ROMANIAN ACADEMY
School of Advanced Studies of the Romanian Academy
Institute of Cellular Biology and Pathology "Nicolae Simionescu"

DOCTORAL THESIS SUMMARY

Study of aortic valve inflammation associated with diabetes using in vivo and in vitro 3D aortic valve models

DOCTORAL ADVISOR:
c.m. RA PhD. MÂNDUȚEANU ILEANA

DOCTORAL STUDENT:
CIORTAN LETIȚIA CRISTINA

Bucharest
2022

TABLE OF CONTENTS

PART II – ORIGINAL CONTRIBUTIONS

Key words: Valvă aortică, inflamație, diabet, glucoză, hidrogel

Aortic valve disease (AVD) is a global health problem and one of the major causes of mortality among people over 65 years of age (Nkomo, Gardin et al. 2006, Yadgir, Johnson et al. 2020, Coffey, Roberts- Thomson et al. 2021). For a long time AVD was considered a passive, degenerative process, as the progression of the disease spans decades. Currently, the disease is recognized as an active process, driven by chronic inflammation, similar in its early stages to atherosclerosis. However, statin therapy proved ineffective in improving AVD, thus showing that the progression mechanisms of the two pathologies are different (Cowell, Newby et al. 2005).

The evolution of AVD includes early sclerosis, characterized by thickening of the cusps but without affecting valve function, and late stenosis, when valve function is compromised due to extensive calcium deposits. Clinical detection of stenotic valves is done by identifying a heart murmur and confirmed by echocardiography, thus relying on symptoms in advanced stages when valve structure and function are affected (Lindman, Bonow et al. 2013). Currently, there are no predictive markers or pharmacological therapies for managing AVD, and the only treatment option for valves in advanced stages of calcification is surgical valve replacement with mechanical or biological prostheses, which in turn can become calcified. It is estimated that, with the aging of the population, the incidence of AVD will significantly increase, especially in developed countries, becoming thus an important socio-economic problem (Kodali, Velagapudi et al. 2018). Diabetes is one of the known risk factors for the development of AVD, and it has recently been associated with accelerated disease progression and disease severity in patients with moderate aortic stenosis (Han, Shi et al. 2021, Manduteanu, Simionescu et al. 2021, Natorska 2021). Thus, in diabetes, the aortic valve (AV) becomes calcified at an accelerated rate, leading to valve and heart dysfunction through mechanisms that are not understood completely.

This thesis was structured in two parts, namely part I, "The current state of knowledge", where an analysis of the literature necessary to put the research into context is presented, and part II, "Original contributions", where I describe the research experiments performed.

Thus, in the first part, I address aspects related to the development of the valve in the embryonic and postnatal stages, the structure and architecture of the aortic valve, resident cells and their physiological role, cellular mechanisms triggered in valve

pathology associated with diabetes and a review of the in vivo and in vitro models used to study AVD.

Development, structure and resident cells in the aortic valve

The aortic valve (AV) is one of four heart valves that ensure one-way blood flow from the left ventricle to the aorta. AV development takes place both in the embryonic stage, when heart anatomycal structures are formed, and in the postnatal stage, when the cell populations in the valve stabilize and the final three layerd architecture is achieved. In the embryonic stage, heart valve formation occurs when endocardial cushions form in the heart tube through signaling mediated by bone morphogenetic proteins (BMPs) of the transforming growth factor β (TGF-β) superfamily. A sub-population of endocardial endothelial cells enters endothelial to mesenchymal transition (EndMT) and migrates into the cardiac jelly where it proliferates and gives rise to the valve interstitial cell (VIC) population of the endocardial cushions (O'Donnell and Yutzey 2020). In these embryonic valves, a complex remodeling process orchestrated by the VIC begins, which is completed in the postnatal stage when the valve acquires the characteristic three-layer structure and the population of resident cells stabilizes (Hinton, Lincoln et al. 2006, Nordquist, LaHaye et al. 2018). Mature valve cusps are avascular structures with a three layer architecture , each layer with a different organization and composition in extracellular matrix (ECM) proteins: (1) fibrosa – located towards the aorta, and mainly made up of type I and III collagen fibers oriented circumferentially, giving mechanical resistance to the valve; (2) ventricularis – located towards the ventricle, composed mainly of radially oriented elastin fibers that give elasticity to the tissue; (3) spongiosa – the middle layer, composed mainly of proteoglycans (PG), glycosaminoglycans (GAG) and collagen fibers, which absorbs mechanical shocks and serves as a buffer between the other two layers. The three layers of the AV cusps are populated by valve resident cells, and recent studies have shown that 80–85% of them are VIC (negative for CD31 and CD45), 5–7% are VEC, and ∼10% are cells of immune origin (Hulin, Anstine et al. 2018, Hulin, Hortells et al. 2019). Valvular endothelial cells (VEC) are arranged in the form of a compact monolayer lining the exterior of the three AV cusps and provide the interface between the blood and the valve interstitium. The endothelium's main is that of a selective and non-adherent barrier, which mediates oxygen and nutrients transport from the blood into the valve interstitial space, but also the immune response. VECs are a population of endothelial cells different from vascular endothelial cells and show high heterogeneity: VECs on the two sides of the cusps are different, so the endothelium on the ventricularis side expresses cardiovascular calcification inhibitors and the one on the fibrous side shows a strong antioxidant profile and increased expression of endothelial nitric oxide synthase (eNOS) (Simmons, Grant et al. 2005). In pathology, VECs are implicated in the initiation and progression of AVD by recruiting immune cells, dysregulating protective nitric oxide (NO) signaling, or initiating EndMT (Ma, Zhao et al. 2020, Majumdar, Manivannan et al. para. 2021). In

the healthy adult valve, the interstitial cell population consists mostly of non-activated VICs (qVICs) distributed in all 3 layers of the cusps, responsible for maintaining the trilaminar structure of the ECM (Bogdanova, Zabirnyk et al. 2019). Interstitial cells form an extensive cellular network in the valve that facilitates unitary response and rapid transmission of information between network members. In response to mechanical or pathological stimuli, qVICs can differentiate into: (1) aVICs, which express metalloproteases and are involved in extracellular matrix remodeling, causing early sclerosis, or (2) obVICs, involved in the accumulation of calcium deposits (Rutkovskiy, Malashicheva et al. al. 2017). Immune cells establish themselves in the valve from the early stages of heart valve development and represent the third major cell population in the AV. T lymphocytes, dendritic cells, mast cells and two subpopulations of macrophages were identified in murine valves: Mφ1 and Mφ2 (Hulin, Hortells et al. 2019). Although the role of immune cells in AV homeostasis has not yet been studied and described in detail, it is assumed that these cells patrol the valve tissue, phagocytose apoptotic cells or pathogens and migrate to lymphoid organs where they initiate the immune response by antigen presentation (Hajdu, Romeo et al 2011).

Diabetes and AVD

Diabetes mellitus induces and exacerbates inflammatory response and lipid accumulation associated with AVD initiation and progression (Manduteanu, Simionescu et al. 2021), and the CANHEART study implicated diabetes as a significant risk factor in the development of aortic stenosis (Yan, Koh et al. 2017). Diabetes not only predisposes to AVD, but is also associated with accelerated disease progression and symptom severity in patients with moderate aortic stenosis (Aronow, Ahn et al. 2001). The pathology of diabetes associated with other cardiovascular diseases is complex and is partly due to the effects of hyperglycemia, which leads to an increase in reactive oxygen species (ROS) levels and promotes pro-inflammatory cytokines and chemokines expression (Dludla, Joubert et al. 2017, Wang, Liu et al. 2021).

Elevated C-reactive protein and diabetes-associated hyperlipemia favor chronic inflammation (Dongway, Faggad et al. 2015). There is ample evidence implicating proteins whose levels are increased in diabetes (TNF-α, MCP-1, IL-1β) in aortic valve pathology (Keane, Calton et al. 2017), so it can be speculated that the inflammatory environment produced in diabetes triggers at least in part the mechanisms responsible for valvular cell dysfunction. In type 2 diabetes, hyperglycemia is also associated with increased ROS production and monocyte adhesion to the endothelium through inhibition of NO production and increased levels of endothelin-1, E-selectin, ICAM-1, and VCAM-1. Monocytes reaching the subendothelial space differentiate into macrophages that secrete proinflammatory cytokines and incorporate modified LDL, transforming themselves into

foam cells (Manduteanu, Voinea et al. 1999, Nandy, Janardhanan et al. 2011, Padilla, Carpenter et al. 2018, Meza, La Favor et al. 2019). Inflammation precedes calcification, and infiltrating immune cells have been identified in the calcified areas of the valve. Macrophage population in the calcified valves is known to be more numerous than that in normal valves and cells express both Mφ1 and Mφ2 characteristic markers (Li, Qiao et al. 2017).

