMP and TIMP in all grades of aortic valve stenosis.

When comparing the MMP-1 levels in the control group and the AVS group, statistically significantly higher ($p = 0.0043$) MMP-1 levels were found in the patient group of aortic valve stenosis, moreover, multimodal distribution of the results was observed (Figure 2.11 and 2.12).

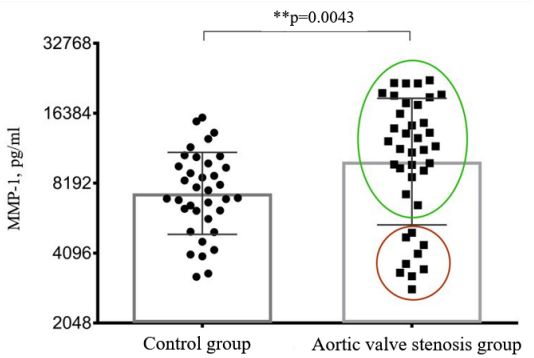

Figure 2.11 **Serum MMP-1 level in the control and AVS groups**

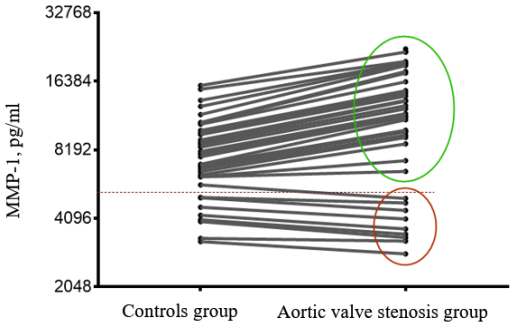

Figure 2.12 **Serum MMP-1 level in the control and AVS stenosis groups, ovals in green and red represent AVS patient clusters of data**

In order to determine the differences between the control and stenosis groups more precisely, distribution analysis was performed. Data distribution histograms determined that the distribution of MMP-1 results in the AVS patient group is trimodal, and in the group of control individual's – monomodal; see Figure 2.13 and 2.14.

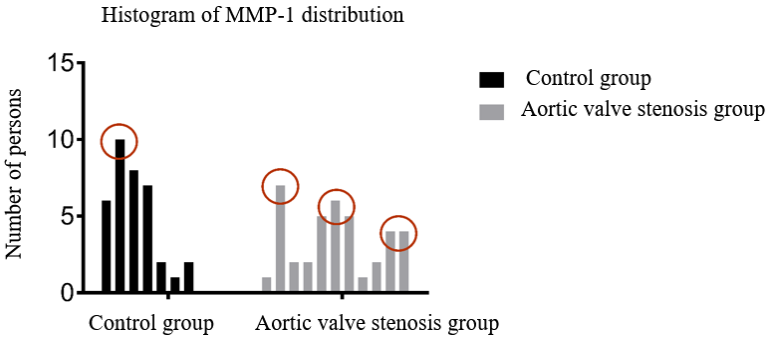

Figure 2.13 **Histograms of MMP-1 distribution, modes are represented by color circles**

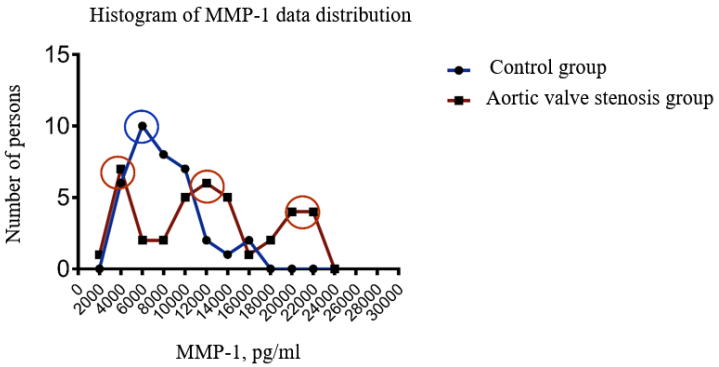

Figure 2.14 **Comparative MMP-1 data distribution between control individuals and AVS patient groups**

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 2.15.

