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EXPRESSION OF TISSUE INHIBITORS OF METALLOPROTEINASES TYPE 1 AND TYPE 2 IN THE LEAFLETS OF EXPLANTED BIOPROSTHETIC HEART VALVES: A NEW PATHOGENETIC PARALLEL BETWEEN STRUCTURAL VALVE DEGENERATION AND CALCIFIC AORTIC STENOSIS

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Objective: to study cellular and lipid infiltration, as well as the expression of tissue inhibitors of metalloproteinases (TIMP) types 1 and 2 in biological prosthetic heart valves (BPHVs) explanted due to dysfunction. **Material and Methods.** We examined 17 leaflets from 6 BPHVs, dissected from the aortic and mitral positions during valve replacement. For microscopic analysis, fragments of the BPHV leaflets were frozen and serial sections were made using a cryotome. In order to study cellular infiltration and the degree of degenerative changes in the prosthetic biomaterial, the sections were stained with Gill's hematoxylin and eosin; Oil Red O stain was used to assess lipid deposition. Immunohistochemistry was used for cell typing and detection of TIMP-1/-2. The stained samples were analyzed by light microscopy. **Results.** Cellular and lipid infiltration of xenogeneic tissues was detected in all BPHV flaps studied. Recipient cells coexpressed pan-leukocyte and macrophage markers PTPRC/CD45 and CD68. Positive staining for TIMP-1/-2 co-localized with cell clusters but was absent in acellular sections. **Conclusion.** Cells infiltrating xenogeneic BPHV tissues express TIMP-1/-2. This suggests that BPHV immune rejection pathophysiology is partially similar to that of calcific aortic stenosis.

Keywords: bioprosthetic heart valves, structural valve degeneration, calcific aortic stenosis, cellular infiltration, lipids, tissue inhibitors of metalloproteinases.

INTRODUCTION

Despite advances in medical technologies, there are still no effective methods of conservative treatment of severe valve pathologies; so affected heart valves are replaced by mechanical or biological prostheses [1]. Bioprosthetic heart valves (BHVs) are made of chemically stabilized animal tissues [2]. They compare favorably with their mechanical counterparts with low thrombogenicity, but their biological component is subject to structural degeneration [3, 4]. The latter is manifested by dissociation, fragmentation and calcification of the fibrous component of the extracellular matrix (ECM) of the flail apparatus, becoming the cause of BHV regurgitation or stenosis [3, 4]. Despite modern achievements in biomaterial chemical modification methods and improvement in BHV design, this problem remains unresolved; up to half of prosthetic valves require replacement as early as 15 years after implantation in patients [1].

Interestingly, the results of modern studies indicate that a number of pathophysiological features of structural degeneration of BHV biomaterial and calcific aortic stenosis (CAS) are similar. Thus, degenerative changes arising in the affected native aortic valve (AV), associated with fibrosis and calcification, are caused by lipid deposition and macrophage infiltration of the leaflets [5]. Recent studies demonstrate that during degeneration of BHV biomaterial, lipids and immune cells also accumulate in the tissue, which is accompanied by production of matrix metalloproteinases (MMPs) [6, 7]. It is assumed that these processes can contribute significantly to degradation of ECM prosthetic biomaterial [8]. In this regard, in-depth study of pathophysiological patterns of BHV degeneration is of significant interest, as it potentially opens the way to development of drug therapy aimed at slowing down degeneration of both native heart valves and their substitutes.

One of the factors regulating ECM remodeling of native AVs includes tissue inhibitors of metalloproteinases (TIMPs) types 1 and 2, overexpressed in CAS [9, 10]. TIMP-1/-2 are multifunctional proteins that, in addition to inhibiting MMPs, act like signaling molecules involved in regulation of apoptosis, cell proliferation and differentiation [11, 12]. Production of TIMP-1/-2 cells, as well as MMPs, indicates their ECM remodeling activity. To date, there have been no published data on TIMP expression in BHV tissues.

The objective of this work is to study cellular and lipid infiltration of leaflets explanted due to BHV dys-

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function, and to detect TIMP-1/-2 expression in the implant tissues.

Material and methods

For this study, we used epoxy-treated BHVs manufactured by NeoCor (Russia), excised from aortic or mitral positions in 6 patients during valve repair operations in 2019–2020. The mean age of recipients with primary valve replacement was 54.5 ± 10.5 years. The studied BHVs lasted for an average of 12 ± 8 years. The study was approved by the local ethics committee of the Research Institute for Complex Issues of Cardiovascular Diseases. BHVs were included in the study subject to the patients' signed voluntary informed consent. Endocarditis as a cause of dysfunction was a criterion for excluding BHVs from this study.