In stenotic valves from diabetic patients, NF-κB, FII and FXa expression is increased in the fibrosa compared to valves from non-diabetic patients. In addition, *in vitro* experiments confirmed that high glucose causes primary VIC inflammation via NF-κB-mediated signaling, leading to cellular calcification (Kopytek, Mazur et al. 2021).

Reduced expression of VE-cadherin was detected on the fibrosa side in calcified valves, and a population of cells co-expressing α-SMA and CD31, but not the hematopoietic marker CD45, was identified in the subendothelium, suggesting that these cells originate from EndMT. This cell population that also shows increased levels of nuclear NF-κB has been identified only in the fibrosa, and is located distal to the calcified area (Mahler, Farrar et al. 2013). Also, endothelial cells isolated from calcified valves have previously been shown to be more prone to osteogenic differentiation *in vitro* than vascular endothelial cells (Ma, Zhao et al. 2020). Diabetes is known to associate with chronic inflammation, and pro-inflammatory mediators TNF-α, IL-6, LPS, IFN-γ and TGF-β have been implicated in EndMT activation in VEC (Mahler, Farrar et al. 2013, Ma , Sanchez-Duffhues et al. 2020). *In vitro*, TNF-α and IL-6 induced EndMT-associated changes in porcine aortic valve endothelial cells (PAVEC) cultured on 3D hydrogels, via NFκB signaling: (1) decreased expression of CD31 and VE-cadherin, (2) cell migration into the collagen matrix and (3) expression of α-SMA. (Mahler, Farrar et al. 2013).

VIC plasticity and its implications for AVD related research

ECM regulates resident valve cells phenotype via integrins, contributing maintaining cell basal tension and providing cells with spatial information (Santa-Cruz Mateos, Valencia-Exposito et al. 2020). ECM can also function as a reservoir of biological signals through proteoglycans, which can locally sequester signaling molecules. Due to the complex role that the ECM has in homeostasis, any change at this level can lead to pathological events. When cultured on rigid substrate or stimulated with specific cytokines (TGF-β), VIC spontaneously transition into myofibroblasts (aVIC) *in vitro* and show high contractile, migratory and proliferative capacity (Liu and Gotlieb 2008, Huang, Yang et al. 2012, Ma, Killaars et al. 2017). Studies where valvular cells were cultured on three-

dimensional matrices of diverse composition and stiffness, highlighted the significant role that the substrate has in modulating cell phenotype, with implications in valve pathology (Hutson, Marohl et al. 2016, Rutkovskiy, Malashicheva et al. 2017, Bogdanova, Zabirnyk et al. 2022). VIC therefore also show plasticity in culture, so one of the biggest problems in the study of aortic valve pathology is the lack of an *in vitro* experimental model that replicates as closely as possible the composition and architecture of the native valve tissue.

In the healthy valve, qVIC to aVIC transition is involved in ECM architecture and composition maintanance through the secretion of ECM proteins (collagen, fibronectin, chondroitin sulfate, etc.) and enzymes that degrade these proteins (MMPs, cathepsins). Once ECM remodeling is complete, aVICs are eliminated by apoptosis. In pathology, however, this signaling is impaired and aVIC continue to synthesise ECM elements, causing tissue fibrosis, leading to dystrophic calcification. When activated, interstitial cells display stress fibers, increased expression of α-SMA, smooth muscle myosin, fibroblast-specific protein (FSP-1) or vimentin, but also ECM proteins and cytokines. Inducers of VIC activation include ECM-associated changes, mechanical stress or TGF-β (Ma, Killaars et al. 2017, Goody, Hosen et al. 2020, Di Vito, Donato et al. 2021).

qVIC transition to obVIC occurs in the presence of factors that promote osteogenesis or chondrogenesis. obVICs are actively involved in the valve calcification and are responsible for late stenosis. At this stage, cell behavior is governed by the transcription factor RUNX2 and bone morphogenetic proteins (BMPs), and obVIC secretes a number of proteins expressed in bone tissue, such as osteopontin, osteocalcin, and alkaline phosphatase (Yip, Chen et al. 2009). Our recent results showed that high glucose induces the overexpression of osteogenic molecules BMP-2, BMP-4, and TGF-β in human aortic valve cells cultured in methacrylate gelatin-based 3D models, with an effect on SMAD1/5/8/9 signaling and the expression of the RUNX2 transcription factor, thus implicating diabetes as a contributor to aortic valve calcification (Vadana, Cecoltan et al. 2020).

In vivo and in vitro research of AVD

The role of valvular cells in AVD in diabetes is still not fully elucidated and is currently being studied in several *in vivo* (Sider, Blaser et al. 2011, Puperi, Kishan et al. 2016, Jung, Ahmad et al. . 2022) and *in vitro* models (Hjortnaes, Goettsch et al. 2016, Porras, Westlund et al. 2018, Zabirnyk, Perez et al. 2020, Kruithof, van de Pol et al. 2021).

The pathology of AVD in diabetes is extremely complex, so an *in vivo* model that can replicate both pathologies as faithfully as possible is needed. *In vivo* models of diabetes

involve the use of animals predisposed to develop diabetes or the induction of diabetes using chemical agents (diabetogenic agents) that destroy pancreatic β-cells. The most widely used diabetogenic agents are streptozotocin and alloxan, which are taken up into pancreatic β-cells via the glucose transporter GLUT2, where they generate ROS and free radicals, causing DNA fragmentation and cell apoptosis.

Numerous *in vivo* diabetes models are described in the literature, but the current models employed for the study of AVD have significant limitations in recapitulating the characteristics of human pathology. Also, models of AVD in diabetes are even fewer, and the initial phases of lesions development in the VA were mostly described *in vivo* at the ultrastructural level (Filip, Nistor et al. 1987, Simionescu, Popov et al. 1996, Tucureanu , Filippi et al. 2019).

In vivo, it was recently shown in a dyslipidemic mouse model prone to developing type 2 diabetes ($LDLr^{-/-}/ApoB^{100/100}$/IGF-II) that diet-induced diabetes causes aortic stenosis (AS) more rapidly and increases osteogenicmolecules gene expression compared to non-diabetic mice ($LDLr^{-/-}/ApoB^{100/100}$) (Le Quang, Bouchareb et al. 2014). Similarly, $LDLr^{-/-}ApoB^{100/100}$ mice kept on a diabetogenic/procalcific diet for 12 months show calcium deposits in the AV and a twofold higher incidence of aortic stenosis confirmed by changes in hemodynamic parameters (Scatena, Jackson et al. 2018). However, when diabetes is induced by streptozotocin injection, in a hyperglycemic-hyperlipemic hamster model, results show that the AV is the first vascular territory affected by diabetes and hyperlipemia (HD) after only 2 weeks of diabetes. Ultrastructural changes in VEC from HD hamsters aortic valves (increased abundance of biosecretory organelles, microfilaments and microtubules), were associated with hyperplasia of the basement membrane at the level of which oxidized low-density lipoproteins (LDL) were identified, suggesting that hyperglycemia induces VEC dysfunction in the initial phase (Simionescu, Popov et al. 1996).

In vitro, the behavior of valvular interstitial cells is significantly influenced by both the substrate on which they are cultured and the architecture of the culture model. Conventional two-dimensional cultures are the least representative of the *in vivo* environment, but are most widely used for VIC research due to practical reasons. Studies have shown that when VICs are cultured on rigid substrate such as polystyrene culture plates, they spontaneously transition into myofibroblasts (Ma, Killaars et al. 2017). As the essential role of ECM in regulating VIC phenotype has become evident (Gould, Matherly et al. 2014), an additional step to 2D culture has been the coating of cell culture surfaces

with ECM proteins, and these types of studies provided important insights into how VIC phenotype is regulated by substrate (Porras, van Engeland et al. 2017). Among all matrix elements tested, laminin coating caused VIC to aggregate into nodules, and type I collagen promoted a non-activated VIC phenotype (Rodriguez and Masters 2009). Thus, *in vitro* research has continued in recent years with attempts to identify a more suitable three-dimensional substrate to maintain the non-activated phenotype of VIC (qVIC), implementation of VIC-VEC co-cultures and, most recently, with the use of human valvular endothelial cells.

In vitro 3D models represent a more representative alternative in terms of structural and functional characteristics of the native cellular environment, favoring interaction with neighboring cells or ECM components and guiding cellular behavior (Kaushik, Ponnusamy et al. 2018, Hong, Kim et al. 2019).

In vitro models for the study of VA pathology are made with cells isolated from patients or from large animals that develop pathology similar to that of humans, the most used of which are porcine (Bowler and Merryman 2015). Although similar in many ways to human cells, porcine interstitial valvular cells (PVICs) still represent a limited *in vitro* model, especially when used at high passages. When maintained in long-term culture, 20% of PVICs are known to exhibit contact inhibition and unstable behavior. Also, the calcification potential of PVIC decreases after three passages following isolation (Chen, Yip et al. 2009). On the other hand, the use of human cells is limited by the availability of biological material. Human cells are most often extracted from valve samples explanted from patients with advanced stages of valve calcification, and control cells are obtained only from patients with aortic insufficiency or undergoing heart transplantation (Goody, Hosen et al. 2020).