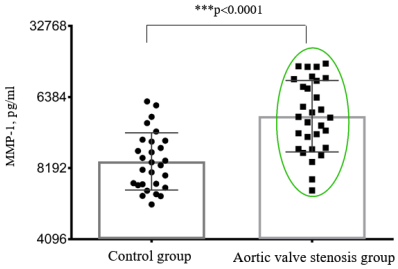

Figure 2.15 **Serum MMP-1 (**upper cluster corresponding to 80 % of patients, see Figure 2.11) **in the control and AVS patient group**

When analysing differences in MMP-1 levels between the control group and three severity grades of AVS, it was 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 2.16.

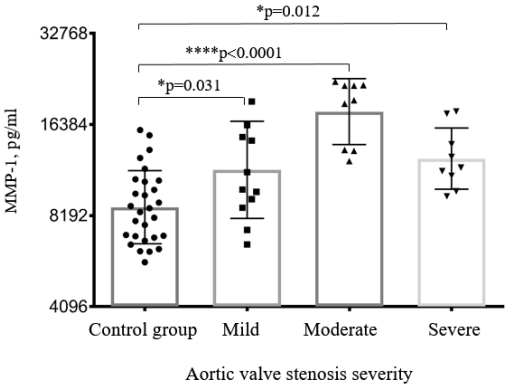

Figure 2.16 **MMP-1 serum levels in the control group and all AVS severity grades**

During the study, matrix metalloproteinases such as MMP-3 and MMP-9 were detected and analysed in the blood serum in the control group and AVS patients. Tissue inhibitors corresponding to these matrix metalloproteinases were also analysed; the corresponding TIMP-1 and TIMP-3. 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 2.17.

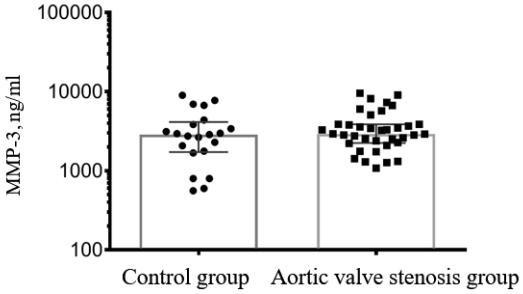

Figure 2.17 **Serum MMP-3 level in the control and AVS groups**

Likewise, no statistically significant differences were found between the individuals of the control group and patients with aortic valve stenosis, when analysing MMP-9 results during the study; see Figure 2.18.

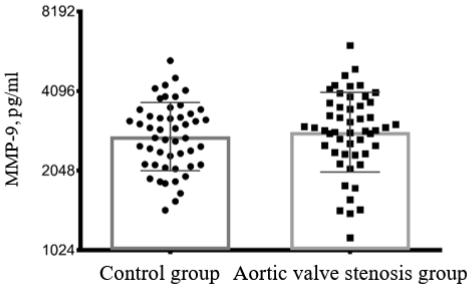

Figure 2.18 **Serum MMP-9 level in the control and AVS groups**

Analysing 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 2.19 and 2.20.

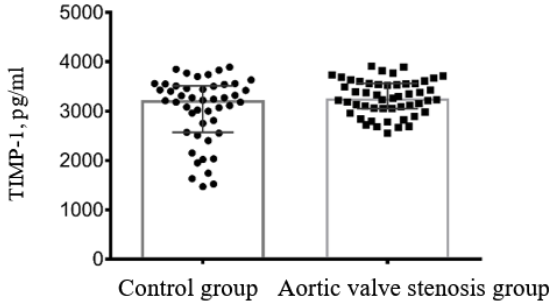

Figure 2.19 **Serum TIMP-1 level in the control and AVS groups**

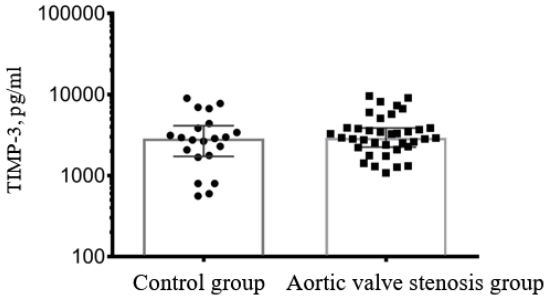

Figure 2.20 **Serum TIMP-3 level in the control and AVS groups**

Possible correlations between MMP-1 and TIMP-1 as well as MMP-3, MMP-9, and TIMP-3 were searched and analysed during the study. Correlation analysis was performed to determine the potential associations between matrix metalloproteinases, tissue inhibitors of matrix metalloproteinases; see Figure 2.21.