After macroscopic description of BHVs obtained during reoperations, samples were prepared for microstructural study of the material. From each BHVs, the central part of 1–3 flaps was taken from the base to the free edge, as well as areas with pronounced degenerative changes, which were frozen at –140 °C. Then, using a Microm HM 525 cryotome (Thermo Scientific, Germany), serial 6 ± 1 μm-thick sections were prepared and placed on slides, 4–6 pieces at a time. A total of 17 specimens from 6 explanted BHVs were studied, including xenoaortic bioprosthetic valves KemCor (n = 2) and PeriCor (n = 2), as well as the pericardial models UniLine (n = 1) and TiAra (n = 1).

In order to study the intensity of infiltration by recipient cells and determine the degree of degenerative changes in the prosthetic biomaterial, the sections were stained with Gill's hematoxylin and eosin in accordance with a protocol optimized for circulatory system tissues [13]. The presence of lipids in the structure of BHV biomaterial was determined by staining the sections with Oil Red O (Abcam, UK). For this purpose, the sections were fixed in 4% paraformaldehyde (Sigma-Aldrich, USA), then washed with phosphate-buffered saline (PBS) (Sigma-Aldrich, USA) and incubated in isopropanol for 5 minutes. Then they were stained with Oil Red O for 15 minutes, washed with 60% isopropanol, and contrasted with Mayer's modified hematoxylin (Abcam, UK). Finally, the sections were washed in tap water and then in double-distilled water and covered with Mowiol (Sigma-Aldrich, USA).

For cell typing and TIMP-1/-2 detection, we used manual immunohistochemical staining using antibodies against PTPRC/CD45, CD68, TIMP-1, and TIMP-2 (Abcam PLC, UK). Before staining, sections were fixed for 10 minutes at room temperature with 4% paraformaldehyde followed by three washes (5 minutes each) in PBS (pH 7.4) on a shaker (Polymax 1040, Heidolph, 25 rpm). Immunohistochemical reaction was performed using NovoLink Polimer Detection System kit (Leica Microsystems Inc., USA) according to the manufacturer's modified protocol. First, endogenous peroxidase was blocked with 4% hydrogen peroxide solution (Peroxidase Block) for 5 minutes. The sections were then washed twice in PBS and blocked for 60 minutes with 0.4% casein saline with adjuvant reagents (Protein Block) for nonspecific antibody binding. Primary antibodies were diluted according to the manufacturer's protocol in 1% bovine serum albumin saline in proportions of 1 to 1000, 1 to 2000, and 1 to 3000 for antibodies against CD68, TIMP-1/2, and PTPRC/CD45, respectively. The sections were incubated with antibodies in the dark at +4 °C overnight, then washed three times in PBS. When stained for CD68 and TIMP-2, the sections were additionally treated for 30 minutes with anti-mouse rabbit antibodies (Post Primary), followed by three washes in PBS. Next, the sections were incubated for 30 minutes with anti-rabbit antibodies (Novolink Polymer), and then again washed three times in PBS and treated for 2 minutes with 0.087% diaminobenzidine solution. Then, the sections were washed with double-distilled water and placed in hematoxylin (from the kit) for 10 minutes. After that, we blued the sections in running water (5 minutes), dehydrated them in three shifts of 95% ethanol (5 minutes each), and cleared them in three shifts of xylene (5 minutes each), concluding with Vitrogel (BioVitrum, Russia) under a coverslip. Intact epoxy-treated xenogeneic tissues were used as controls: porcine aortic valve leaflets and bovine pericardium purchased from Neocor (Kemerovo, Russia). Also, one slice was isolated on each glass with negative control of primary and secondary antibodies.

Samples with histological and immunohistochemical stains were analyzed using an AxioImager.A1 light microscope (Zeiss, Germany). The images were processed using AxioVision software (Zeiss, Germany).

RESULTS

Macroscopic description of explanted BHVss

BHVs included in this study showed signs of primary tissue failure in the form of leaflet flail ruptures in the commissural area, perforations as well as signs of calcification (Fig. 1). Pannus fixation of the cusps along the frame struts on the outflow side was noted, which contributed to some limitation in their mobility. Pannus was noted in 4 out of 6 BHVs, while calcification was observed in varying degrees for all the studied implants.