Another essential element for replicating AV conditions *in vitro* is the use of VIC and VEC co-culture to study the interaction between the two cell types. Numerous studies have associated culturing PVICs in the presence of PVECs with inhibition of their transition into aPVICs or obPVICs (Richards et al., 2013; Gould et al., 2014 (Butcher and Nerem 2006, Kennedy, Hua et al. 2009). Also, dysfunction or endothelial injury has been reported as the initiating event for pathological transformation of VIC (Leopold, 2012; Gomel et al., 2018; Hulin et al., 2018), making it evident that the two cell populations mutually regulate their behavior.

3D in vitro models obtained from native tisues

Many of the 3D *in vitro* models originate from tissue engineering, where the main goal is to develop a biological variant for valve prosthesis. To this end, a variety of synthetic or natural scaffolds have been tested for compatibility with valvular cells. Systems that mimic native ECM are preferred because they provide an environment similar to the architecture and conditions of the native valve (sequestration of growth factors and other molecules, cell adhesion, etc.) (Scott, Simon et al. 2021). One of the options successfully used for the three-dimensional culture of cells *in vitro* hydrogels obtained from natural polymers (collagen, gelatin) or from decellularized tissues. Decellularized tissues can in turn be (1) repopulated with cells, or (2) processed to obtain hydrogels from native MEC.

Hydrogels are highly hydrated polymer networks that retain their structural integrity when the polymer chains, which may be synthetic or natural in nature, are polymerized by physical or chemical methods. Hydrogels are considered the analogue of native MEC due to their capacity to retaining large volumes of fluids inside, their elasticity, but also the fact that the interface between the hydrogel and fluid phase resembles that observed in most soft tissues (Geckil, Xu et al. 2010). The advantages of 3D culture models have become evident in recent years, so the development of compatible hydrogels for the culture of human valvular cells is currently being attempted. In the past, different proteins or protein mixtures (Matrigel) present in the valve matrix have been used for valve cell culture with promising results. Currently, the use of hydrogels obtained from native ECM for the *in vitro* study of AVD is still very limited. Promising results were obtained with a hydrogel obtained from decellularized ovine aortic valves, which retains a protein composition similar to that of the native valve (collagen and laminin), but a relatively low GAG content. This hydrogel showed similar biocompatibility to collagen I-based scaffolds regarding cell proliferation and had no cytotoxic effect on VIC (Nehrenheim, Raschke et al. 2019).

Natural polymers such as agarose, alginate, collagen, fibrin, gelatin, chitosan or hyaluronic acid can also be used to obtain hydrogels and generate biological scaffolds. Due to the fact that it represents the most abundant component of valve ECM, type I collagen was among the first materials used for three-dimensional *in vitro* culture of valvular cells, and VICs grown in 2% collagen gels showed a less activated phenotype (Cushing, Mariner et al. 2008). Despite the biological advantages of collagen, this material also presents a number of limitations, such as the fact that VIC contracts these matrices. For these reasons, modified gelatin represents an alternative to the use of collagen. Gelatin methacrylation

(GelMA) leads to hydrogels with easily adjustable stiffness, which favor cell adhesion and can be easily combined with other types of materials. GelMA hydrogels have been successfully used for PVIC culture or porcine VIC-VEC co-culture in organ-on-a-chip systems (Chen, Srigunapalan et al. 2013). To obtain more complex patterns, the combination of GelMA with other AV-specific ECM proteins, such as hyaluronic acid (AH), is frequently used.

Internationally, research AVD is mainly focused on: (1) improving the understanding of the basic biological processes associated with aortic valve pathology, including the signaling pathways involved and the role of inflammation in the initiation and propagation of the disease; (2) determining the unique contributions associated with comorbidities in AVD (3) developing imaging methods to identify subclinical manifestations associated with AVD and (4) investigating the feasibility of pharmacological intervention in the initial stages of AVD (Rajamannan, Evans et al. 2011).

The analysis presented in Part I "The Current State of Knowledge" highlights three particularly important issues, namely: (1) AVD is accelerated in diabetes by mechanisms that have not yet been described in detail, (2) molecular changes in the initial stages of AVD in diabetes have not been addressed in *in vivo* models and (3) existing 3D *in vitro* models do not include human valvular cells and the communication between them, essential for mimicking the native AV environment.

In this context, the hypothesis from which this research started is that hyperglycemia associated with diabetes induces specific changes in the inflammatory process in the AV, thus contributing to the initiation and accelerated progression of AVD. Thus, the main objective of the study was to investigate the mechanisms by which hyperglycemia induces and propagates inflammation of aortic valve cells.

In this sense, the following scientific objectives were established:

1. Characterization of the inflammatory process in the aortic valve associated with early diabetes in an *in vivo* model of diabetic, hyperlipemic mice;

2. Development of 3D *in vitro* models populated with human valve cells in order to investigate the mechanisms involved in valve cell inflammation following exposure to high glucose concentrations.

Thus, the second part of the thesis consists of the Original Contributions made in order to achieve these objectives, and is divided into two chapters.

Chapter I had as its main objective the investigation of inflammatory mediators in early diabetes in the aortic valve in an *in vivo* diabetic, hyperlipemic mouse model. For this

purpose, male ApoE$^{-/-}$ C57BL/6J mice, induced with type I diabetes by injection of streptozotocin (STZ), and control mice (C), injected only with citrate buffer, were used., Histological staining of lipids, collagen and GAG uncovered lesions with lipid infiltrates characteristic of the early stages of valvular disease in Avs as early as day 4. Immunofluorescence analysis showed a very rapid increase, from day 4, in the expression of the adhesion molecules P-selectin, VCAM-1, ICAM-1 and PECAM-1 in mice in the STZ4 group, which was maintained at least equally high on day 7 after diabetes induction in the STZ7 group. Also, the mesenchymal markers, α-SMA and FSP-1, were significantly increased in STZ4 and STZ7 mice relative to C4 and C7 controls, suggesting the role of diabetes in valvular cell activation. Vimentin, another mesenchymal cell marker, was identified to be significantly increased in the 7-day (C7 and STZ7) versus 4-day experimental groups, implicating the high-lipid diet in the transition of valvular cells to mesenchymal phenotypes.

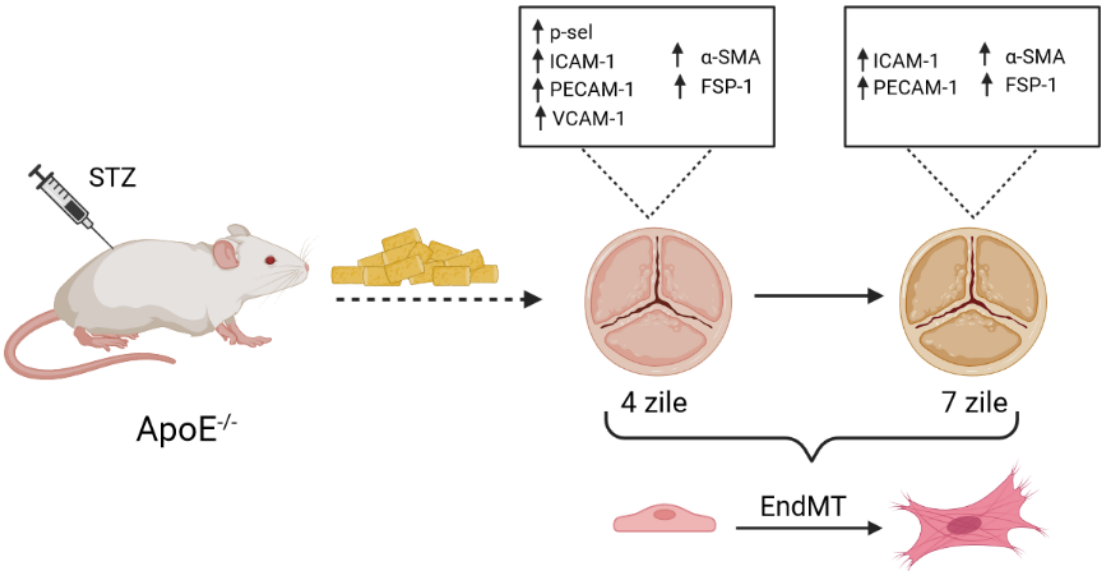

Figure 1. *Schematic representation of in vivo study conclusions.* ApoE-/- mice injected with STZ and fed a high-lipid diet show overexpression of markers of VEC activation (P-selectin, VCAM-1, ICAM-1 and PECAM-1) and VIC activation (α-SMA and FSP- 1) at 4 and 7 days and also cells involved in EndMT.