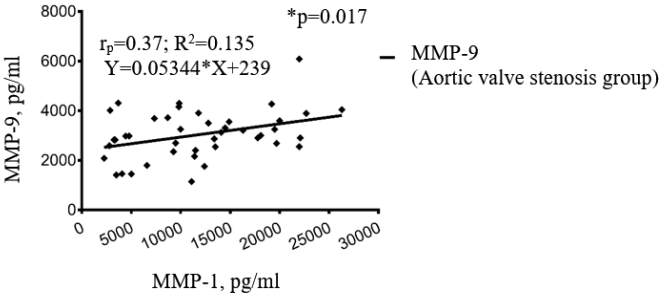

Figure 2.21 **Correlation between MMP-1 and MMP-9 concentrations in AVS patient group**

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

The correlation between MMP-1 and MMP-3, as well as MMP-1 and TIMP-1 was analysed during the study. No associations between MMP-1 and MMP-3, as well as between MMP-1 and TIMP-1 were found; see Figures 2.22 and 2.23.

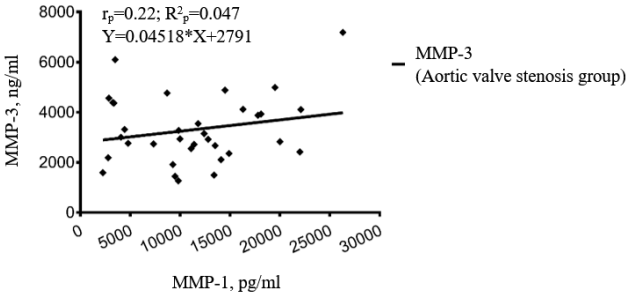

Figure 2.22 **Correlation between MMP-1 and MMP-3 concentrations in AVS patient group**

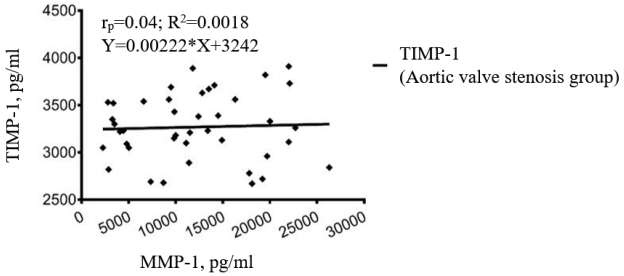

Figure 2.23 **Correlation between MMP-1 and TIMP-1 concentrations in AVS patient group**

In the current study, no associations between MMP-9 and TIMP-1 in the blood serum were identified; see Figure 2.24.

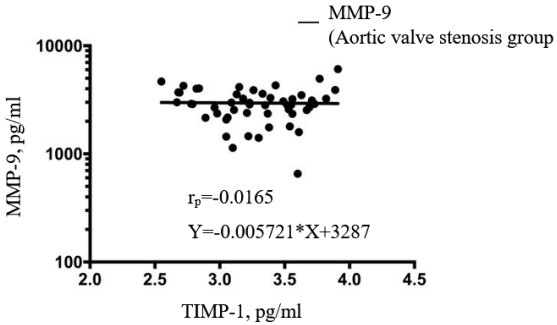

Figure 2.24 **Correlation between TIMP-1 and MMP-9 concentrations in AVS patient group**

There was also correlation analysis between MMP-9, MMP-3, MMP-1, and chemerin; see Figures 2.25, 2.26 and 2.27.

The following correlation ($p = 0.0084$; $r_p = 0.362$) was obtained that higher chemerin serum levels were associated with higher MMP-9 serum

levels. This further confirms the role and presence of MMP-9 in aortic valve stenosis process.