Histological examination of flaps of explanted BHVs

At the microstructural level, degenerative changes in the biomaterial were in the form of loosening and fragmentation of collagenous fibers. Infiltration of prosthetic biomaterial by recipient cells was detected in all examined samples (Fig. 2). Cellular infiltrates were localized predominantly on the surface or in the loosened pre-surface layers of the xenogeneic ECM near the base

of the flaps. Cell penetration deep into the biomaterial was noted only for xenoaortic prostheses, and the most massive cellular infiltrates were recorded near perforations and large calcifications, where pronounced ECM debridement was observed. The biomaterial was affected to a greater extent by cellular infiltration on the outflow side.

Lipid infiltration in varying degrees was noted in all samples studied. Lipid localization did not depend on the presence of cells in the ECM. Samples of intact epoxy-treated xenogeneic tissues showed no positive staining with Oil Red O dye.

Immunohistochemical analysis of flaps of explanted BHVs

Immunohistochemical staining of sections demonstrated that most cells in the infiltrates coexpressed the panleukocyte and macrophage markers PTPRC/CD45

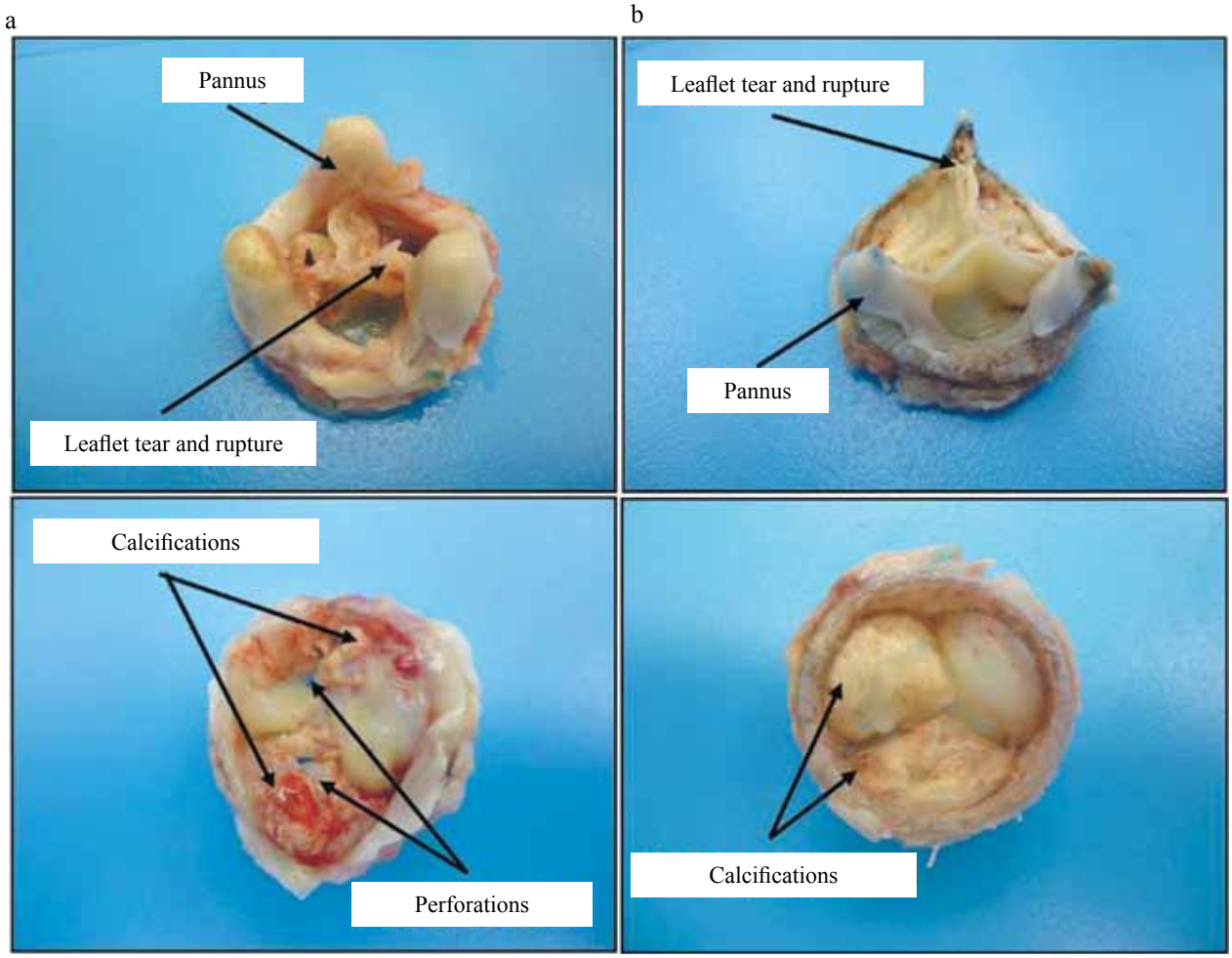

Fig. 1. Heart valve bioprostheses removed due to dysfunction: a – xenoaortic prosthesis (PeriCor); b – xenopericardial prosthesis (UniLine); Upper and lower rows – view from the outflow and inflow side, respectively

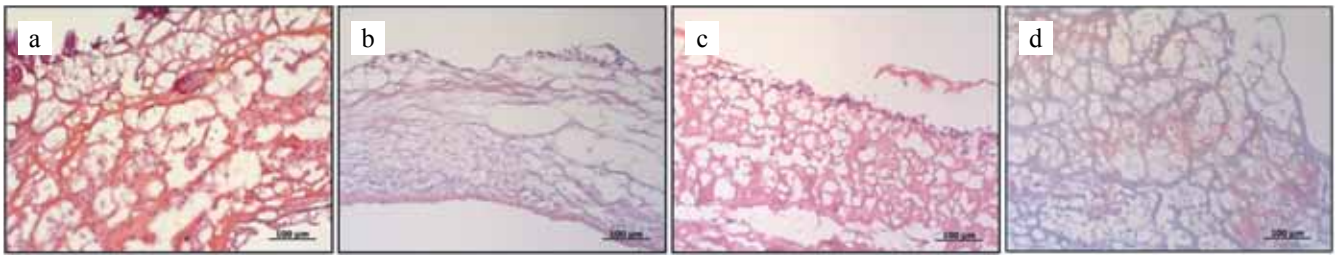

Fig. 2. Cellular and lipid infiltration of heart valve bioprosthesis leaflets: a – large cellular infiltrate near the calcific (Gill's hematoxylin and eosin staining); b and c – cellular infiltration of the surface layers of xenogeneic tissue of the xenoaortic and xenopericardial prosthesis respectively (Gill's hematoxylin and eosin); d – lipid spots in the thickness of the prosthetic leaflets (Oil Red O stain)

and CD68, allowing them to be identified as macrophages (Fig. 3). It was also possible to identify these cells as a source of TIMP-1/-2. Positive staining for these molecular agents was noted in all samples studied in co-localization with cellular infiltrates (Fig. 4). No staining for TIMP-1/-2 was observed in acellular ECM.

No positive staining for PTPRC/CD45, CD68, or TIMP-1/-2 was observed in all controls.

DISCUSSION

Evidence obtained is consistent with the results of other original studies that investigated the cellular and