Cells involved in EndMT were identified by immunohistochemistry in AVs from diabetic mice in the same area where lipid-rich lesions were observed, suggesting that diabetes is involved in the overexpression of aortic valvular cells dysfunction markers. The inflammatory mediators found to be affected by diabetes were used to generate a matrix of

correlations together with other proteins found to be involved in valve remodeling and calcification in diabetes, and also with plasmatic and functional parameters associated with the diabetic state (Tucureanu, Filippi et al. 2019, Vadana, Cecoltan et al. 2020). Significant correlations were identified between inflammatory, osteogenic and remodeling mediators and plasma parameters, suggesting specific molecular associations that may contribute to early valve dysfunction detected in the hyperlipemic diabetic mouse model.

Research described in Chapter II aimed to investigate the mechanisms involved in valve cell inflammation under conditions of chronic exposure to high glucose concentrations *in vitro*, and had three specific objectives: (1) the isolation and characterization of primary human aortic valvular cells (2) the development of *in vitro* 3D models populated with human valvular cells and (3) to describe the mechanisms involved in valvular cell inflammation under conditions of chronic exposure to high glucose concentrations in the developed 3D models.

In order to fulfill the **first specific objective (1),** human valvular cells were isolated by enzymatic digestion from human aortic valves obtained from the Central Military Hospital (Bucharest) following valve replacement surgery. Pure cultures of primary endothelial and interstitial valvular cells were established by separation with CD31+ microbeads, and the specific VIC and VEC markers were investigated by immunohistochemistry.

Within **the second objective (2),** several 3D *in vitro* models were investigated: decellularized porcine cusps repopulated with human cells (subchapter II.1.2.), bioprinted model based on commercial hydrogels (subchapter II.1.3.) and models based on hydrogels developed in our laboratory from methacrylated gelatin (subchapter II.1.4.) and from decellularized porcine aortic roots (subchapter II.1.5.).

The decellularized porcine cusp model repopulated with human cells (subchapter II.1.2.) involved 2 steps: (1) decellularization of the native tissue and (2) repopulation of the matrix with human cells. For the first step, decellularization was confirmed by DNA quantification and histological staining in order to identify cells and ECM proteins. For the second part, multiple techniques to repopulate the matrix with human cells were tested. On the outside, a uniform monolayer of endothelial cells was achieved, but repopulation yield with fibroblasts/VIC on the matrix inside was not good enough to obtain an *in vitro* model suitable for investigating aortic valve pathology.

Among the commercial hydrogel formulas tested to obtain a bioprinted *in vitro* model (subchapter II.1.3.), gelatin-based hydrogels showed good cell compatibility

confirmed by viability tests and microscopy. VICs encapsulated in BioInk and Gel4Cell hydrogels showed high viability and formed a three-dimensional network inside the 3D constructs. On the other hand, VECs are not compatible with encapsulated culture, but when cultured on top of the gelatin-based hydrogels they proliferate and form a characteristic monolayer. Among the 3 formulas tested, gelatin-based hydrogels showed the highest biocompatibility but were also the most difficult to bioprint due to variability of properties and instability in culture at 37°C. CellinkRGD was the most compatible with the bioprinting protocol, but not with human valvular cells.

Starting from the promising biocompatibility results of gelatin based commercial hydrogels with valvular cells and of information from the literature, in the next stage of the research (subchapter II.1.4.) we developed a hydrogel formula based on methacrylated gelatin (GP-1) in our laboratory. Biocompatibility and phenotypic characterization analysis showed that the GP-1 hydrogel developed in our laboratory is not cytotoxic, allows cell proliferation, and valvular cells encapsulated in the hydrogel or cultured on its surface retain their characteristic cell markers. Also, α-SMA (marker of VIC activation) gene and protein expression quantified by RT-PCR and Western Blot, decreases when cells are cultured in the GP-1 hydrogel compared to classical 2D culture, thus replicating VIC phenotype characteristic to the native valve. Increased α-SMA gene expression in the first week is comparable to that in 2D culture, and is associated with increased expression of MMPs and some ECM elements (collagen I, collagen III, elastin and laminin), suggesting an initial process of cell remodeling and adaptation in the new matrix (Ciortan, Macarie et al. 2020).

In order to obtain a model with protein composition closer to that of native valve ECM, we developed a second hydrogel formulation from porcine native extracellular matrix (HDRA). As with the first studies, the hydrogel was evaluated in terms of protein composition by histological staining and SDS-PAGE. Thus, it was shown that the main component of HDRA is type I and/or III collagen, similar to the fibrosa layer in the valve. Histological analysis showed that the structure of the HDRA-based constructs is very similar in terms of density and porosity to that of native decellularized valve tissue. Biocompatibility analysis with human valvular cells performed by immunohistochemistry and viability assay showed that VEC form a continuous monolayer on the surface of HDRA constructs, express the endothelial marker CD31 and proliferate in culture, and VIC are uniformly distributed inside the hydrogel where they form a complex network.

To achieve objective (3), *in vitro* models based on methacrylated gelatin hydrogel (GP-1) and decellularized porcine aortic root hydrogel (HDRA) developed in our laboratory were used to investigate human aortic valvular cells inflammation when exposed to high glucose conditions.

In the GP-1 hydrogel-based *in vitro* model populated with human valvular cells in co-culture, RT-PCR results showed that when exposed to high glucose (HG) concentrations, VECs become dysfunctional by overexpressing pro-inflammatory cytokines and chemokines (MCP-1, TNF-α and IL-8 at 7 days and MCP-1 and IL-1β at 14 days of HG) and adhesion molecules (VCAM-1 and E -selectin at 7 and 14 days of HG). In contrast, exposure to HG for up to 14 days does not alter α-SMA expression in VICs, suggesting that glucose is not involved in VIC transition to an activated myofibroblast-like phenotype. However, VICs respond to increased glucose concentrations by assuming an inflammatory phenotype, characterized by increased expression of pro-inflammatory cytokines and chemokines, at 7 (IL-1β) and 14 days (TNF-α, IL-8 and IL-1β) of exposure to HG. VEC activation therefore occurs mainly on day 7 of HG exposure, after which VIC activation follows on day 14. Protein expression of the inflammatory mediators IL-1β and MCP-1 secreted into the medium by valvular cells cultured in HG significantly increases only on day 14, thus confirming that valve cell inflammation is more pronounced at a longer experimental time.

At the molecular level, using the GP-1 hydrogel co-culture model, we identified a potential mechanism of IL-1β expression under conditions of chronic high glucose exposure, mediated by pPKC. Thus, we observed that the expression of pPKC significantly increases in VECs at 7 days and in VICs at 14 days under HG conditions, and its inhibition significantly reduces the concentration of secreted Il-1β in the culture medium under HG conditions. Thus, the results obtained in the GP-1 model suggest that, under HG conditions, the inflammatory process in the valve is first triggered by VEC, after which it propagates to VIC, where inflammation becomes chronic and may promote later valve calcification.

In the HDRA hydrogel-based *in vivo* model, VEC phenotypic changes with implications in valvular pathology were mainly investigated. Gene expression analysis and immunohistochemistry results showed that when HDRA constructs populated with human valvular cells are exposed to pathological concentrations of glucose, EndMT triggering occurs, also observed in the *in vivo* model, characterized by the overexpression of mesenchymal markers (α-SMA and vimentin), decreased endothelial markers expression

(CD31 and VE-cadherin) and increased co-localization of α-SMA and CD31 in valvular cells. It can thus be concluded that a pathological glucose concentration, similar to that in diabetes, favors EndMT, possibly by creating a pro-inflammatory environment in the valve, as we have shown to happen in the GP-1 model. Previously, Mahler et al. (2013) demonstrated that inflammatory cytokines (TNFα and IL-6) induce EndMT in ovine valve endothelial cells, a process by which myofibroblasts involved in pathological valve remodeling are generated.