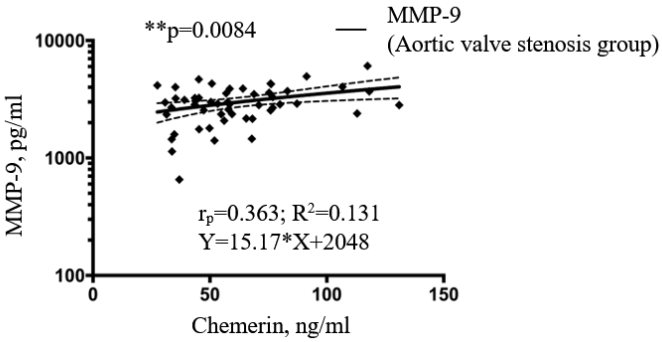

Figure 2.25 **Correlation between chemerin and MMP-9 concentrations in AVS patient group**

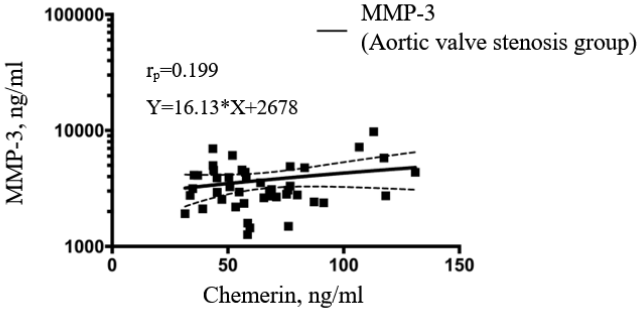

Figure 2.26 **Correlation between chemerin and MMP-3 concentrations in AVS patient group**

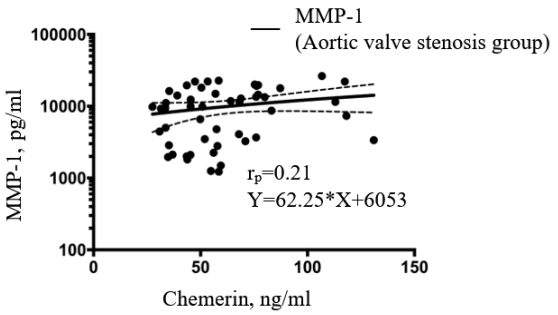

Figure 2.27 **Correlation between chemerin and MMP-3 concentrations in AVS patient group**

2.2.5 Thioredoxin reductase-1

Analysis of antioxidant thioredoxin reductase-1 (TrxR1) in the blood plasma was also performed. When determining the level of TrxR1 in the plasma in the subjects of the control group and in the patients with AVS, a statistically significantly higher level in the AVS group ($p = 0.0016$) was obtained; see Figure 2.28.

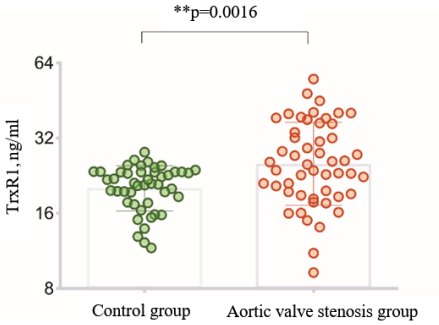

Figure 2.28 **Plasma TrxR1 level in the control and AVS groups**

When analysing differences of the TrxR1 level between the control group and three severity grades of aortic valve stenosis, a statistically significant ($p = 0.0001$) higher TrxR1 level in patients with mild aortic valve stenosis and severe aortic valve stenosis ($p = 0.039$) was obtained; see Figure 2.29.

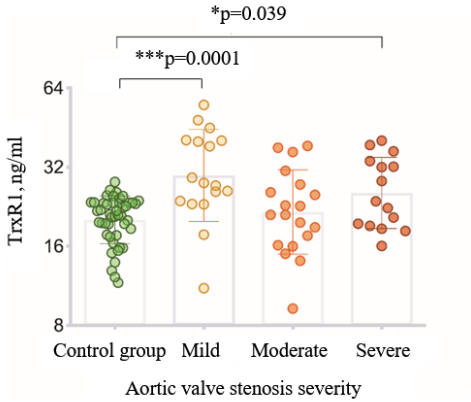

Figure 2.29 **Plasma TrxR1 level in the control group and in all AVS severity grades**

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

In the group of aortic valve stenosis, a statistically significant positive correlation between TrxR1 and MMP-3 ($p = 0.013$; $r_p = 0.37$) was obtained; see Figure 2.30.