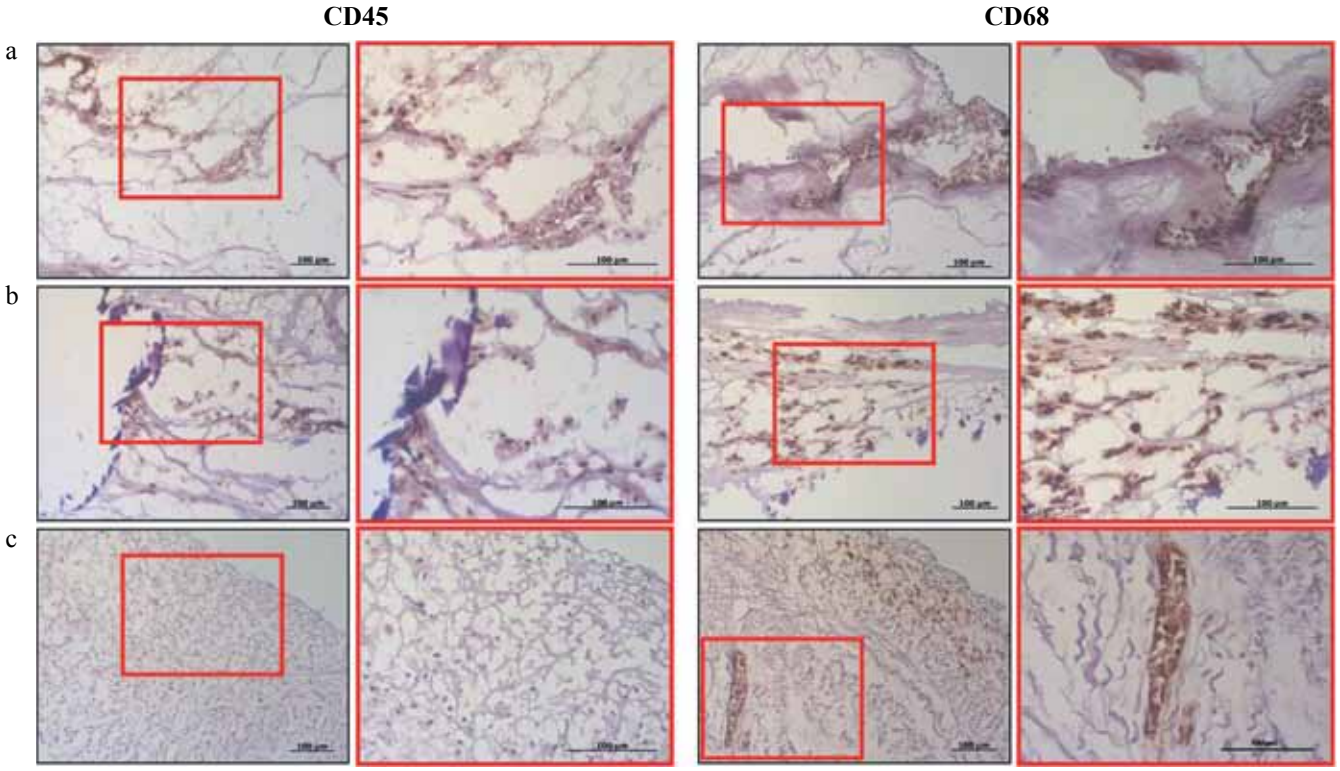

Fig. 3. Results of immunohistochemical staining of cardiac valve bioprosthesis leaflets for PTPRC/CD45 and CD68: a – clusters of immune cells in the loosened subsurface layers of xenogeneic prosthesis leaflets; b – large inflammatory infiltrates near calcium deposits in xenogeneic prosthesis leaflets; c – diffuse cellular infiltration of the surface layers of xenogeneic prosthesis leaflets. Pay attention to the penetration of macrophages deep into the xenopericardium due to the pseudovessels