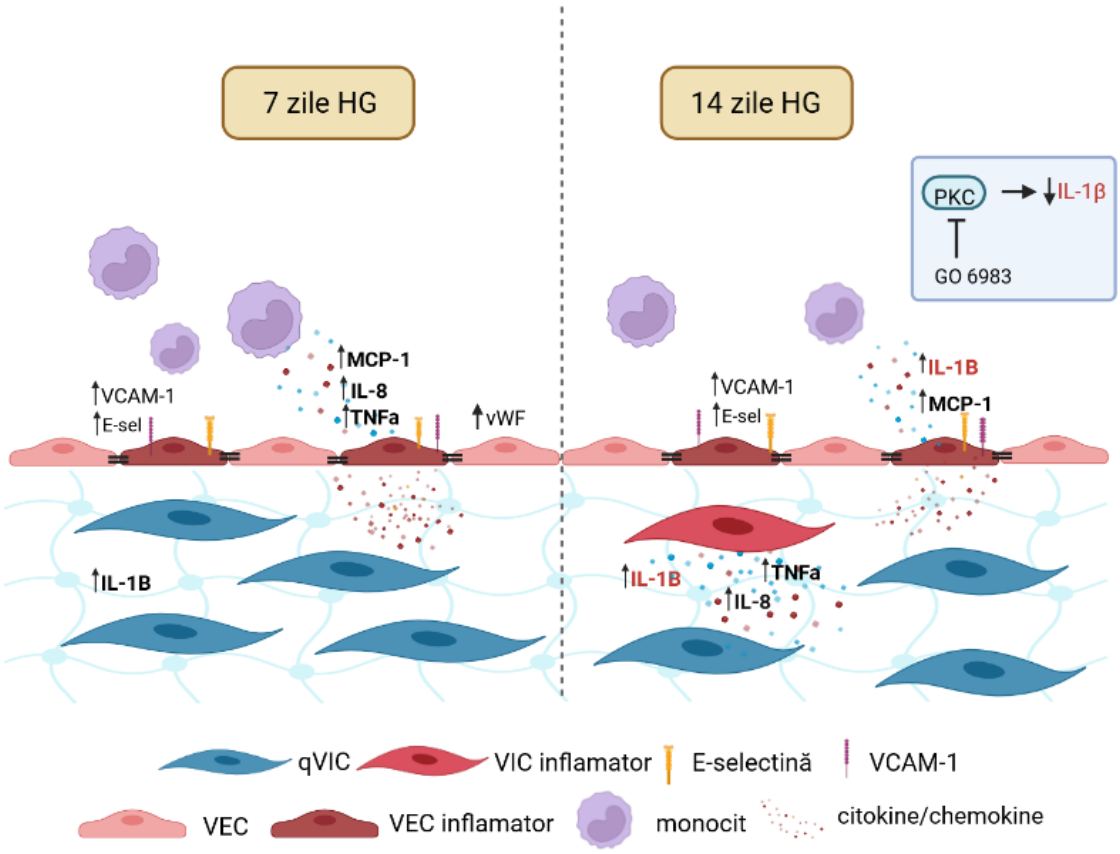

Figure 2. Schematic representation of conclusions using the GP-1 in vitro model*. Chronic exposure to high glucose concentrations generates a sequential inflammatory response of valvular cells: at 7 days, VEC inflammatory response is dominant, and is characterized by the overexpression of vWF, MCP-1, IL-1β, VCAM-1 and E-selectin. At 14 days, VIC transition to an inflammatory phenotype (overexpression of IL-1β, TNF-α, IL-8), suggesting that chronic VIC inflammation may be due in part to VEC, thus following in later during AVD progression; A potential mechanism involved in the expression and release of IL-1β under high glucose conditions is mediated by the PKC signaling pathway.*

Another molecule whose increased expression is associated with VEC dysfunction and which has been identified as a pro-inflammatory agent in early diabetes is ET-1 (Iglarz and Clozel 2007, Saleh, Boesen et al. 2011). The importance of ET-1 in diabetes has been highlighted by studies showing that HG increases ET-1 expression in primary cells and aortic endothelial cell lines (Manea, Todirita et al. 2013) and that dysregulation of the ET-1 system plays an important role in the establishment and progression of macro- and microvasculature pathology in diabetes (Pernow, Shemyakin et al. 2012). ET-1 and its receptors have been shown to be overexpressed in aortic stenosis, suggesting that a number of pathological changes associated with atherosclerotic lesions and valve calcification may be a consequence of ET-1 signaling (Peltonen, Taskinen et al. 2009). For these reasons, we investigated the effect of HG on ET-1 expression in VECs cultured in HDRA. RT-PCR results showed that the expression of ET-1 and its receptors (ET-A and ET-B) is increased in VECs from HDRA constructs exposed for 7 days to HG compared to the normal glucose (NG) controls. In addition, ELISA results show that the level of IL-1β and MCP-1 secreted in the culture medium of constructs exposed to HG is significantly reduced when constructs are exposed to HG in the presence of ET-1 receptor antagonists. The ET-B receptor antagonist reduces pro-inflammatory cytokines and chemokines secretion almost to the level observed in the NG control, thus demonstrating the involvement of ET-1 signaling in HG-initiated VEC inflammation. Since IL-1β is known to be involved in the phenotypic transition of vascular endothelial cells in EndMT, it can be hypothesized that IL-1β might be involved in EndMT in valvular endothelial cells. Both ET-1 and IL-1β have been previously shown to be involved in the initiation of EndMT in endothelial cells from other vascular beds (Chaudhuri, Zhou et al. 2007, Widyantoro, Emoto et al. 2010). The role of ET-1 in EndMT was studied *in vivo* in diabetic mice deficient for the gene encoding the ET-1 protein, where ET-1 was shown to promote cardiac fibrosis development of (Cho, Lee et al. 2018).

Exposure of HDRA-cultured VECs to high glucose also resulted in overexpression of ICAM-1 and VCAM-1 adhesion molecules involved in monocyte recruitment to the subendothelial space.

Data obtained in the HDRA model suggest that high glucose promotes EndMT in VEC, possibly through ET-1 signaling and IL-1β secretion. Also, the inflammatory environment created may contribute to increased adhesion of immune cells to the endothelium and inflammation propagation in the valve.

The studies presented in this thesis regarding the inflammatory process in diabetes, together with those carried out in our research group (Tucureanu, Filippi et al. 2019, Vadana, Cecoltan et al. 2020) regarding calcification markers, showed that the inflammatory process and the osteogenic transition of valve interstitial cells occur concurrently in early diabetes, with implications for valve dysfunction. Also, the effect of high glucose on human valvular cells cultured in 3D *in vitro* models has not been investigated by other groups, so the results obtained in this research are original.

Achieving these results was possible through the use of animal handling techniques to establish the *in vivo* model, tissue decellularization and the development of hydrogels to obtain the *in vitro* models, molecular biology, immunohistochemistry or flow adhesion techniques, thus underlining the interdisciplinary character and complexity of the presented research.

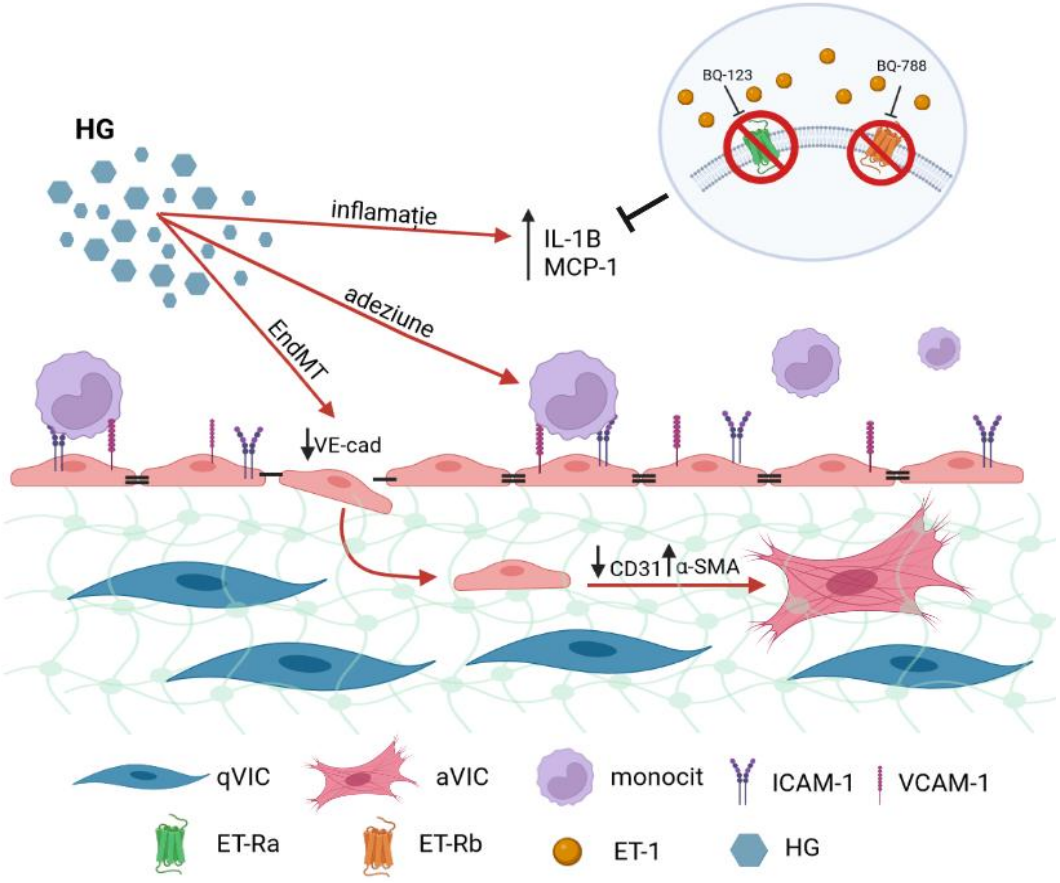

Figure 3. Schematic representation of conclusions using the HDRA in vitro model. *Chronic exposure to high glucose concentrations favours (1) EndMT initiation, characterized by decreased expression of endothelial markers (CD31 and VE-cadherin), overexpression of mesenchymal markers (α-SMA and vimentin) and increased co-localization of*