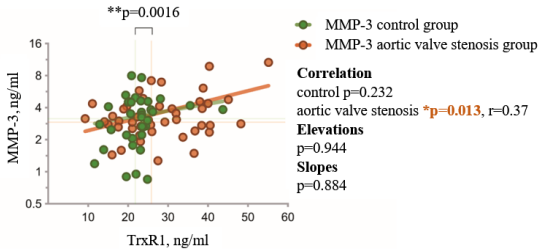

Figure 2.30 **Correlation between TrxR1 and MMP-3 concentrations in the control group and AVS patients**

In contrast, it was absent between TrxR1 and MMP-9, or was not statisticaly significant between TrxR1 and MMP-9. However, in the case of TrxR1 and MMP-1, a significant difference was found between the slopes of the curves characterising the correlation (p = 0.026) when comparing control group with the AVS group; see Figure 5.31. Furthermore, the correlation in case of AVS, 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, Δr, reaches 0.51 or [0.24 - (− 0.27)], that is, increasing TrxR1 level, MMP-1 level also increase; see Figure 2.31.

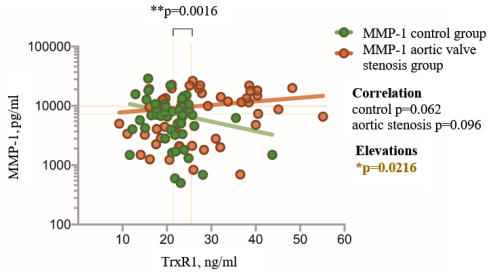

Figure 2.31 **Correlation between thioredoxin reductase-1 and MMP-1 concentrations in the control group and AVS patients**

Association between TrxR1 and chemerin was analysed by the correlation method and a statistically significant ($p = 0.006$; $r_p = 0.32$) positive correlation was obtained; see Figure 2.32.

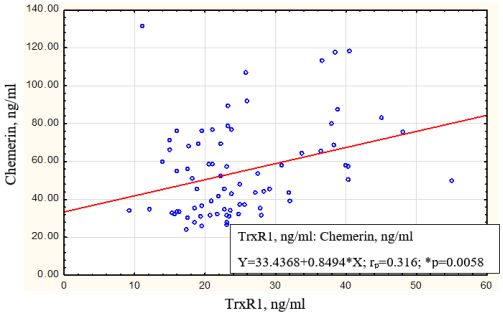

Figure 2.32 **Correlation analysis between thioredoxin reductase-1 and chemerin concentrations in the control group and AVS patients**

Correlation between TrxR1 and FGF-21 was also analysed, and a statistically reliable ($p = 0.031$; $r_p = 0.25$) positive correlation was also obtained; see Figure 2.33.

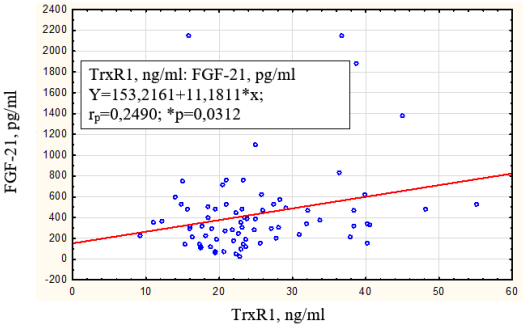

Figure 2.33 **Correlation analysis between thioredoxin reductase-1 and FGF-21 concentrations in the control group and AVS patients**

2.2.6 Myeloperoxidase (MPO)

MPO levels were determined in the control group and in patients with AVS in all three severity grades of the aortic valve stenosis. A statistically significant result was obtained that the plasma myeloperoxidase levels are higher in patients with aortic valve stenosis compared to the control group ($p < 0.00003$). When performing in–depth analysis and comparison of the myeloperoxidase plasma levels between severity grades of the aortic valve stenosis, 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) were obtained; see Figure 2.34. The results show that myeloperoxidase levels increase with the increase in severity of aortic valve stenosis and is the highest in the patient group with severe stenosis.