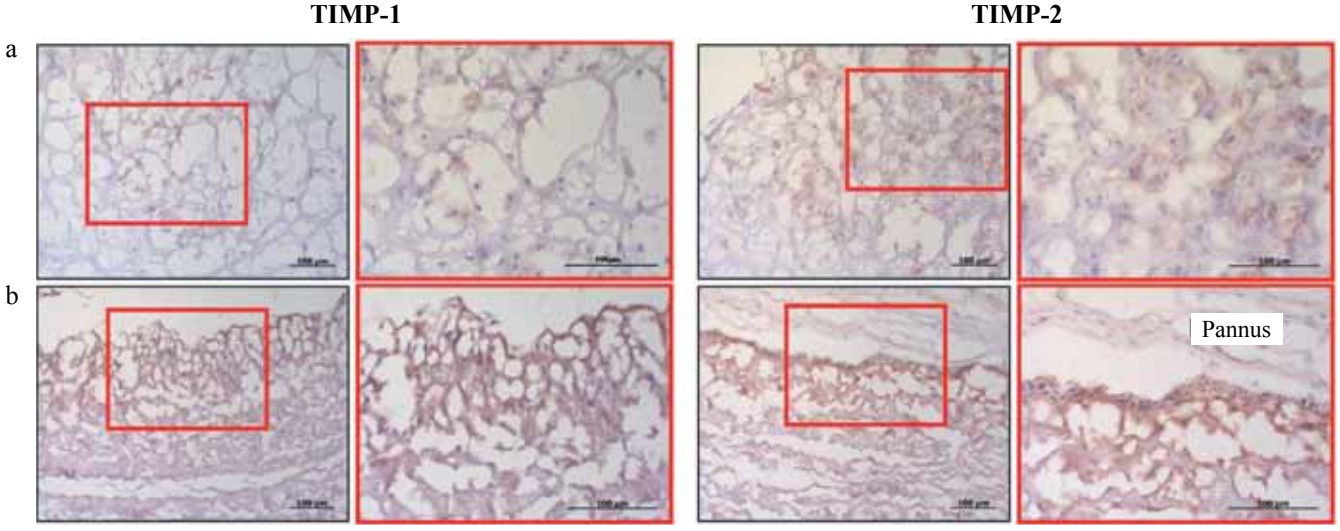

Fig. 4. The results of immunohistochemical staining of heart valve bioprosthesis leaflets for TIMP-1/-2: a – staining of infiltrates in the thickness of xenoaortic prosthesis leaflets; b – staining of infiltrates located in the surface layers of the xenopericardial prosthesis biomaterial

lipid infiltration of BHV xenogeneic tissues [6, 7]. It also confirms the conclusions made by us earlier [14]. Staining of most cells for PTPRC/CD45 and CD68 markers indicates the predominance of macrophages in the composition of cell infiltrates formed in the BHV flaps. It is important to note that macrophages dominate regardless of the timing of implant functioning. This indicates a chronic type of the inflammatory response arising in response to BHV implantation. Interestingly, inflammation in the native AV leaflets, which is the driving force of CAS, is also chronic in nature [5].

Lipid deposition in the flaps of BHVs of foreign and domestic models has been previously studied, although the exact contribution to the structural destruction of the prosthetic biomaterial has not yet been established [6, 15]. Potentially, lipid accumulation can promote activation of oxidative processes in the flaps. It has been demonstrated that the biological component of BHVs is highly susceptible to degradation through oxidation-dependent mechanisms [16]. In addition, lipid uptake by macrophages stimulates the latter to degenerate into foam cells, which are found in the tissues of BHVs [15]. Foam cells secrete a wide range of bioactive compounds, including chemokines, mitogens and growth factors, so they can stimulate fibroblast migration, proliferation and synthesis of connective tissue. This process is intrinsic to the pathogenesis of CAS, where it is responsible for fibrosis and sclerosing of AV leaflets [5]. Apparently, it is also partly responsible for pannus formation on the surface of BHVs.

We found for the first time that cells infiltrating BHV flaps express TIMP-1/-2 cells, but the clinical significance of this phenomenon is still unknown. Experiments on knockout mice show that overexpression of TIMP-1/-2 has an antiatherogenic effect by suppressing the activity of MMPs and reducing the inflammatory response in the vessels [17]. It is suggested that TIMP-1/-2 may play a similar role in native AV lesions, with increased expression of these factors being a reflex mechanism protecting ECMs against proteolytic degradation [10]. TIMP-1/-2 expression in BHV tissues can have both positive and negative significance. We previously found that macrophages infiltrating BHVs produce MMPs [14]. Thus, TIMP-1/-2 can protect the prosthetic biomaterial from proteolysis. Meanwhile, decreased activity of proteolytic enzymes may promote proliferation of connective tissue over the BHV flaps. Further research is needed to test the hypotheses presented.

CONCLUSION

The xenogeneic biomaterial of BHVs is subject to lipid accumulation as well as infiltration by immune cells, which in turn express TIMP-1/-2. These observations suggest that there is a partial similarity in the pathophysiology of structural degeneration of BHV biomaterial with that of CAS. The direct role of TIMP-1/-2 in the development of BHV dysfunction is currently unknown and requires to be studied.

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The authors declare no conflict of interest.