Bibliography

1. Aronow, W. S., C. Ahn, I. Kronzon and M. E. Goldman (2001). "Association of coronary risk factors and use of statins with progression of mild valvular aortic stenosis in older persons." Am J Cardiol **88**(6): 693-695.
2. Bogdanova, M., A. Zabirnyk, A. Malashicheva, K. Z. Enayati, T. A. Karlsen, M. L. Kaljusto, J. P. Kvitting, E. Dissen, G. J. Sullivan, A. Kostareva, K. O. Stenslokken, A. Rutkovskiy and J. Vaage (2019). "Interstitial cells in calcified aortic valves have reduced differentiation potential and stem cell-like properties." Sci Rep **9**(1): 12934.
3. Bogdanova, M., A. Zabirnyk, A. Malashicheva, D. Semenova, J. E. Kvitting, M. L. Kaljusto, M. D. M. Perez, A. Kostareva, K. O. Stenslokken, G. J. Sullivan, A. Rutkovskiy and J. Vaage (2022). "Models and Techniques to Study Aortic Valve Calcification in Vitro, ex Vivo and in Vivo. An Overview." Front Pharmacol **13**: 835825.
4. Bowler, M. A. and W. D. Merryman (2015). "In vitro models of aortic valve calcification: solidifying a system." Cardiovasc Pathol **24**(1): 1-10.
5. Butcher, J. T. and R. M. Nerem (2006). "Valvular endothelial cells regulate the phenotype of interstitial cells in co-culture: effects of steady shear stress." Tissue Eng **12**(4): 905-915.
6. Chaudhuri, V., L. Zhou and M. Karasek (2007). "Inflammatory cytokines induce the transformation of human dermal microvascular endothelial cells into myofibroblasts: a potential role in skin fibrogenesis." J Cutan Pathol **34**(2): 146-153.
7. Chen, J. H., C. Y. Yip, E. D. Sone and C. A. Simmons (2009). "Identification and characterization of aortic valve mesenchymal progenitor cells with robust osteogenic calcification potential." Am J Pathol **174**(3): 1109-1119.
8. Chen, M. B., S. Srigunapalan, A. R. Wheeler and C. A. Simmons (2013). "A 3D microfluidic platform incorporating methacrylated gelatin hydrogels to study physiological cardiovascular cell-cell interactions." Lab Chip **13**(13): 2591-2598.
9. Cho, J. G., A. Lee, W. Chang, M. S. Lee and J. Kim (2018). "Endothelial to Mesenchymal Transition Represents a Key Link in the Interaction between Inflammation and Endothelial Dysfunction." Front Immunol **9**: 294.

10. Ciortan, L., R. D. Macarie, S. Cecoltan, M. Vadana, M. M. Tucureanu, A. C. Mihaila, I. Droc, E. Butoi and I. Manduteanu (2020). "Chronic High Glucose Concentration Induces Inflammatory and Remodeling Changes in Valvular Endothelial Cells and Valvular Interstitial Cells in a Gelatin Methacrylate 3D Model of the Human Aortic Valve." Polymers (Basel) **12**(12).
11. Coffey, S., R. Roberts-Thomson, A. Brown, J. Carapetis, M. Chen, M. Enriquez-Sarano, L. Zuhlke and B. D. Prendergast (2021). "Global epidemiology of valvular heart disease." Nat Rev Cardiol **18**(12): 853-864.
12. Cowell, S. J., D. E. Newby, R. J. Prescott, P. Bloomfield, J. Reid, D. B. Northridge, N. A. Boon, S. Scottish Aortic and I. o. R. I. Lipid Lowering Trial (2005). "A randomized trial of intensive lipid-lowering therapy in calcific aortic stenosis." N Engl J Med **352**(23): 2389-2397.
13. Cushing, M. C., P. D. Mariner, J. T. Liao, E. A. Sims and K. S. Anseth (2008). "Fibroblast growth factor represses Smad-mediated myofibroblast activation in aortic valvular interstitial cells." FASEB J **22**(6): 1769-1777.
14. Di Vito, A., A. Donato, I. Presta, T. Mancuso, F. S. Brunetti, P. Mastroroberto, A. Amorosi, N. Malara and G. Donato (2021). "Extracellular Matrix in Calcific Aortic Valve Disease: Architecture, Dynamic and Perspectives." Int J Mol Sci **22**(2).
15. Dludla, P. V., E. Joubert, C. J. F. Muller, J. Louw and R. Johnson (2017). "Hyperglycemia-induced oxidative stress and heart disease-cardioprotective effects of rooibos flavonoids and phenylpyruvic acid-2-O-beta-D-glucoside." Nutr Metab (Lond) **14**: 45.
16. Dongway, A. C., A. S. Faggad, H. Y. Zaki and B. E. Abdalla (2015). "C-reactive protein is associated with low-density lipoprotein cholesterol and obesity in type 2 diabetic Sudanese." Diabetes Metab Syndr Obes **8**: 427-435.
17. Filip, D. A., A. Nistor, A. Bulla, A. Radu, F. Lupu and M. Simionescu (1987). "Cellular events in the development of valvular atherosclerotic lesions induced by experimental hypercholesterolemia." Atherosclerosis **67**(2-3): 199-214.
18. Geckil, H., F. Xu, X. Zhang, S. Moon and U. Demirci (2010). "Engineering hydrogels as extracellular matrix mimics." Nanomedicine (Lond) **5**(3): 469-484.
19. Goody, P. R., M. R. Hosen, D. Christmann, S. T. Niepmann, A. Zietzer, M. Adam, F. Bonner, S. Zimmer, G. Nickenig and F. Jansen (2020). "Aortic Valve Stenosis: From Basic Mechanisms to Novel Therapeutic Targets." Arterioscler Thromb Vasc Biol **40**(4): 885-900.

20. Gould, S. T., E. E. Matherly, J. N. Smith, D. D. Heistad and K. S. Anseth (2014). "The role of valvular endothelial cell paracrine signaling and matrix elasticity on valvular interstitial cell activation." Biomaterials **35**(11): 3596-3606.
21. Hajdu, Z., S. J. Romeo, P. A. Fleming, R. R. Markwald, R. P. Visconti and C. J. Drake (2011). "Recruitment of bone marrow-derived valve interstitial cells is a normal homeostatic process." J Mol Cell Cardiol **51**(6): 955-965.
22. Han, K., D. Shi, L. Yang, M. Xie, R. Zhong, Z. Wang, F. Gao, X. Ma and Y. Zhou (2021). "Diabetes Is Associated With Rapid Progression of Aortic Stenosis: A Single-Center Retrospective Cohort Study." Front Cardiovasc Med **8**: 812692.
23. Hinton, R. B., Jr., J. Lincoln, G. H. Deutsch, H. Osinska, P. B. Manning, D. W. Benson and K. E. Yutzey (2006). "Extracellular matrix remodeling and organization in developing and diseased aortic valves." Circ Res **98**(11): 1431-1438.
24. Hjortnaes, J., C. Goettsch, J. D. Hutcheson, G. Camci-Unal, L. Lax, K. Scherer, S. Body, F. J. Schoen, J. Kluin, A. Khademhosseini and E. Aikawa (2016). "Simulation of early calcific aortic valve disease in a 3D platform: A role for myofibroblast differentiation." J Mol Cell Cardiol **94**: 13-20.
25. Hong, S., J. S. Kim, B. Jung, C. Won and C. Hwang (2019). "Coaxial bioprinting of cell-laden vascular constructs using a gelatin-tyramine bioink." Biomater Sci **7**(11): 4578-4587.
26. Huang, X., N. Yang, V. F. Fiore, T. H. Barker, Y. Sun, S. W. Morris, Q. Ding, V. J. Thannickal and Y. Zhou (2012). "Matrix stiffness-induced myofibroblast differentiation is mediated by intrinsic mechanotransduction." Am J Respir Cell Mol Biol **47**(3): 340-348.
27. Hulin, A., L. J. Anstine, A. J. Kim, S. J. Potter, T. DeFalco, J. Lincoln and K. E. Yutzey (2018). "Macrophage Transitions in Heart Valve Development and Myxomatous Valve Disease." Arterioscler Thromb Vasc Biol **38**(3): 636-644.
28. Hulin, A., A. Hego, P. Lancellotti and C. Oury (2018). "Advances in Pathophysiology of Calcific Aortic Valve Disease Propose Novel Molecular Therapeutic Targets." Front Cardiovasc Med **5**: 21.
29. Hulin, A., L. Hortells, M. V. Gomez-Stallons, A. O'Donnell, K. Chetal, M. Adam, P. Lancellotti, C. Oury, S. S. Potter, N. Salomonis and K. E. Yutzey (2019). "Maturation of heart valve cell populations during postnatal remodeling." Development **146**(12).