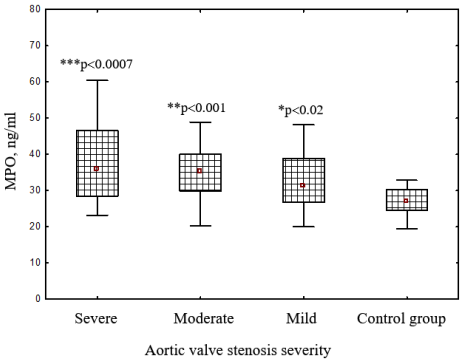

Figure 2.34 **Plasma MPO level in the control group and in all AVS severity grades**

A correlation analysis was performed with the creation of the linear regression line to analyse the correlation between myeloperoxidase (MPO) and thioredoxin reductase-1 (TrxR1); see Figure 2.35.

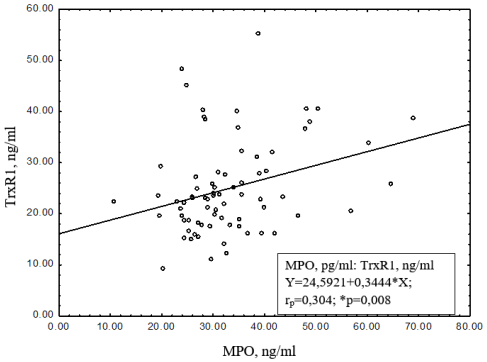

Figure 2.35 **Correlation analysis between MPO and TrxR1 concentrations in the control group and AVS patients**

Statistically significant positive correlation ($p = 0.008$; $r_p = 0.304$) was obtained. Increases in myeloperoxidase plasma levels lead to increase in thioredoxin reductase-1 levels.

Association between MPO and chemerin was also investigated and a statistically significant positive ($p = 0.0057$; $rp = 0.316$) correlation obtained. Increase in serum chemerin levels increases myeloperoxidase levels; see Figure 2.36.

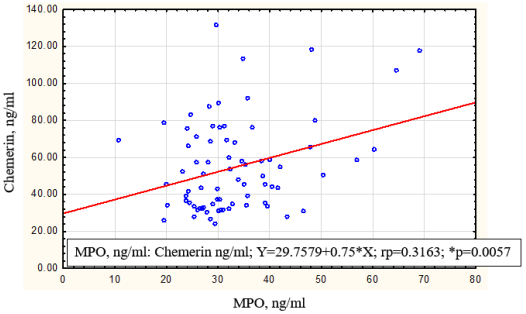

Figure 2.36 **Correlation analysis between MPO and TrxR1 concentrations in the control group and AVS patients**

When analysing the possible association between MPO and FGF-21, a statistically significant correlation ($p < 0.05$) between these biomarkers was not obtain neither in individuals of the control group nor in the patients with aortic valve stenosis; see Figure 2.37.

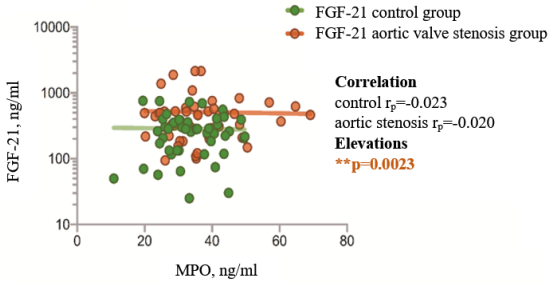

Figure 2.37 **Correlation analysis between MPO and FGF-21 concentrations in the control group and AVS patients**

When evaluating the associations between myeloperoxidase and matrix metalloproteinases, a statistically significant, positive ($p = 0.007$; $r_p = 0.37$) correlation between MPO and MMP-9 was obtained: by increase in the myeloperoxidase plasma levels in patients with aortic valve stenosis the MMP-9 level also increases; see Figure 2.38.