REFERENCES

1. *Pibarot P, Dumesnil JG.* Prosthetic heart valves: selection of the optimal prosthesis and long-term management. *Circulation.* 2009; 119 (7): 1034–1048. doi: 10.1161/CIRCULATIONAHA. 108.778886.
2. *Ovcharenko EA, Klyshnikov KYu, Glushkova TV, Nushtaev DV, Kudryavtseva YuA, Savrasov GV.* The choosing of the xenopericardial patch for transcatheter heart valve. *Medical equipment.* 2015; 5: 1–4. [In Russ].
3. *Capodanno D, Petronio AS, Prendergast B, Eltchaninoff H, Vahanian A, Modine T et al.* Standardized definitions of structural deterioration and valve failure in assessing long-term durability of transcatheter and surgical aortic bioprosthetic valves: a consensus statement from the European Association of Percutaneous Cardiovascular Interventions (EAPCI) endorsed by the European Society of Cardiology (ESC) and the European Association for Cardio-Thoracic Surgery (EACTS). *Eur Heart J.* 2017; 38 (45): 3382–3390. doi: 10.1093/eurheartj/ehx303.
4. *Dvir D, Bourguignon T, Otto CM, Hahn RT, Rosenhek R, Webb JG et al.* Standardized definition of structural valve degeneration for surgical and transcatheter bioprosthetic aortic valves. *Circulation*. 2018; 137 (4): 388–399. doi: 10.1161/CIRCULATIONAHA.117.030729.
5. *Lindman BR, Clavel MA, Mathieu P, Iung B, Lancellotti P, Otto CM et al.* Calcific aortic stenosis. *Nat Rev Dis Primers.* 2016; 2: 16006. doi: 10.1038/nrdp.2016.6.
6. *Shetty R, Pibarot P, Audet A, Janvier R, Dagenais F, Perron J et al.* Lipid-mediated inflammation and degeneration of bioprosthetic heart valves. *Eur J Clin Invest.* 2009; 39 (6): 471–480. doi: 10.1111/j.1365-2362.2009.02132.x.
7. *Simionescu A, Simionescu DT, Deac RF.* Matrix metalloproteinases in the pathology of natural and bioprosthetic cardiac valves. *Cardiovasc Pathol.* 1996; 5 (6): 323–332. PMID: 25851789.
8. *Cote N, Pibarot P, Clavel MA.* Incidence, risk factors, clinical impact, and management of bioprosthesis structural valve degeneration. *Curr Opin Cardiol.* 2017; 32 (2): 123–129. doi: 10.1097/HCO.0000000000000372.
9. *Bosse Y, Miqdad A, Fournier D, Pepin A, Pibarot P, Mathieu P.* Refining molecular pathways leading to calcific aortic valve stenosis by studying gene expression profi-

le of normal and calcified stenotic human aortic valves. *Circ Cardiovasc Genet.* 2009; 2 (5): 489–498. doi: 10.1161/CIRCGENETICS.108.820795.

10. *Fondard O, Detaint D, Iung B, Choqueux C, Adle-Biassette H, Jarraya M et al.* Extracellular matrix remodelling in human aortic valve disease: the role of matrix metalloproteinases and their tissue inhibitors. *Eur Heart J.* 2005; 26 (13): 1333–1341. doi: 10.1093/eurheartj/ehi248.
11. *Arpino V, Brock M, Gill SE.* The role of TIMPs in regulation of extracellular matrix proteolysis. *Matrix Biol.* 2015; 44–46: 247–254. doi: 10.1016/j.matbio.2015.03.005.
12. *Ries C.* Cytokine functions of TIMP-1. *Cell Mol Life Sci.* 2014; 71 (4): 659–672. doi: 10.1007/s00018-013-1457-3.
13. *Bogdanov LA, Kutikhin AG.* Optimization of hematoxylin and eosin staining of heart, blood vessels, liver, and spleen. *Fundamental and Clinical Medicine.* 2019; 4 (4): 70–77. [in Russian]. doi: 10.23946/2500-0764-2019-4-4-70-77.
14. *Kostyunin AE, Glushkova TV.* The first results of the study of matrix metalloproteinases-1/-2/-9/-12 expression in xenogenic tissues of epoxy-treated bioprosthetic heart valves explanted due to dysfunctions. *Russian Journal of Cardiology.* 2020; 25 (10): 49–55. doi: 10.15829/1560-4071-2020-3978.
15. *Kostyunin A, Mukhamadiyarov R, Glushkova T, Bogdanov L, Shishkova D, Osyaev N et al.* Ultrastructural pathology of atherosclerosis, calcific aortic valve disease, and bioprosthetic heart valve degeneration: commonalities and differences. *Int J Mol Sci.* 2020; 21 (20): E7434. doi: 10.3390/ijms21207434.
16. *Lee S, Levy RJ, Christian AJ, Hazen SL, Frick NE, Lai EK et al*. Calcification and oxidative modifications are associated with progressive bioprosthetic heart valve dysfunction. *J Am Heart Assoc.* 2017; 6 (5). pii: e005648. doi: 10.1161/JAHA.117.005648.
17. *Di Gregoli K, George SJ, Jackson CL, Newby AC, Johnson JL.* Differential effects of tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 on atherosclerosis and monocyte/macrophage invasion. *Cardiovasc Res.* 2016; 109 (2): 318–330. doi: 10.1093/cvr/cvv268.

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