30. Hutson, H. N., T. Marohl, M. Anderson, K. Eliceiri, P. Campagnola and K. S. Masters (2016). "Calcific Aortic Valve Disease Is Associated with Layer-Specific Alterations in Collagen Architecture." PLoS One **11**(9): e0163858.
31. Iglarz, M. and M. Clozel (2007). "Mechanisms of ET-1-induced endothelial dysfunction." J Cardiovasc Pharmacol **50**(6): 621-628.
32. Jung, J. J., A. A. Ahmad, S. Rajendran, L. Wei, J. Zhang, J. Toczek, L. Nie, G. Kukreja, M. Salarian, K. Gona, M. Ghim, R. Chakraborty, K. A. Martin, G. Tellides, D. Heistad and M. M. Sadeghi (2022). "Differential BMP Signaling Mediates the Interplay Between Genetics and Leaflet Numbers in Aortic Valve Calcification." JACC Basic Transl Sci **7**(4): 333-345.
33. Kaushik, G., M. P. Ponnusamy and S. K. Batra (2018). "Concise Review: Current Status of Three-Dimensional Organoids as Preclinical Models." Stem Cells **36**(9): 1329-1340.
34. Keane, K. N., E. K. Calton, R. Carlessi, P. H. Hart and P. Newsholme (2017). "The bioenergetics of inflammation: insights into obesity and type 2 diabetes." Eur J Clin Nutr **71**(7): 904-912.
35. Kennedy, J. A., X. Hua, K. Mishra, G. A. Murphy, A. C. Rosenkranz and J. D. Horowitz (2009). "Inhibition of calcifying nodule formation in cultured porcine aortic valve cells by nitric oxide donors." Eur J Pharmacol **602**(1): 28-35.
36. Kodali, S. K., P. Velagapudi, R. T. Hahn, D. Abbott and M. B. Leon (2018). "Valvular Heart Disease in Patients >/=80 Years of Age." J Am Coll Cardiol **71**(18): 2058-2072.
37. Kopytek, M., P. Mazur, M. Zabczyk, A. Undas and J. Natorska (2021). "Diabetes concomitant to aortic stenosis is associated with increased expression of NF-kappaB and more pronounced valve calcification." Diabetologia **64**(11): 2562-2574.
38. Kruithof, B. P. T., V. van de Pol, T. Los, K. Lodder, B. M. Gourabi, M. C. DeRuiter, M. J. Goumans and N. Ajmone Marsan (2021). "New calcification model for intact murine aortic valves." J Mol Cell Cardiol **156**: 95-104.
39. Le Quang, K., R. Bouchareb, D. Lachance, M. A. Laplante, D. El Husseini, M. C. Boulanger, D. Fournier, X. P. Fang, R. K. Avramoglu, P. Pibarot, Y. Deshaies, G. Sweeney, P. Mathieu and A. Marette (2014). "Early development of calcific aortic valve disease and left ventricular hypertrophy in a mouse model of combined

dyslipidemia and type 2 diabetes mellitus." Arterioscler Thromb Vasc Biol **34**(10): 2283-2291.

40. Li, G., W. Qiao, W. Zhang, F. Li, J. Shi and N. Dong (2017). "The shift of macrophages toward M1 phenotype promotes aortic valvular calcification." J Thorac Cardiovasc Surg **153**(6): 1318-1327 e1311.
41. Lindman, B. R., R. O. Bonow and C. M. Otto (2013). "Current management of calcific aortic stenosis." Circ Res **113**(2): 223-237.
42. Liu, A. C. and A. I. Gotlieb (2008). "Transforming growth factor-beta regulates in vitro heart valve repair by activated valve interstitial cells." Am J Pathol **173**(5): 1275-1285.
43. Ma, H., A. R. Killaars, F. W. DelRio, C. Yang and K. S. Anseth (2017). "Myofibroblastic activation of valvular interstitial cells is modulated by spatial variations in matrix elasticity and its organization." Biomaterials **131**: 131-144.
44. Ma, J., G. Sanchez-Duffhues, M. J. Goumans and P. Ten Dijke (2020). "TGF-beta-Induced Endothelial to Mesenchymal Transition in Disease and Tissue Engineering." Front Cell Dev Biol **8**: 260.
45. Ma, X., D. Zhao, P. Yuan, J. Li, Y. Yun, Y. Cui, T. Zhang, J. Ma, L. Sun, H. Ma, Y. Zhang, H. Zhang, W. Zhang, J. Huang, C. Zou and Z. Wang (2020). "Endothelial-to-Mesenchymal Transition in Calcific Aortic Valve Disease." Acta Cardiol Sin **36**(3): 183-194.
46. Mahler, G. J., E. J. Farrar and J. T. Butcher (2013). "Inflammatory cytokines promote mesenchymal transformation in embryonic and adult valve endothelial cells." Arterioscler Thromb Vasc Biol **33**(1): 121-130.
47. Majumdar, U., S. Manivannan, M. Basu, Y. Ueyama, M. C. Blaser, E. Cameron, M. R. McDermott, J. Lincoln, S. E. Cole, S. Wood, E. Aikawa, B. Lilly and V. Garg (2021). "Nitric oxide prevents aortic valve calcification by S-nitrosylation of USP9X to activate NOTCH signaling." Sci Adv **7**(6).
48. Manduteanu, I., D. Simionescu, A. Simionescu and M. Simionescu (2021). "Aortic valve disease in diabetes: Molecular mechanisms and novel therapies." J Cell Mol Med **25**(20): 9483-9495.
49. Manduteanu, I., M. Voinea, G. Serban and M. Simionescu (1999). "High glucose induces enhanced monocyte adhesion to valvular endothelial cells via a mechanism involving ICAM-1, VCAM-1 and CD18." Endothelium **6**(4): 315-324.

50. Manea, S. A., A. Todirita and A. Manea (2013). "High glucose-induced increased expression of endothelin-1 in human endothelial cells is mediated by activated CCAAT/enhancer-binding proteins." PLoS One **8**(12): e84170.
51. Meza, C. A., J. D. La Favor, D.-H. Kim and R. C. Hickner (2019). "Endothelial Dysfunction: Is There a Hyperglycemia-Induced Imbalance of NOX and NOS?" International Journal of Molecular Sciences **20**(15): 3775.
52. Nandy, D., R. Janardhanan, D. Mukhopadhyay and A. Basu (2011). "Effect of hyperglycemia on human monocyte activation." J Investig Med **59**(4): 661-667.
53. Natorska, J. (2021). "Diabetes mellitus as a risk factor for aortic stenosis: from new mechanisms to clinical implications." Kardiol Pol **79**(10): 1060-1067.
54. Nehrenheim, L., S. Raschke, A. Stefanski, M. Barth, J. Isabel Selig, A. Barbian, A. Fernandez-Colino, K. Stuhler, P. Mela, A. Albert, A. Lichtenberg and P. Akhyari (2019). "Native aortic valve derived extracellular matrix hydrogel for three dimensional culture analyses with improved biomimetic properties." Biomed Mater **14**(3): 035014.
55. Nkomo, V. T., J. M. Gardin, T. N. Skelton, J. S. Gottdiener, C. G. Scott and M. Enriquez-Sarano (2006). "Burden of valvular heart diseases: a population-based study." Lancet **368**(9540): 1005-1011.
56. Nordquist, E., S. LaHaye, C. Nagel and J. Lincoln (2018). "Postnatal and Adult Aortic Heart Valves Have Distinctive Transcriptional Profiles Associated With Valve Tissue Growth and Maintenance Respectively." Front Cardiovasc Med **5**: 30.
57. O'Donnell, A. and K. E. Yutzey (2020). "Mechanisms of heart valve development and disease." Development **147**(13).
58. Padilla, J., A. J. Carpenter, N. A. Das, H. K. Kandikattu, S. Lopez-Ongil, L. A. Martinez-Lemus, U. Siebenlist, V. G. DeMarco and B. Chandrasekar (2018). "TRAF3IP2 mediates high glucose-induced endothelin-1 production as well as endothelin-1-induced inflammation in endothelial cells." Am J Physiol Heart Circ Physiol **314**(1): H52-H64.
59. Peltonen, T., P. Taskinen, J. Napankangas, H. Leskinen, P. Ohtonen, Y. Soini, T. Juvonen, J. Satta, O. Vuolteenaho and H. Ruskoaho (2009). "Increase in tissue endothelin-1 and ETA receptor levels in human aortic valve stenosis." Eur Heart J **30**(2): 242-249.
60. Pernow, J., A. Shemyakin and F. Bohm (2012). "New perspectives on endothelin-1 in atherosclerosis and diabetes mellitus." Life Sci **91**(13-14): 507-516.