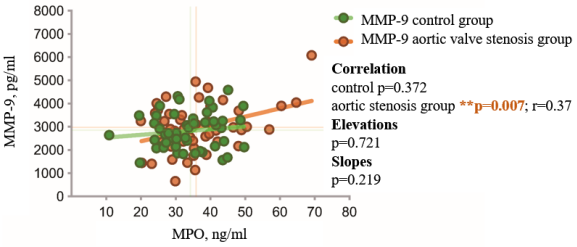

Figure 2.38 **Correlation analysis between MPO and MMP-9 concentrations in the control group and AVS patients**

By the correlation analysis, weak, but statistically significant negative ($p = 0.047$; $r_p = -0.28$) correlation between MPO and HDL-C was obtained: the higher the MPO level, the lower the level of HDL-C; see Figure 2.39.

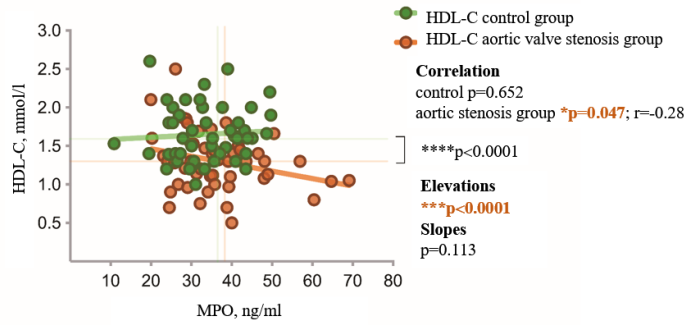

Figure 2.39 **Correlation analysis between MPO and HDL-C concentrations in the control group and AVS patients**

3. 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, Ehokg 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. The current study included severe AVS patients with 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 ml/m^2). Following the above-mentioned guidelines of 2017, the determination of the amount of calcium (calcium score) by multislice DT is a first–line additional examination in patients with PG mean. < 40 mm/Hg, AVA ≤ 1.0 cm^2 and SVI ≤ 35 ml/m^2. Since the study did not include any such patients, the determination of the calcium score was not indicated as patients with normal left ventricle function were initially included in order to avoid the examination, the availability of which is limited and, during the formation of patient groups, insufficiently developed to determine the precise calcium score. Etiopathogenesis of calcific AVS by comparing guidelines and analysing 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 the highest in the mild AVS patients suggest that the action takes place more when chemerin binds to the Chem23 receptor. If chemerin binded to GPR-1, then its level would have to 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.

Statistically significant differences between the control group and the AVS group when determining MMP-9 serum levels were not defined, but a relationship between chemerin and MMP-9 was obtain: 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. The obtained data further support the association with inflammatory processes in AVS patients and indicate remodelling of extracellular space already in the mild degree of aortic valve stenosis when there is the highest level of the chemerin. This connection could also indicate that chemerin acts as an inflammatory promoter.

Positive association between chemerin and antioxidant thioredoxin reductase-1 (TrxR1) was also found: 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 between chemerin and TrxR1 indicates that there is both oxidative stress and inflammation in the development of AVS from its beginning.

When analysing pro-oxidant enzyme myeloperoxidase (MPO), a positive correlation between chemerin and MPO was obtained: with increase in chemerin levels, MPO levels increase. Chemerin and MPO have a known common property of influencing NO: chemerin reduces NO release; MPO reduces bioavailability of NO and by binding NO forms ROS. Both chemerin and MPO contribute to the release of MMP and remodelling of extracellular space. This may indicate that chemerin in 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 serum FGF-21, the role of this biomarker in the development of AVS cannot be unequivocally evaluated. FGF-21 results allow to suspect 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 the current 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. More can be learnt about FGF-21 by determining the level of IL-6 and TNF-α 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 the current study, CRP cannot be used to predict the rate of progression of AVS. The obtained 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 analysed in the reserch showed no statistically significant differences between the control group and AVS patients.

Analysing 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 middle 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 remodelling of the extracellular space with the degradation of the collagen fibers and osteoblast differentiation and calcification.

In the 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 analysing these patients that everybody had mild aortic valve stenosis. 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 valve 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, as these patients could theoretically expect a slow progression of aortic valve stenosis.

The data obtained in the current study on changes in the MMP-1 levels in different grades of aortic valve stenosis (Figure 2.16), 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.

No correlation between MMP-1 and TIMP-1 serum levels was found. This suggests that the level and function of MMP-1 involved in remodelling extracellular space of aortic valve stenosis is not regulated by TIMP-1. No statistically significant differences in the TIMP-1 serum levels between patients of aortic valve stenosis and the control group were found. 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, the correlation was obtained 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 these 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 the current study it did not have a 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. Localisation of MMP-9 around the calcification nodules was observed.

Correlation was found that higher chemerin serum levels are associated with higher MMP-9 serum levels. This further substantiates the role and 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, lower in the severe aortic valve stenosis, and 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 the heart failure is, the higher is the thioredoxin level (Jekell et al., 2004).

Positive correlation was found 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, no correlations that would demonstrate the role of MMP-3 in the aortic valve stenosis were found. The fact that a statistically significant, positive correlation between the thioredoxin reductase-1 and MMP-3 in AVS patients was find suggest that MMP-3 also plays a role in the process of aortic valve stenosis.

Some association was determined 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 localised around the calcification zones.

When determining the MPO level, it was obtained 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:

- the positive correlation with TrxR1 and chemerin could be explained by endothelial dysfunction, ROS formation and active participation in inflammatory and oxidative stress processes;
- 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 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 oxidises 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. There is no ability to directly influence the activity of MPO, but the

higher the level of HDL-C will be in patients, the better will be the protective role of HDL-C (Perrot et al., 2018).

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

The fact that the oxidative stress level is relatively higher in 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.

4. CONCLUSIONS

1. Distribution of patients with aortic valve stenosis into three severity grades and evaluation against 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. Remodelling of the extracellular space begins at the mild degree of aortic valve stenosis, and is most pronounced in the moderate severity degree of the aortic stenosis. This is indicated by a significant increase in MMP-1 concentrations, both in the mild AVS grade and the more pronounced in moderate grade of AS, and in a positive correlation between MMP-1 and MMP-9, chemerin and MMP-9.
4. The obtained results of the analysis 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 severe AVS degree than in moderate AVS degree are attributed to the fact that both oxidative stress continues and most 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. The first hypothesis on the role of inflammatory factors and cytokines in the development of AVS is demonstrated: by the results of Chemerin, FGF-21, CRP, MMP-1, TrxR1, MPO levels; by correlations between MMP-1 and MMP-9 and chemerin, also between TrxR1 and MMP-3. It is not yet possible to judge whether these factors and cytokines determine AVS prognosis. This requires further genetic studies.
7. The second hypothesis has been confirmed: a significant role of HDL-H in AVS patients – a negative correlation was found between MPO and HDL-H, where higher MPO levels are associated with lower levels of HDL-H. Patients with severe AVS have the highest MPO level, which can be explained by the activity of MPO, forming ox-HDL-H and reducing the protective activity of HDL-H, so the higher the level of HDL-H in patients, the better the protective role of HDL-H.
8. It is determined that oxidative stress is present in all severity levels of AVS and is related to the inflammatory process, also confirming the third hypothesis.

5. PRACTICAL RECOMMENDATIONS

1. Chemerin and its detection in the blood serum is a good diagnostic marker for 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 detection could be performed in patients in whom due to the reduced left ventricular systolic function it is difficult to distinguish between mild and moderate degrees of stenosis in the diagnosis based on the maximum flow rate and mean pressure gradient. Blood serum detection could be used theoretically in patients with visual AV sclerosis and in patients with elevated pressure gradient to AV but without AVA and indexed AVA reduction for dynamic observation.
2. A program of monitoring and dynamic examination of the patients with mild AVS should be developed 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 patients 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 analysis, both ox-LDL-C and HDL-C are associated with AVS. Efforts should be made to maximise the level of HDL-C in 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; the higher it will be, 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. *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. *23rd World Congress on Advances in Oncology and 22nd International Symposium on Molecular Medicine.* Athens, Greece, 2018, September 20–22

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