61. Porras, A. M., N. C. van Engeland, E. Marchbanks, A. McCormack, C. V. Bouten, M. H. Yacoub, N. Latif and K. S. Masters (2017). "Robust Generation of Quiescent Porcine Valvular Interstitial Cell Cultures." J Am Heart Assoc **6**(3).
62. Porras, A. M., J. A. Westlund, A. D. Evans and K. S. Masters (2018). "Creation of disease-inspired biomaterial environments to mimic pathological events in early calcific aortic valve disease." Proc Natl Acad Sci U S A **115**(3): E363-E371.
63. Puperi, D. S., A. Kishan, Z. E. Punske, Y. Wu, E. Cosgriff-Hernandez, J. L. West and K. J. Grande-Allen (2016). "Electrospun Polyurethane and Hydrogel Composite Scaffolds as Biomechanical Mimics for Aortic Valve Tissue Engineering." ACS Biomater Sci Eng **2**(9): 1546-1558.
64. Rajamannan, N. M., F. J. Evans, E. Aikawa, K. J. Grande-Allen, L. L. Demer, D. D. Heistad, C. A. Simmons, K. S. Masters, P. Mathieu, K. D. O'Brien, F. J. Schoen, D. A. Towler, A. P. Yoganathan and C. M. Otto (2011). "Calcific aortic valve disease: not simply a degenerative process: A review and agenda for research from the National Heart and Lung and Blood Institute Aortic Stenosis Working Group. Executive summary: Calcific aortic valve disease-2011 update." Circulation **124**(16): 1783-1791.
65. Richards, J., I. El-Hamamsy, S. Chen, Z. Sarang, P. Sarathchandra, M. H. Yacoub, A. H. Chester and J. T. Butcher (2013). "Side-specific endothelial-dependent regulation of aortic valve calcification: interplay of hemodynamics and nitric oxide signaling." Am J Pathol **182**(5): 1922-1931.
66. Rodriguez, K. J. and K. S. Masters (2009). "Regulation of valvular interstitial cell calcification by components of the extracellular matrix." J Biomed Mater Res A **90**(4): 1043-1053.
67. Rutkovskiy, A., A. Malashicheva, G. Sullivan, M. Bogdanova, A. Kostareva, K. O. Stenslokken, A. Fiane and J. Vaage (2017). "Valve Interstitial Cells: The Key to Understanding the Pathophysiology of Heart Valve Calcification." J Am Heart Assoc **6**(9).
68. Saleh, M. A., E. I. Boesen, J. S. Pollock, V. J. Savin and D. M. Pollock (2011). "Endothelin receptor A-specific stimulation of glomerular inflammation and injury in a streptozotocin-induced rat model of diabetes." Diabetologia **54**(4): 979-988.
69. Santa-Cruz Mateos, C., A. Valencia-Exposito, I. M. Palacios and M. D. Martin-Bermudo (2020). "Integrins regulate epithelial cell shape by controlling the

architecture and mechanical properties of basal actomyosin networks." PLoS Genet **16**(6): e1008717.

70. Scatena, M., M. F. Jackson, M. Y. Speer, E. M. Leaf, M. C. Wallingford and C. M. Giachelli (2018). "Increased Calcific Aortic Valve Disease in response to a diabetogenic, procalcific diet in the LDLr(-/-)ApoB(100/100) mouse model." Cardiovasc Pathol **34**: 28-37.
71. Scott, A. J., L. R. Simon, H. N. Hutson, A. M. Porras and K. S. Masters (2021). "Engineering the aortic valve extracellular matrix through stages of development, aging, and disease." J Mol Cell Cardiol **161**: 1-8.
72. Sider, K. L., M. C. Blaser and C. A. Simmons (2011). "Animal models of calcific aortic valve disease." Int J Inflam **2011**: 364310.
73. Simionescu, M., D. Popov, A. Sima, M. Hasu, G. Costache, S. Faitar, A. Vulpanovici, C. Stancu, D. Stern and N. Simionescu (1996). "Pathobiochemistry of combined diabetes and atherosclerosis studied on a novel animal model. The hyperlipemic-hyperglycemic hamster." Am J Pathol **148**(3): 997-1014.
74. Simmons, C. A., G. R. Grant, E. Manduchi and P. F. Davies (2005). "Spatial heterogeneity of endothelial phenotypes correlates with side-specific vulnerability to calcification in normal porcine aortic valves." Circ Res **96**(7): 792-799.
75. Tucureanu, M. M., A. Filippi, N. Alexandru, C. Ana Constantinescu, L. Ciortan, R. Macarie, M. Vadana, G. Voicu, S. Frunza, D. Nistor, A. Simionescu, D. T. Simionescu, A. Georgescu and I. Manduteanu (2019). "Diabetes-induced early molecular and functional changes in aortic heart valves in a murine model of atherosclerosis." Diab Vasc Dis Res **16**(6): 562-576.
76. Vadana, M., S. Cecoltan, L. Ciortan, R. D. Macarie, M. M. Tucureanu, A. C. Mihaila, I. Droc, E. Butoi and I. Manduteanu (2020). "Molecular mechanisms involved in high glucose-induced valve calcification in a 3D valve model with human valvular cells." J Cell Mol Med **24**(11): 6350-6361.
77. Voicu, G., D. Rebleanu, C. A. Mocanu, G. Tanko, I. Droc, C. M. Uritu, M. Pinteala, I. Manduteanu, M. Simionescu and M. Calin (2022). "VCAM-1 Targeted Lipopolyplexes as Vehicles for Efficient Delivery of shRNA-Runx2 to Osteoblast-Differentiated Valvular Interstitial Cells; Implications in Calcific Valve Disease Treatment." Int J Mol Sci **23**(7).
78. Wang, M., Y. Liu, Y. Liang, K. Naruse and K. Takahashi (2021). "Systematic Understanding of Pathophysiological Mechanisms of Oxidative Stress-Related

Conditions-Diabetes Mellitus, Cardiovascular Diseases, and Ischemia-Reperfusion Injury." Front Cardiovasc Med **8**: 649785.

79. Widyantoro, B., N. Emoto, K. Nakayama, D. W. Anggrahini, S. Adiarto, N. Iwasa, K. Yagi, K. Miyagawa, Y. Rikitake, T. Suzuki, Y. Y. Kisanuki, M. Yanagisawa and K. Hirata (2010). "Endothelial cell-derived endothelin-1 promotes cardiac fibrosis in diabetic hearts through stimulation of endothelial-to-mesenchymal transition." Circulation **121**(22): 2407-2418.
80. Yadgir, S., C. O. Johnson, V. Aboyans, O. M. Adebayo, R. A. Adedoyin, M. Afarideh, F. Alahdab, A. Alashi, V. Alipour, J. Arabloo, S. Azari, C. M. Barthelemy, C. P. Benziger, A. E. Berman, A. Bijani, J. J. Carrero, F. Carvalho, A. Daryani, A. R. Duraes, A. Esteghamati, T. A. Farid, F. Farzadfar, E. Fernandes, I. Filip, M. M. Gad, S. Hamidi, S. I. Hay, O. S. Ilesanmi, S. S. Naghibi Irvani, M. Jurisson, A. Kasaeian, A. P. Kengne, A. R. Khan, A. Kisa, S. Kisa, D. Kolte, N. Manafi, A. Manafi, G. A. Mensah, E. M. Mirrakhimov, Y. Mohammad, A. H. Mokdad, R. I. Negoi, H. L. Thi Nguyen, T. H. Nguyen, M. R. Nixon, C. M. Otto, S. Patel, T. Pilgrim, A. Radfar, D. L. Rawaf, S. Rawaf, W. F. Rawasia, A. Rezapour, L. Roever, A. M. Saad, S. Saadatagah, S. Senthilkumaran, K. Sliwa, B. E. Tesfay, B. X. Tran, I. Ullah, M. Vaduganathan, T. J. Vasankari, C. D. A. Wolfe, N. Yonemoto, G. A. Roth and C. Global Burden of Disease Study Nonrheumatic Valve Disease (2020). "Global, Regional, and National Burden of Calcific Aortic Valve and Degenerative Mitral Valve Diseases, 1990-2017." Circulation **141**(21): 1670-1680.
81. Yan, A. T., M. Koh, K. K. Chan, H. Guo, D. A. Alter, P. C. Austin, J. V. Tu, H. C. Wijeysundera and D. T. Ko (2017). "Association Between Cardiovascular Risk Factors and Aortic Stenosis: The CANHEART Aortic Stenosis Study." J Am Coll Cardiol **69**(12): 1523-1532.
82. Yip, C. Y., J. H. Chen, R. Zhao and C. A. Simmons (2009). "Calcification by valve interstitial cells is regulated by the stiffness of the extracellular matrix." Arterioscler Thromb Vasc Biol **29**(6): 936-942.
83. Zabirnyk, A., M. D. M. Perez, M. Blasco, K. O. Stenslokken, M. D. Ferrer, C. Salcedo and J. Vaage (2020). "A Novel Ex Vivo Model of Aortic Valve Calcification. A Preliminary Report." Front Pharmacol **11**: 568764.